CHEMICAL INHIBITION OF β-GALACTOSIDASE ACTIVITIES AND DEVELOPMENT OF NEW PROTOCOLS FOR β-GALACTOSIDASE ACTIVITY ASSAY IN MICROBIAL CELLS

BY

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CERTIFICATION

This is to certify that this work," Chemical Inhibition of β-galactosidase Activities and Development of New Protocols for β-galactosidase Activity Assay in Microbial Cells" was carried out by Nwangwu, Oluchukwu Roseline, Reg number (20204252548) in partial fulfilment for the award of the degree of Masters of Science (M.Sc.) in Food and Industrial Microbiology in the Department of Microbiology of the Federal University of Technology, Owerri.

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DEDICATION

This project is devoted to the Almighty God, who, in his abundant grace and compassion, guided me to complete my project successfully.

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ABSTRACT

Inhibitory effects of surfactants, organic solvents and solvent mixtures on cell-free and cell-bound β-galactosidases were assessed to establish their sub-inhibitory concentrations for the development of methods for cell permeabilization and rapid *in situ* determination of β-galactosidase activity in *Escherichia coli* and *Kluyveromyces marxianus*. The concentration-response relationships of the individual surfactants, solvents and mixtures were fitted to a Gormpertz model to estimate the median inhibitory concentrations (EC_{50}) and No-Observable-Effect-Concentration (NOEC) thresholds. The inhibitory effects of solvents on cell-free β-galactosidase were in the order: N, N-dimethylformamide (DMF) > dimethylsulfoxide (DMSO) > ethanol (*E. coli*) and DMF > DMSO ≥ ethanol (*K. marxianus*). The inhibitory effects of surfactants on cell-free β-galactosidases were in the order: 1-cetylpyridinium chloride (CPC) > Cetyltrimethylammonium bromide (CTAB) > sodium dodecyl sulphate (SDS). Triton X-100, sarcosyl, Tween 20, Tween 80 and sodium deoxycholate (SDC) are not inhibitory to β-galactosidases. The established range of concentrations of these permeabilizing agents that are not inhibitory were used for whole cell β-galactosidase activity assay in *E. coli* and *K. marxianus*. In this *in situ* β-galactosidase activity assay, the cells were not washed, and permeabilization agents remained integral part of the reaction mixtures. Some of the selected concentrations of permeabilizing agents used for both organisms were compared with the classical methods in the literature. For *E. coli*, assay with a 5% 9:1 ethanol-chloroform mixture and 0.0008% CPC resulted in higher β-galactosidase activity than Miller's SDS-chloroform treatment. For *K. marxianus*, the β-galactosidase activity with 0.15% sarcosyl, 0.4% SDC and 15% of 9:1 ethanol-chloroform mixture resulted in higher β-galactosidase activity than Kippert's 0.133% sarcosyl treatment. This current method was found to be less time-consuming and accurate. The treatments could be used as an alternative procedure for *in situ* assay of β-galactosidase activity in *E. coli* and *K. marxianus* cells.

Keywords: β-galactosidase, *O*NPG, Toxic index, Concentration addition, Permeabilization, *Kluyveromyces marxianus*, Binary solvent mixtures.

CHAPTER ONE
INTRODUCTION

1.1 Background Information

Lactose is a disaccharide of glucose and galactose connected by a β-1, 4-glycosidic bond. In cheese whey, lactose accounts for 70–80% of the solid components, whereas 3-8% (w/v) of lactose is found in mammalian milk (Anisha, 2016). The author also mentioned that the limited solubility, low sweetness, and problems associated with lactose intolerance limit the direct use of this sugar. Lactose can be hydrolyzed to produce glucose and galactose, which are superior sugars in sweetness, solubility, and digestibility compared to lactose. It has been estimated that over 70% of people worldwide, and across all age groups, are intolerant to lactose. The symptoms of lactose intolerance include abdominal pain, loose stool (Perini, Souza, Kelbert, & Giannini, 2013), nausea, flatulence, and bloating after consuming lactose-containing foods. It has been proposed that lactose breakdown in milk before ingestion aids in absorbing the monosaccharide components of the sugar. The β-galactosidase is frequently used to break down β-galactopyranosides, or lactose, to create milk and dairy products with a lower lactose content. Lactose-intolerant people are the target market for these products (Xavier, Ramana, & Sharma, 2018). Thus, lactose intolerance is no longer an issue due to the usage of this enzyme. Lactose hydrolysis has significant implications for medicine, technology, and the environment. Hydrolysis of disaccharides improves the solubility and sweetness of the product because of monosaccharides, glucose, and galactose, in addition to relieving lactose intolerance. Moreover, β-galactosidase is responsible for carrying out the transgalactosylation reaction apart from the hydrolysis of lactose (Vera, Guerrero, Aburto, Cordova, & Illanes, 2020).

β-galactosidases are produced from many sources, such as plants, animals and microorganisms (Oliveira, Guimarães, & Domingues, 2011; Xavier et al., 2018). Nonetheless, microorganism-produced enzymes are utilized in food technology to hydrolyze lactose in milk and milk byproducts. Microorganisms generate enzymes more efficiently than animals and plants, resulting in lower prices for β-galactosidase (Mlichová & Rosenberg, 2006). The enzyme has received much attention due to lactose intolerance in the human population and the importance of milk in the human diet. Furthermore, this enzyme synthesizes lactose-based sweeteners from high lactose-containing effluents in cheese-making firms. Apart from food

or industrial and medical applications of β-galactosidase, as explained above, Husain (2010) highlighted some other applications of β-galactosidase, such as biotechnological and analytical applications. Anisha (2016) pointed out that submerged fermentation is the most often used technique for producing β-galactosidase (crude or whole cell) and batch cultivation results in lower enzyme production than lactose-based fed-batch culture.

The high extraction and downstream processing costs of intracellular β-galactosidase hinder its industrial use. To circumvent the difficulty of intracellular localization, β-galactosidase can be supplied from entire cells or released through cell disintegration or disruption. The cell disruption techniques are of two types: mechanical cell disruption and non-mechanical cell disruption. Non-mechanical cell disruption is categorized into three types: physical cell disruption, chemical cell disruption, and biological cell disruption. Ultra-sonication, high-pressure homogenizers, and bead mills are mechanical techniques that break microbial cells. Chemical techniques employ chemical agents, including organic solvents, antibiotics, detergents, chelating agents, hydroxides, and hypochlorides, to damage cellular integrity. The biological or enzymatic techniques for cell disruption encompass autolysis, lytic enzymes, and phage lysis.

Poor permeability of microbial cell walls is the fundamental downside of employing entire cells for β-galactosidase production. Researchers have found that permeabilizing microbial cells with organic solvents like toluene, chloroform, or ethanol can release β-galactosidase (Princely, Saleem Basha, Kirubakaran, & Dhanaraju, 2013; Prasad et al., 2013). The incubation period, temperature, solvent, and cell concentration affect how well organic solvents increase cell permeability. The permeabilization method may hydrolyze lactose in milk or whey at minimal cost, utilizing yeast cells (Panesar, Panesar, Singh, Kennedy, & Kumar, 2006). Ethanol-permeabilized yeast cells hydrolyzed milk lactose well under optimal conditions. The Ethylenediaminetetraacetic acid (EDTA) treatment is another approach that facilitates β-galactosidase production in gram-negative bacteria. According to Prasad et al. (2013), lytic enzymes and EDTA treatment work together to make it easier for β-galactosidase to be released from gram-negative bacteria.

B-galactosidase activity can be quantified by monitoring the hydrolysis rate of its chromogenic substrate, *o*-nitrophenyl-β-D-galactopyranoside (*o*NPG). The levels of β-galactosidase and the duration of the reaction are directly related to the quantity of o-

nitrophenol generated. Upon the addition of sodium carbonate (Na_2CO_3), the reaction is terminated, and the pH of the reaction mixture is adjusted to 11. Most of the o-nitrophenol at this pH is converted to a yellow anionic form, and β-galactosidase becomes inactive. Measuring the absorbance at 420 nm accurately assesses the amount of o-nitrophenol produced (Kippert, 1995). Miller in the year 1972 was the first to delineate the standard quantitative assessment of β-galactosidase activity in bacterial cell cultures by utilizing a permeabilization solution to rupture the cell membrane rather than preparing cell extracts. Apart from Miller's β-galactosidase assay procedures, many researchers have improved the assay methods (Griffith & Wolf, 2002; Thibodeau, Fang, & Joung, 2004), which led to better procedures. There are many types of β-galactosidase assays, including colorimetric assay, fluorometric β-galactosidase assay, and chemiluminescent β-galactosidase assay (Gao et al., 2021) and a single-step β-galactosidase assay(Schaefer, Jovanovic, Kotta-loizou, & Buck, 2016), etc. The in *vitro* assays for β-galactosidase have been described in some publications, particularly those that permeabilize cells for use as a whole-cell biocatalyst (Rodriguez-Colinas et al., 2011; Viana, Pedrinho, Morioka, & Suguimoto, 2018). The elevated concentration of the permeabilizing agent markedly diminished the enzyme's activity. However, when the concentration of the agent was low, it was not enough to properly permeate the membrane (Kumari, Panesar, Bera, & Singh, 2011). This caused the enzyme activity to decline even further.

Depending on the concentration, chemical agents have an impact on the stability and β-galactosidase activity. Guven and Bashan (1998) have reported the toxicity of ionic and non-ionic detergents on β-galactosidase activity, which, at all doses, the ionic detergent SDS inhibits the activity of β-galactosidase. In contrast, the non-ionic detergents Triton-X 100 and Tween 80 have no impact.

1.2 Problem Statement

The chromogenic substrate, *o*-nitrophenyl-β-D-galactopyranoside (*o*NPG), commonly employed to measure the activity of β-galactosidase, cannot penetrate intact microbial cells. It is necessary to permeabilize the cells to allow *o*NPG entry when assaying for the activity of β-galactosidase in microbial cells. Several chemical permeabilization agents including, surfactants and solvents have been used to permeabilize microbial cells for β-galactosidase activity assay including, chloroform and SDS (Sodium Dodecyl Sulfate) (Miller, 1972), Sodium lauroyl sarcosinate(Sarcosyl) (Kippert, 1995), toluene (Kumari et al., 2011), DMSO (Dimethylsulfoxide), ethanol (Panesar, Panesar, Singh, & Bera, 2007; de Faria et al., 2013; Panesar, 2008), lysozyme-EDTA treatment (Prasad et al., 2013), Cetyltrimethylammonium bromide (CTAB) (Kaur, Panesar, Bera, & Singh, 2009) etc.

Some permeabilizing agents inhibit β-galactosidase activity from microbial cells (Bell, Magill, Hallsworth, & Timson, 2013; Kippert, 1995). When applied, the cell permeabilizing agents could penetrate the cell envelop in bacteria and yeast or cause leakage of β-galactosidase, which decreases the enzyme activity (Kumari et al., 2011). During the permeabilization of the cell for the production of whole-cell biocatalyst, higher concentrations of these chemical permeabilizing agents could be employed. Cells are usually harvested and washed before determining the β-galactosidase activity of the permeabilized cells. This could result in loss of enzyme and concomitant underestimation of β-galactosidase activity in whole microbial cells. However, in the *in situ* assay of β-galactosidase activity, the cell-permeabilizing agents remained an integral part of the reaction mixture, where it could interact with β-galactosidase that possibly leaked out of cells. This underlined the need to investigate the inhibitory effects of cell-permeabilizing agents on microbial β-galactosidase activity.

The traditional SDS-chloroform method of Miller (1972) for β-galactosidase activity assay is still in contemporary use. Miller's SDS-Chloroform method could be challenging due to the water-insolubility of chloroform that results in interferences with spectrophotometer readings and variability in measured β-galactosidase activity. Several alternative approaches for β-galactosidase assay in microbial cells have been explored, where specific steps of the Miller method have been adapted for use with plate readers (Arvidson, Youderian, & Stormo, 1991; Griffith & Wolf, 2002; Thibodeau et al., 2004). However, these methods have inherent limitations associated with the first method and still necessitate substantial physical labor.

Thibodeau et al. (2004) devised an alternative test approach that utilizes a detergent-based proprietary reagent (PopCulture reagent) and lysozyme to break down bacteria, reducing the time-consuming Miller's method. Nevertheless, employing a detergent-based solution (PopCulture reagent and lysozyme) to lyse bacteria and extract β-galactosidase from cells is an expensive method due to the high cost of the Popculture reagent and lysozyme.

Therefore, there is a need to develop simple, rapid and in-expensive methods for assay of β-galactosidase activity in microbial cells.

1.3 Objectives of the Study

The aim of the study was to assess the chemical inhibition of β-galactosidase activities and develop new protocols for β-galactosidase activity assay in microbial cells.

The specific objectives of the study are to:

1. Isolate and screen the test organisms for β-galactosidase production
2. Identify the test organisms.
3. Produce crude β-galactosidase enzymes from the test organisms.
4. To assess inhibition of cell-free and cell-bound β-galactosidase activities in microbial cells.
5. To determine EC_{50} and No-Observable-Effect Concentrations (NOEC) for individual surfactants, organic solvents, or solvent mixtures.
6. Evaluate microbial cell permeabilization abilities at varying concentrations of 1-cetylpyridinium chloride monohydrate (CPC), other surfactants, solvents and solvent mixtures for *in-situ* β-galactosidase activity assays in whole microbial cells.
7. Ascertain the effect of mercaptoethanol and EDTA on β-galactosidase activity in microbial cells permeabilized with solvents and surfactants.
8. To develop new protocols for β-galactosidase activity assay in microbial cells and compare the new protocols with the existing protocols in the literature.

1.4 Justification of the Study

Inhibition of β-galactosidase biosynthesis and activity have been used as end-points in the assessment of chemical toxicity to microorganisms (Dutton, Bitton, Koopman, & Agami, 1990; Nweke & Okpokwasili, 2011a, 2011b). Additionally, research has been conducted on the effects of various substances, such as ionic and non-ionic detergents, on the activity of β-galactosidase (Guven & Bashan, 1998). When dairy products are produced industrially, lactose is hydrolyzed by β-galactosidase-producing microbial cells acting as whole-cell

biocatalysts (Choi, Song, & Yoo, 2004; Panesar et al., 2006). Since *O*NPG cannot penetrate the intact cell, there is a need to permeabilize the microbial for β-galactosidase assay with permeabilizing agents. Some permeabilizing agents may inhibit the β-galactosidase activity. Many studies have assessed the effectiveness of different organic solvents and surfactants in permeabilizing microbial cell membranes for the β-galactosidase assays over the years. However, a heuristic assessment of the inhibitory impacts of surfactants, solvents, or their mixture on the activity of β-galactosidase has not widely been conducted. Loss of enzyme activity may result from the permeabilization of microbial cells with high concentrations of organic solvents and subsequent cell washing (Panesar et al., 2007; Kippert, 1995; Kumari et al., 2011). The goal of the current work was to design rapid and effective method(s) for β-galactosidase assay in intact cells by evaluating the inhibitory effects of several chemical permeabilizers on β-galactosidase enzyme activity and their permeabilization capabilities. In this study, solvents and surfactants should be included in the reaction mixture during the permeabilization of the microbial cells and in the *in situ* enzyme assay since the absence of washing did not influence the enzyme's activity (Kippert, 1995).

1-cetylpyridinium chloride monohydrate (CPC), a cationic surfactant, was investigated as part of the current research to assess its ability as a cell permeabilizing agent for β-galactosidase activity measurement. The potential of these chemicals to permeabilize cells for β-galactosidase tests has not been widely studied. CPC is soluble in water at 0.5 M, appearing clear and colourless. Thus, CPC does not have solubility and colour interference in aqueous system.

In summary, this study would provide a range of alternative methods for a rapid and effective determination of β-galactosidase activity in microbial cells.

1.5 Scope of the Study

This study focused on the isolation and screening of test organisms for the production of β-galactosidase, Identification of test organisms, production of crude enzyme from microbial cells, β-galactosidase activity inhibition tests using the crude β-galactosidases to establish No-Observable-Effect Concentration (NOEC) ranges for the individual surfactants, organic solvents or their mixtures. The information obtained from the prior assays was used to design experiments for chemical permeabilization of microbial cells for *in situ* β-galactosidase activity assay using concentrations of selected chemical agents. Determination of the effect of

mercaptoethanol and EDTA on β-galactosidase activity in microbial cells permeabilized with solvents and surfactants. The newly suggested protocol(s) data was compared with existing protocols using statistical tools in IBM SPSS version 20.

CHAPTER TWO

LITERATURE REVIEW

2.1 Overview of β-Galactosidase

B-galactosidase (EC 3.2.1.23), also called lactase, is one of the most frequent hydrolytic enzymes. Lactase is an essential member of β-glycosidases, which hydrolyze glycosides into oligosaccharides, polysaccharides, and glycoconjugates with minimal expenses. β-glycosidases are carbohydrate-active enzymes that play a significant role in the breakdown and production of carbohydrates (Movahedpour et al., 2022). Lactase catalyzes the conversion of lactose into glucose and galactose to be used as carbon/energy sources. Besides this, the breakdown of lactose into a monosaccharide reduces crystallization in meals, including sugar, preventing grittiness (Wolf, Gasparin, & Paulino, 2018). Hence, to improve the national diet structure and fully utilize the health benefits of milk, it is essential to develop new types of β-galactosidase and research the hydrolysis of lactose in milk by β-galactosidase (Luan & Duan, 2022). The enzymatic breakdown of lactose is a frequently used technique for producing related dairy products and lactose-reduced milk for consumption by those experiencing lactose intolerance.

Silanikove et al. (2015) highlighted that fermented dairy products with little or no lactose can be consumed by those who are lactose intolerant. The β-galactosidase output globally is estimated to reach around 5.75 million tons annually. In the food processing industry, it has a wide range of uses. It is commonly employed in the food industry to enhance the taste, sweetness, solubility, and digestibility of dairy products since lactose has limited industrial applications due to its poor solubility and sweetness. Again, the significant uses of the enzyme are lactose hydrolysis and galactosylated product synthesis (Neri et al., 2008).

Besides hydrolyzing lactose, β-galactosidase transfers galactose, linked to glucose by a glycosidic bond, from lactose to other acceptor molecules (Anisha, 2016). The author also observed that these acceptor molecules include lactose, butanol, and antibiotics such as chlorphenesin and chloramphenicol. Transgalactosylation and transglycosylation facilitate the transfer of galactose from lactose to an acceptor molecule.

The products of these reactions are known as galactooligosaccharides (GOSs), and they are beneficial to human health since they are prebiotic food ingredients.

According to Prasad et al.(2013), β-galactosidase is an intracellular enzyme; one of the critical obstacles to successfully synthesizing this enzyme is its release in sufficient amounts from cells. It could be seen as a brilliant idea to employ complete cells as a source of the enzyme. Saqib et al. (2017) opined that β-galactosidase must be active for the small intestine to absorb undigested lactose; hence, lactose intolerance results from an enzyme deficit. Furthermore, molecular biology has also grown to value this enzyme's capacity to generate a colourful product during a chemical reaction.

The structure of β-galactosidase was initially deduced from a monoclinic crystal form that contained four independent tetramers in the asymmetric unit (Matthews, 2005). According to the author, it was feasible to figure out the structure by taking the average of the 16 polypeptide chains found in each of the four tetramers. β-galactosidase is a tetramer of four identical polypeptide chains(labelled A–D), each of which has 1023 amino acid monomers that are joined to form five distinct structural domains (1-5, which are respectively colored blue, green, yellow, red, and cyan in fig 2.1)(Matthews, 2005). The domains are jelly roll barrels, a central domain with a TIM-type barrel that also serves as the active site and the remaining one comprised of fibronectin and b-sandwich. The active site is rendered inactive by breaking the tetramer into dimers (Saqib et al., 2017). The amino terminal has a complementation-related peptide sequence that serves as a subunit interface. The most critical factor is the ability of β-galactosidases to produce other oligosaccharides or glycosides in addition to GOS (Galactooligosaccharides). Therefore, they have a broad spectrum of substrate specificity.

According to the Carbohydrate Active Enzymes database (CAZy), β-galactosidases are currently categorized within glycoside hydrolase (GH) families 1, 2, 35, 42, 59, and 147, specifically GH1, GH2, GH35, GH42, GH59, and GH147, based on structural similarity (Movahedpour et al., 2022; Vera et al., 2020). The GH2, GH35, and GH42 enzyme families encompass most of them. The GH1 and GH2 β-galactosidases are located inside the same evolutionary branch of the phylogenetic tree, whereas the GH35, GH42, GH59, and GH147 enzymes are situated on a distinct branch. The GH35 and GH42 enzymes are inside the same subbranch, indicating a potential common ancestry. Clan-A is the only superfamily that contains all families of β-galactosidases, and all of these enzymes have an 8-barrel shape. Both glycoside hydrolase 35 (GH35) and glycoside hydrolase 2 (GH2) belong to the two

prominent families of β-galactosidases. Although most β-galactosidases are members of the GH2 family, the vast majority of these enzymes are produced by microbes (Lu, Guo, Wang, Liu and Xiao (2019) claimed that the GH family two (2) encompasses the β-galactosidase gene known as lacZ from *Escherichia coli*. This enzyme has been the subject of the most comprehensive research. Over 70 per cent of GH35 is derived from plants. The lactase enzyme was first mentioned in 1889 by Martinus W. Beijerinck, who also named it lactase. The history of lactase's discovery is riddled with false starts, but eventually, it was discovered by Martinus W. Beijerinck.

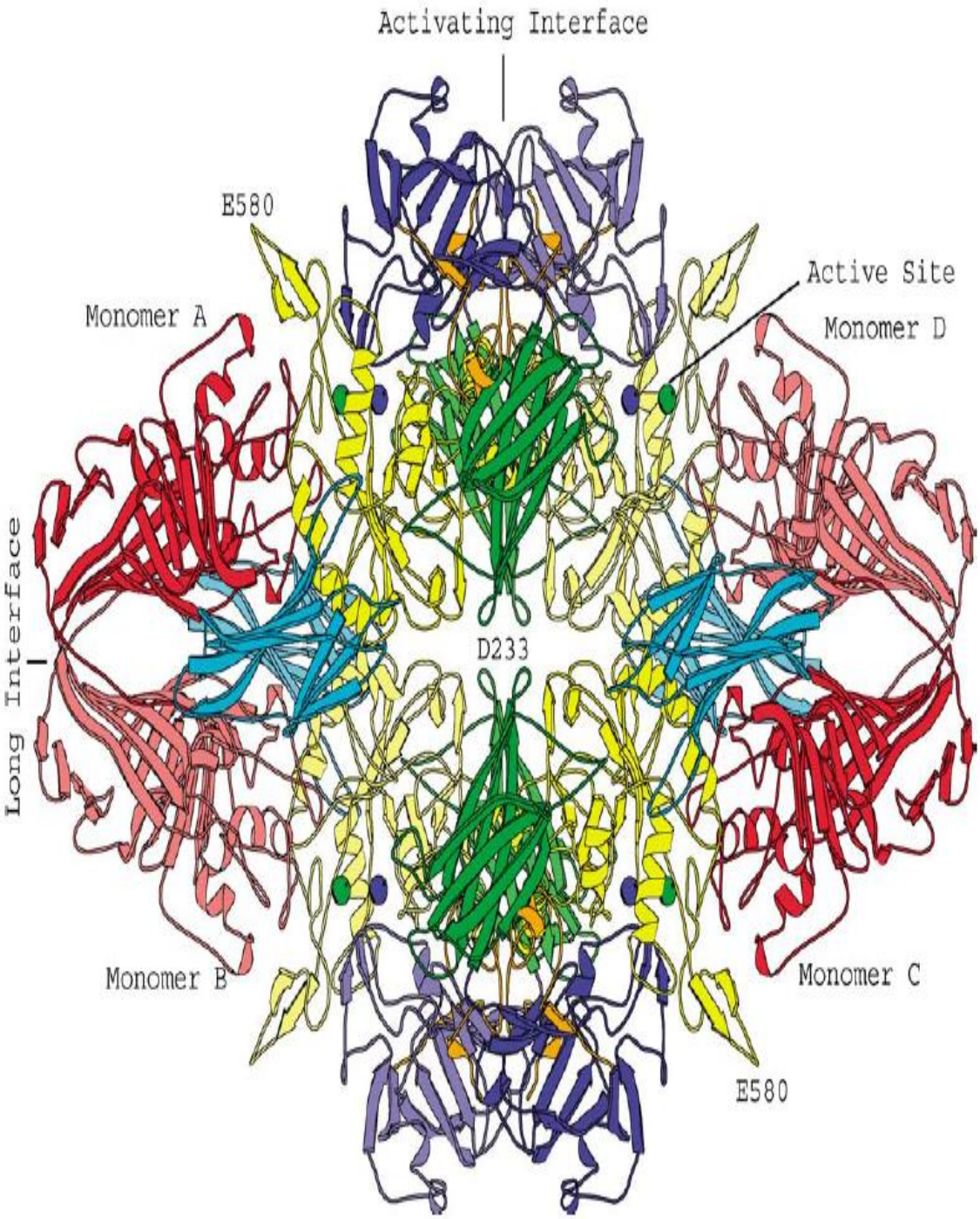

Figure 2.1: Structure of β-galactosidase from *Escherichia coli*

Source: (Matthews, 2005).

2.2 Sources of β-galactosidases

β-galactosidases are found in plants, animals and microbes (Husain, 2010; Nivetha & Mohanasrinivasan, 2017). Nevertheless, only microbial β-galactosidase is technologically significant since it can be manufactured cheaply with high yield and productivity through an extensive fermentation process. Also, those produced from microbes are potent instruments in fundamental research, organic molecule synthesis, pharmaceutical production, and numerous biotransformation processes for mass chemical production (Xavier et al., 2018). The microbial sources include (bacteria, fungi (especially yeasts and mold) and microalgae). Plant sources are found in almonds, peaches, apples, and apricots, whereas the animal sources include animal organs like the intestine, brain, placenta and testis of dogs, rabbits, snails, calves, sheep, goats, and rats (Nivetha & Mohanasrinivasan, 2017). However, enzymatic characteristics of the each organism are distinct. A new β-galactosidase exhibiting significant hydrolytic and transgalactosylation activity was extracted from infant faeces (Xin, Guo, Zhang, Wu, & Kong, 2019) and mammalian milk. This enzyme is prevalent in the human colon, where it aids in lactose fermentation, and its activity is assessed to gauge the ability of microbiota to ferment intestinal lactose. They are predominantly located in the stems, leaves (comprising cytoplasm and cell wall), and seeds of plants. The selection of sources primarily relies on the required reaction circumstances, including the sources utilized for extraction and their properties.

β-galactosidase properties vary depending on the organism they come from. The pH range within which lactase operates determines its use. Enzymes can be classified into two groups based on their pH: those from fungus with acidic conditions and those from bacteria and yeast with neutral conditions (Nivetha & Mohanasrinivasan, 2017). β-galactosidases are active in a wide pH range. For instance, fungal β-galactosidase is active in the pH range of 2.5–5.4, whereas yeast and bacterial enzymes are active in the pH range of 6.0–7.0 (Husain, 2010; Temuujin et al., 2012) Also, fungal β-galactosidases have a generally high optimum temperature between 55-60°C. Fungal β-galactosidase is not pure compared to yeast enzymes because they may contain other enzymes, such as amylase, lipase and Protease. In contrast, yeast and bacterial enzymes have the best temperature from 50-60°C (Nivetha & Mohanasrinivasan, 2017).

2.2.1 Microbial Sources of β-galactosidases

According to Oliveira et al. (2011)**,** lactase can be obtained from microbial sources such as bacteria, fungi and microalgae. β-galactosidase derived from microorganisms displays diverse functional properties that may be augmented by isolating appropriate microbial strains, generating enzymes suitable for immobilization, responsive to chemical mutagenesis, and facilitating enhanced enzyme secretion and gene expression through continually advancing recombinant DNA techniques. Moreover, microbial sources demonstrate enhanced productivity, leading to cost reductions. β-galactosidases sourced from yeast and moulds are increasingly employed in industry because of their exceptional efficiency and resistance to pH and temperature variations.

2.2.1.1 Bacterial sources of β-galactosidase

Due to the high activity, simple fermentation, and stability, β-galactosidases isolated from bacteria (either gram-positive or gram-negative bacteria) are utilized to hydrolyze lactose than other sources, especially the fungal sources (Husain, 2010; Picard et al., 2005). β-galactosidases can be produced from gram-negative bacteria such as *Escherichia coli*. It can also be produced by other bacteria especially gram-positive bacteria including *Lactobacillus, Bifidobacterium*, *Bacillus* sp., and *Bacillus aryabhattai* Gel-09 (CCTCC M2017320), *Lactobacillus* and *Bifidobacterium* (a probiotic, *Bifidobacterium infantis* strain CCRC 14633, *B. longum* strain CCRC 15708, and *B. longum* CCRC15708) (Iqbal, Nguyen, Nguyen, Maischberger, & Haltrich, 2010). *Bifidobacterium* and lactic acid bacteria, both regarded as harmless organisms, are good sources of β-galactosidases, particularly for functional food applications. Lactic acid bacteria include a variety of *lactococci, streptococci, and lactobacilli* (Husain, 2010). Also, thermotolerant β-galactosidases are also reported. Chen et al. (2008) reported that *Pyrococcus woesei*, *Thermus* sp., and *Bacillus stearothermophilus* also produce thermostable β-galactosidase. The cold-active and cold-adapted β-galactosidase from psychrophilic microorganisms like *Arthrobacter psychrolactophilus* and *Pseudoalteromonas haloplanktis*, respectively, have been reported (Nakagawa, Ikehata, Myoda, Miyaji, & Tomizuka, 2007; Van De Voorde, Goiris, Syryn, Van Den Bussche, & Aerts, 2014). Also, some of the other bacteria that produce the enzymes are reported, such as *Beijerinckia indica, Bifidobacterium infantis, Bifidobacterium longum* (Hsu, Yu, Lee, &

Chou, 2007), *Lactobacillus reuteri*(Alazzeh, Ibrahim, Song, Shahbazi, & AbuGhazaleh, 2009), *Enterobacter cloacae* (Lu, Xiao, Li, & Wang, 2009), *Bacillus megatherium, Bacillus circulans* (Guerrero, Vera, Conejeros, & Illanes, 2015), *Lactobacillus plantarum* (Iqbal et al., 2010) *Streptococcus thermophilus*(Ustok, Tari, & Harsa, 2010), *Propionibacterium acid-ipropionic*i (Zárate & Pérez Chaia, 2012), *Lactobacillus crispatus* (Nie et al., 2013).

Lactic acid bacteria (LAB), encompassing various *lactococci, streptococci, and lactobacilli*, have garnered scientific attention for their production of β-galactosidase, attributed to their GRAS (generally regarded as safe) classification, which facilitates the use of the resulting enzymes without extensive purification. Furthermore, individuals with lactose intolerance can typically consume fermented dairy products with minimal adverse effects, and the probiotic properties of LAB enhance lactose digestion (Husain, 2010). The author also noted that *Bifidobacterium* sp. and *Lactobacillus* sp. are the most commonly employed probiotics in food and food systems due to their potential health benefits.

The β-galactosidase of a yoghurt mixed culture comprising gram-positive rod bacteria; *Lactobacillus delbrueckii subsp. Bulgaricus* (*Lactobacillus bulgaricus)* and *Streptococcus thermophilus,* formerly known as *Streptococcus salivarius subsp. thermophilus* has been studied(Greenberg & Mahoney, 1982; Ustok et al., 2010; Vasiljevic & Jelen, 2001), which maintains their stability and exhibits a high activity level at temperatures beyond 50 degrees Celsius. These conditions can potentially speed up the lactose hydrolysis process while inhibiting the growth of unwanted bacteria. However, due to the demanding nature of LAB, the manufacture of enzymes requires a growth medium that contains not just supplies of carbohydrates and nitrogen but also various other growth elements such as minerals and vitamins. The β-galactosidase that is produced by thermophilic LAB is an enzyme that is found inside cells. To apply it using a culture grown in-house, the media containing the culture will need to be removed, and the microbial cells will need to be disrupted to release the enzyme.

2.2.1.2 Microalgal sources of β-galactosidases

According to Odjadjare et al. (2015), microalgae (microphytes, or microscopic algae) are a diversified group of microorganisms that live in a variety of ecological environments., but only a tiny fraction of the species are used for the benefit of man. Since yeast and bacteria produce β-galactosidases in large quantities, microalgae produce β-galactosidase also.

Microalgae like *Chlorella vulgaris*, *Dunaliella tertiolecta*, Nannochloropsis *oculata*, *Tetraselmis gracilis*, *A. platensis*, *Synechococcus subsalsus*, and *Scenedesmus ecornis* were grown in a mixotrophic manner to produce β-galactosidase (Zanette, Mariano, Yukawa, Mendes, & Spier, 2019). Compared to autotrophic cultures on CO_2, the biomass productivity of these microalgae in the presence of lactose increased. Throughout growth, a drop in lactose concentration was seen, along with a concurrent rise in glucose and galactose concentrations, indicating the action of β-galactosidase. This degree of activity, meanwhile, was not measured at the time (Girard, Deschênes, Tremblay, & Gagnon, 2013). Eventually, the mixotrophic cultivation of green microalgae on cheese whey permeate for biodiesel production has been investigated(Girard et al., 2014).

2.2.1.3 Fungal sources of β-galactosidases

The sources of β-galactosidase from fungi can be broken down into two categories: yeast sources and mold sources. There have been reports of *Kluyveromyces lactis* (Frenzel, Zerge, Clawin-Rädecker, & Lorenzen, 2015), *Kluyveromyces marxianus* (Cortés, Trujillo-Roldán, Ramírez, & Galindo, 2005), and *Saccharomyces cerevisiae* producing β-galactosidase Also, Bakri, Researcher and Hajmustafa (2015) noted that commercial β-galactosidases were derived from yeasts such as *Kluyveromyces lactis* and *Kluyveromyces marxianus* (formerly known as *Kluyveromyces fragilis* and *Saccharomyces fragilis*), as well as molds such as *Aspergillus niger* and *Aspergillus oryzae*. The authors emphasized the numerous advantages of *Kluyveromyces marxianus*, including its high growth yield, which has significant economic implications in the food industry. Additionally, it is considered a safe microorganism, making it suitable for use in fermented products with food or pharmaceutical applications. Furthermore, it exhibits higher β-galactosidase activity compared to other yeasts. It has been shown that *Geobacillus stearothermophilus* produces β-glucosidase in conjunction with other enzymes like amylase for co-producing β-galactosidase. Movahedpour et al.(2022) noted that the yeast *Kluyveromyces lactis* is an essential source of β-galactosidases, an enzyme that is generally exploited in the dairy industry. *Kluyveromyces lactis* is a yeast that produces one of the β-galactosidases (β-gals) most widely used in neutral milk products (Husain, 2010; Mlichová & Rosenberg, 2006). The enzyme, commonly known as lactase, is produced on an industrial scale by selected yeast strains containing extra copies of the LAC4 gene (Coenen, Bertens, De Hoog, & Verspeek-Rip, 2000). Cell disruption is necessary to release the enzyme

into solution since the β-gal is located in the cytoplasm of yeasts. *K. marxianus* can produce homologous β-galactosidases and other heterologous enzymes, allowing it to thrive on many substrates; however, lactose remains its exclusive energy source. Furthermore, cold-active acid β-galactosidases were extracted from a strain of the psychrophilic yeast species, *Guehomyces pullulans* and *K. fragilis*, both of which are utilized as commercial yeasts. Smith and Gross (2000) identified the predominant sources of β-galactosidases in fungi as species from the genus, *Aspergillus*, particularly *A. niger* and *A. oryzae*, which have been designated as "generally recognised as safe" (GRAS) by the Food and Drug Administration. Several researchers have utilized the filamentous fungus, *Aspergillus niger* for the manufacture of multiple extracellular enzymes, including lactase. In *Aspergillus* spp., β-galactosidase is secreted into the extracellular environment. Domingues, Guimarães and Oliveira (2010) asserted that the β-galactosidase found in *Kluyveromyces* spp. is an intracellular enzyme. Their findings indicated that lactose is initially carried into the yeast cell by a permease, followed by hydrolysis to produce glucose and galactose. In addition, since the enzyme resides within the yeast cells, it is necessary to disrupt or render the cells permeable using chemical and mechanical methods.

2.2.1.4 Plant sources of β-galactosidase

β-Galactosidases are widespread in plants, where they participate in the growth of plants, the breakdown of lactose, and the ripening of fruit. Seddigh and Darabi (2014) emphasized that softening-associated β-galactosidases were isolated from various fruits, including avocado, Japanese pear, tomato, apple, mango, muskmelon, papaya, strawberry (*Fragaria ananassa*), coffee, wild rose tips, soybean seeds, alfalfa, and kiwifruit. Reports indicated that this enzyme facilitates the ripening of persimmons by reducing the galactosyl content in the cell wall, hence promoting fruit maturation.

2.3 Microbial Cell Disruption Techniques

Cell disruption or cell lysis is the act of breaking up the microbial cells to acquire a desired biological product, which might be inside of cells or periplasmic space. According to Islam, Aryasomayajula and Selvaganapathy (2017), it is a technique that involves breaking down or destroying the outer boundary or cell membrane of a cell to release intracellular components such as protein, RNA, DNA, enzymes or organelles from the cell.

Microalgae, yeasts and bacteria serve as sources of various biological molecules, including lipids, proteins, pigments, enzymes, and organic acids, which are utilized in numerous industrial processes (Drévillon, Koubaa, & Vorobiev, 2018; Peternel, 2013). These biological molecules with high added value frequently accumulate inside cells, and their extraction requires many subsequent procedures, the most critical step being the breakdown of the cell wall(Gomes, Zanette, & Spier, 2020). Since the structure of various cells varies, it is necessary to use various techniques to disrupt them. Because yeast cell walls block the access of the solvent to the products you seek, yeast cells are rigid to destroy. Other types of cells, such as bacteria, mould, plant, mammalian, and ground tissue, must be disrupted to be studied. Gomes et al.(2020) also added that when choosing the most suitable disruption method, it is essential to examine parameters such as the degree of purity, the composition of the cell wall, scalability, the nature of the biomolecule, and energy input.

Before selecting a method, it is crucial to take into account other factors such as the sample size of the cell, the toughness of the cell (be it plant, animal, bacterial, or fungal), the efficiency of the disruption method, the stability of the product to the method used, the ease of extraction and purification, the biohazardous nature of the cell, time and cost considerations, and whether or not expertise or training is required. Islam et al. (2017) pointed out that, depending on the application, cell lysis can be classified as partial or complete.

2.3.1 Methods of Microbial Cell Disruption

There are two main cell disruption methods. They include the mechanical method of microbial cell disruption and the non-mechanical method of microbial cell disruption.

2.3.1.1 Mechanical methods of microbial cell disruption

The mechanical approaches are termed severe methods due to their lack of specificity and dependence on mechanical principles, which may disrupt the cell wall, particularly the degradation of components that confer strength to the cell wall (Geciova, Bury, & Jelen, 2002). Direct physical force must be applied to the cell wall to induce rupture. In employing a mechanical method, it is crucial to ensure that cell membranes are adequately disrupted without diminishing the activity of enzymes. Husain (2010) highlighted that the ultrasonication approach is predominantly utilized for laboratory-scale applications due to its lack of necessity for complex apparatus or extensive technical proficiency. Mechanical cell disruption techniques encompass ultrasonication (Liu, Zeng, Sun, & Han,

2013), high-pressure homogenization (HPH), bead milling, French pressing, microfluidizer, etc.

(i) Ultrasonication

This method is extensively investigated because of its adaptability and minimal operational costs (Üstün-Aytekin, Arisoy, Aytekin, & Yildiz, 2016). Ultrasonic vibrators or ultrasonicators are most commonly used for this method. It is a speedy method; it is done mainly on a laboratory scale to disrupt a relatively tiny amount of cell biomass. It does so by causing cavitation effects, resulting in cell disruption. In this technique, high-frequency sonic waves generate a strong shear action, leading to the cavitation phenomena. Cavitation is characterized by the production of vapour bubbles, their development, and their eventual collapse. According to Zhang, Grimi, Marchal, Lebovka and Vorobiev (2019), the collapse encourages the production of powerful elastic shockwaves, which, owing to the elevated pressure (up to 200 MPa), have the potential to destroy cell walls. Disadvantages of this technique include local high temperatures, resulting in low yields, scalability challenges, and noise.

i) High-pressure homogenization (HPH)

High-pressure homogenization (HPH) is a mechanical method used to cause cell breakdown. The efficacy of this approach in disrupting microbial cells has been extensively studied, particularly in large sample volumes (Drévillon, Koubaa, Nicaud, & Vorobiev, 2019). High-pressure homogenization is an essential process used to break apart fat globules or cells that carry bioproducts inside them. This technique involves subjecting the cell suspension to high pressure (about 20-120 MPa) using a pump that displaces the fluid. The suspension is subsequently compelled through a constricted aperture, where the pressure is rapidly released. This technique results in fluid velocities reaching up to 400 m/s. The impact rings function to safeguard the chamber against damage caused by high pressure, while shear stress, turbulence, and friction lead to cellular fragmentation (Drévillon, Koubaa, & Vorobiev, 2018; Floury, Bellettre, Legrand, & Desrumaux, 2004). In addition to the working pressure, the frequency of valve passage is a critical operational variable that may fluctuate depending on the microbial cell. This approach allows for the control of ripping force by modifying the flow rate, which is influenced by the applied pressure and the dimensions of the small gap. High-pressure homogenizer systems are efficient for large-scale operations; nonetheless, they

require substantial energy input, especially in ultra-high-pressure homogenization (UHPH) systems. Furthermore, the thermal treatment of the sample may lead to the degradation of thermolabile biomolecules, constituting an additional disadvantage. Therefore, it is essential to equip the device with a cooling system.

ii) Glass-beads and bead milling

Bead milling is a complex method with numerous parameters, including bead diameter, bead density, bead filling, agitator speed and feed rate: the impact and shear stress of beads damage cells. A popular and easy method for encouraging microbial cell disruption is using glass beads. The properties of the cells that are going to be crushed are what decide the dimensions and weight of the beads that are going to be employed in the bead mill. The size of the beads is one of the most critical factors that determines how efficiently the cell disruption process works. Depending on the type of cell, a different bead diameter is chosen. For instance, 0.1 mm glass beads are suggested for breaking up bacterial cells.

In contrast, glass beads of 0.5 mm to 1.25 mm are recommended for disrupting yeast or fungi, while those measuring 0.3 mm to 0.4 mm are advised for microalgae (Montalescot et al., 2015). Commercially accessible beads are offered in solid form or within dense material that incorporates the beads. A cooled buffer solution can be utilized to amalgamate the cellular biomass before the disruption process. Furthermore, it is recommended that glass beads undergo pretreatment with concentrated HCl for several hours, followed by rinsing, drying for 16 hours at 150°C, and subsequent cooling to 4°C before use. Vortex-type agitators or grinding chambers can utilize various materials, such as quartz sand, zirconia, and ceramic, as abrasives for cellular lysis (Montalescot et al., 2015). The following are some significant benefits of bead milling that make it a necessary tool in many applications: Processing smaller materials is an advantage of using a bead mill. In addition to efficiently breaking down and disrupting cells, the mill's tiny beads also grind materials into tiny bits. Furthermore, bead mills provide operational versatility by supporting both continuous and batch processing modes. They are therefore quite flexible. Bead mills are frequently used to break up yeast cells. Bead mills are commonly used to grind animal tissues in addition to yeast cells. However, a drawback of bead milling is that as the amount of beads used in the mill increases, the temperature tends to rise. One important consideration when using bead mills is the possibility of contamination. Inadequate process scaling is one of the main problems with

bead milling. Maintaining characteristics like the regularity of the disruption process, temperature control, and the effectiveness of bead-to-bead contacts may be more challenging at bigger scales.

iii) **French pressing**

French presses are commonly employed to extract small quantities of intracellular substances, including proteins and DNA, from bacterial and plant cells. A French press consists of a steel cylinder featuring a small aperture and a needle valve at the base. The French press operates on the same concept of high-pressure homogenization, as it is classified as a high-pressure homogenization device. The French press operates by introducing a cell suspension into a cylindrical chamber and expelling it through a valve. The suspension is propelled through an annular gap or a small aperture at pressures reaching 1500 bar. At this juncture, as it traverses the discharge valves, the pressure abruptly decreases to atmospheric levels. Jaschke, Drake and Beatty (2009) asserted that shear stress and decompression result in cellular disruption, leading to cell rupture or disintegration due to rapid pressure reduction. The abrupt decrease in pressure leads to rupture, resulting in the release of intracellular constituents and the breakdown of cellular membranes or walls. It is imperative to pre-cool both the apparatus and the sample to a low temperature before the procedure to liberate thermolabile biomolecules.

2.3.1.2 Non- Mechanical methods of cell disruption

Non-mechanical methods do not employ physical force to break the cell wall. They use alternate methods to break the cells instead. Anand, Balasundaram, Pandit and Harrison (2007) stated that gentle methods are less energy-intensive than mechanical alternatives, with some demonstrating the ability to achieve selective product release. Peralta, Bergamini and Hynes (2019) facilitated the permeability of cell membranes, enabling the ingress and egress of low-molecular-weight solutes. Non-mechanical procedures, by definition, do not employ physical force to breach the cell wall. They use alternate methods to disrupt cells instead. Physical, chemical, and enzymatic or biological processes are categorized as non-mechanical approaches.

(a) Physical methods

They entail breaking down the cell wall and releasing the intracellular fluid by exposing cells to extremely high temperatures (thermal shock) and high-frequency sound waves. Osmotic shock, thermolysis, freeze-thaw, etc., are a few examples of physical methods of non-mechanical cell disruption techniques.

i) Osmotic shock

Osmotic shock is a method of disrupting cells that includes exposing them to varying salt concentrations, which results in cell death. The rapid movement of water from environments with low salt concentration to those with high salt concentration causes cell volume and pressure alterations, ultimately resulting in cell rupture; this is the fundamental idea of osmotic shock.

Osmotic pressure arises from a difference in solute concentration across a semi-permeable membrane. Due to partial permeability of the cell membranes, swiftly introducing a cell from an isotonic environment into a hypotonic medium such as distilled water, would lead to an influx of water into the cell at an accelerated rate. This would later result in a fast increase in cell volume followed by its breakdown. Prior to the administration of an osmotic shock, bacterial and fungal cell walls must be broken down first. Osmotic shock is mainly used in cell membrane permeabilization, primarily done in Gram-negative bacteria. Gram-positive bacteria, which have a elevated internal osmotic pressure, are susceptible to the method since it is not effective against them. Fungi and yeasts have cell walls that are strong to mechanical injury, the same thing happens to them. Nonetheless, osmotic shock can be used for as a pretreatment for lipid extraction in microalgae and permeabilization of proteins with low molecular weight. Show, Lee, Tay, Lee and Chang (2015) noted that osmotic shock has not been used extensively since it needs a cooling system, uses significant amounts of water, and thus has high operating expenses. The osmotic pressure method can be employed with either a hypotonic or a hypertonic solution. Osmotic shock is produced by placing the cell suspension on either of two different solutions: a hypotonic solution or a hypertonic solution. Plasmolysis occurs when a hypotonic solution is present, which results in water entering the cytoplasm of the cell and well bursting. In hypotonic situations, when solute concentrations are low, water enters the cell in significant volumes, causing the cell to swell and either rupture or undergo apoptosis. On the other hand, plasmolysis occurs in the presence of a hypertonic solution, and the cell contracts as a result of the water being lost. This is usually used in

conjunction with other strategies because it does not ensure success. Even though single-celled creatures are more vulnerable to osmotic shock due to their direct contact to their surroundings, large animals like mammals can however experience it under specific conditions (Ho, 2006).

According to recent studies by Brocker, Thompson and Vasiliou (2012), osmotic stress in cells and tissues may significantly contribute to several human disorders

ii) **Thermolysis**

Thermolysis is a method for cell disruption that employs heat generated by microwave radiation to lyse cells and release their internal constituents. It is also known as microwave treatment or heat shock. To induce protein release and cell lysis, cells must be subjected to elevated temperatures for a specified duration; this is the principle of thermolysis. Extensive uses of thermolysis are feasible (Middelberg, 1995), particularly for enzyme liberation. Thermolysis has been employed to extract hyperthermophilic enzymes from bacteria and Archaea species. High heat inactivates cells by rupturing the cell wall and liberating intracellular components. The effect of heat is determined by various factors, including pH, temperature, chelating agents, ionic strength, the presence of enzymes (hydrolytic and proteolytic), time, and more variables. Thermolysis is often used in research institutions and industrial environments to extract intracellular components, including proteins and enzymes. It can be utilized to expel the contents of several cell types, including bacteria. Thermolysis is particularly advantageous for large-scale production as it offers a rapid and efficient method for destroying cellular structures. The advantages of this cell disruption method include i) the deactivation of proteases and the facile separation of soluble biomolecules from cellular debris, hence enhancing operational efficiency. ii) This process is simple and cost-effective for a heat-stable product. Nonetheless, an elevation in the viscosity of the fermented broth may transpire. In instances where the biomolecule is solid, substantial cell debris may result in intricate separation, necessitating additional mechanical procedures (Middelberg, 1995). Additional drawbacks encompass: i) Inapplicability to heat-labile substances, ii) Resistance of spore-forming bacteria to this technique, iii) The expense of thermolysis, necessitating specialized apparatus such as microwave ovens or reactors that can produce controlled heat, iv) Although it holds promise for large-scale production, thermolysis is frequently confined

to small-scale laboratories owing to its associated costs and specific infrastructure prerequisites. v) Inconsistent enzymatic activity.

iii. Freeze-thawing

Gomes et al. (2020) described the freeze-thaw approach as a process that entails the recurrent formation of ice crystals and the facilitation of cell growth during thawing to induce cellular disruption. Liquid nitrogen may be employed in the freezing process to get quick freezing, while temperatures ranging from -15 to -80 °C can be utilized for delayed freezing. Thawing is a progressive procedure occurring at room temperature or in a refrigerator (4°C) (Dixon & Wilken, 2018). According to Lee, Show, Ling and Chang (2017), multiple cycles are required to disrupt cells successfully, which can be time-consuming. Bacterial and mammalian cells are commonly subjected to lysis in laboratories using the freeze-thaw technique. It involves the repeated freezing and thawing of a cell solution, resulting in the rupture of cells and the release of their contents. The formation of ice crystals during freezing and the subsequent cellular expansion, followed by cellular disintegration during thawing, are the fundamental principles of freeze-thaw lysis.

The extraction of intracellular enzymes, particularly thermolabile biomolecules, is recommended using this technique (Kar & Singhal, 2015; Mayerhoff, Franco, & Roberto, 2008). Peralta, Bergamini and Hynes (2019) averred that although this physical technique possesses the potential for industrial application, its efficacy is limited by temporal constraints and substantial energy demands. The freeze-thaw method is frequently used in conjunction with or as a precursor to other treatments. A recent work by Kumar, Rao and Arumugam (2015) employed the freeze-thaw procedure as a pretreatment for subsequent techniques (detergent and bead milling) to permeabilize the cell wall of *Kluyveromyces lactis* for the release of β-galactosidase. The author indicated that the freeze-thawing process led to minimal permeabilization (about 20%) in the control cells. Thus, this strategy may improve future release methods and result in superior recovery efficiency. The primary advantage of this treatment is its relative affordability compared to alternative cell lysis methods. Researchers with limited resources can utilize it as it does not require costly tools or reagents.

Nonetheless, it is subject to the following limitations: The freeze-thaw lysis technique often requires multiple cycles of freezing and thawing to lyse cells effectively. The entire procedure can be laborious, mainly when dealing with substantial volumes or dense cellular

populations. Cellular components susceptible to temperature variations may not be obtained during freeze-thaw lysis. During the freeze-thaw cycle, specific proteins or biomolecules may undergo denaturation or degradation, leading to a loss of activity or functionality.

(b) Chemical methods

Chemical methods are often used for microbial cell disruption and lysis, employing specific chemical agents, including permeabilizers, antibiotics, chartrops, chelating agents, hydroxides, hypochlorides, isoamyl alcohol, toluene, ammonia, sodium hydroxide, benzene, ether, acetone, methanol, hexane, and detergents such as Triton X-100, sodium dodecyl sulphate (SDS), and N-lauroyl sarcosine (sarcosyl) (Kippert, 1995; (Voget, 2018). These chemical compounds may facilitate cell breakdown or permeabilization, resulting in the release of intracellular contents. These chemical techniques provide several alternatives for cell disruption and lysis, although they include inherent limits. Considerations such as elevated chemical expenses, stability issues in alkaline environments, protein denaturation, downstream processing disruptions, and the necessity for supplementary purification procedures must be considered when choosing and implementing these approaches. Nevertheless, chemical techniques are crucial in laboratory research and particular applications necessitating focused cell lysis. Gomes et al. (2020) claimed that the chemical disruption techniques can also be employed as a pretreatment to enhance the effectiveness of other cell disruption techniques like bead milling and ultrasonication. The type of microbe, biomolecules, and location of the microbe play a role in the choice of chemical agents. Although it is readily available and only requires a small amount of energy, its application is nevertheless limited due to the problem of highly associated breakdown, particularly for enzymes and pigments. The chemical agents used for cell disruption are elucidated as follows:

i) Chemical permeabilizers

They are also referred to as solvents and are pore-forming lipid solubilizers that allow the release of cellular materials. Toluene, ether, phenylethyl alcohol, benzene, methanol, pentanol, N, N-dimethylformide (DMF), chloroform, ethanol, Dimethyl sulphoxide (DMSO), and other organic solvents are some of the chemical permeabilizers. These solvents are commonly utilized to disrupt cell walls to extract bio-pigments, enzymes, and intracellular biological molecules. Toluene, a prevalent organic solvent, functions by dissolving the hydrophobic components of the phospholipids in the inner membranes of Gram-negative

bacteria (Middelberg, 1995). Chloroform can be used to extract periplasmic proteins from Gram-negative bacteria. Cytoplasmic proteins from Gram-positive bacteria can be extracted employing acetone and SDS detergent (Bhaduri & Demchick, 1983). The application of solvents presents certain disadvantages, such as low selectivity and the tendency to disrupt cell walls, thereby damaging sensitive proteins and enzymes. Geciova et al. (2002) concluded that using organic solvents in food industry applications hinders downstream processes, as these solvents are generally not food-grade.

ii) Detergents

Detergents are amphipathic molecules or substances that possess hydrophobic (tails, which are often polar hydrocarbon) and hydrophilic(heads) parts in their structures (Trawcznska, 2020). They are also known as surfactants because they decrease the surface tension of water. They are frequently employed in laboratories to penetrate or lyse cells by disrupting protein-lipid bonds, aiding in the solubilization of lipids and denaturing of proteins. Depending on the concentration of the detergent, these characteristics enable their interaction with biological membranes. Geciova et al. (2002) indicated that detergents can be absorbed by the membrane at low concentrations, resulting in fast permeability alterations, while at elevated concentrations, they may induce membrane fusion and lysis. All detergents have one thing in common: they immediately destroy the cell wall or membrane, which causes the intracellular substance to leak.

Trawcznska (2020) stated that there are typically four different types of detergents: ionic (cationic or anionic), nonionic, and zwitterionic. i) anionic detergents are negatively charged detergents that frequently disorganize cell membranes. They include SDS, sarcosyl, and sodium deoxycholate (CPC); ii) Cationic detergents are positively charged detergents that alter the lipopolysaccharide component of the cell membrane. They include cetyltrimethylammonium bromide (CTAB) and 1-cetylpyrinidium chloride (CPC). CTAB is a surfactant that enhances the permeability of cell membranes. It is often employed at ambient temperature, with concentrations ranging from 0.1% to 2% for 30 minutes. iii) Non-ionic detergents include, Tween-80, Tween-20, Triton X-100, and Triton X-400, and pluronic F-68. Proteins in the inner structure of the membrane are partially solubilized by pluronic F-68 and other compounds, causing permeabilization. Non-ionic detergents lack any charge in the hydrophilic head region, unlike ionic detergents. iv) Zwitterionic detergents are compounds

with a net charge of zero that have both positive and negative charges simultaneously. The zwitterionic detergent 3-[(3-cholamidopropyl) dimethylammonio] is one example.3-[(3-cholamidopropyl)dimethylammonio] and -1-propanesulfonate (CHAPS)CHAPSO, or 2-hydroxy-1-propanesulfonate. They do not alter the charge of the solubilized protein and can be utilized to lyse cells. Because they may inhibit or reverse electroosmotic flow in later electrophoretic separation procedures, caution is required (Brown & Audet, 2008). However, detergents have the potential to denaturize proteins and require further purifying procedures, which limits their application in large-scale processes. Since cell death occurs quickly, non-ionic detergents are frequently used. Furthermore, it is well known that the non-ionic detergents listed above can dissolve phospholipid bilayer membranes and are mostly used to permeabilize bacteria. These detergents prevent cell lysis, prevent protein denaturation, and maintain protein structure. Proteins and enzymes are therefore preferred for uses involving their action. Despite their ability to disrupt cells quickly (within seconds), strong ionic detergents like SDS usually denature cellular proteins. Because they require an extra step to remove, detergents are rarely employed to stop microbial cell disruption, which leads to a complicated and expensive downstream procedure. Once more, when combined with additional methods like ultrasonication, enzymes, and bead milling, surfactants can speed up the release of intracellular biomolecules (Islam, Aryasomayajula, & Selvaganapathy, 2017). One disadvantage of using detergents is that many proteins will become denatured during cell lysis. Detergents may also obstruct subsequent downstream processing steps.

Thus, further purification steps would be necessary after cell lysis, which restricts their application in large-scale procedures. Other drawbacks include the possibility of insufficient interruption and the necessity of repetition.

(iii) Chelating agent, antibiotics, peroxide and hypochlorite

EDTA is an efficacious chelating agent that chelates or binds to bivalent cations such as Mg^{2+} and Ca^{2+}, leaving them unavailable for cellular use and causing disruption of the cell membrane. Antibiotics such as polymyxin, azoles, and nystatin, which impede cell membranes, are employed to disrupt membrane formation and facilitate the release of cellular contents alongside the application of chelating chemicals to lyse cells; additionally, peroxide and hypochlorite (e.g., H_2O_2 and HClO) are utilized. Examples of this include calcium hypochlorite (a constituent of bleaching powder and swimming pool "chlorine") and sodium

hypochlorite (household bleach); these hypochlorites and peroxides damage lipid bilayers and inhibit -SH proteins by oxidizing cellular components. Cellular components are liberated due to the oxidation of cellular membranes.

c) Biological or enzymatic method of cell disruption

Enzymatic techniques, often known as biological techniques, are used to lyse and disrupt cells. These methods include using specific enzymes to degrade the cell walls and membranes of the test organisms. Zheng, Xiao and Roberts (2016) noted that the enzymatic disruption of microbial cells is a promising and highly sustainable technique. Enzymes can be a valuable substitute for mechanical methods when the biomolecule is easily influenced by outside stimuli like pressure and speed forces. High selectivity, favourable pH, and temperature are some of the benefits of enzymatic lysis. According to de Carvalho, Medeiros, Letti, Kirnev and Soccol (2016) and Islam et al. (2017), Peptidoglycan hydrolases, often referred to as murein hydrolases, are the enzymes that encourage the breakdown of the peptidoglycan layer in bacteria. Commercial enzymes that can be deployed on a large scale, primarily when immobilized enzymes are used, include lysozyme, proteases, cellulases, and glucanases.

Additionally, the authors noted that depending on the bond specificity, these enzymes might be categorized as amidases, endopeptidases, or glycosidases (lysozymes, muramidases, and glucosaminidases) (Salazar & Asenjo, 2007). Furthermore, different cell types and strains have distinct forms for their cell walls and membranes; hence, the type of enzyme used varies according to the microorganism. For example, lysozyme is commonly used to digest gram-positive bacterial cell walls. Lysozyme is in charge of hydrolyzing the β-1-4-glucosidic bonds in the peptidoglycan. In contrast to gram-positive bacteria, gram-negative bacteria have a distinct cell wall composition. As a result, lysozyme performs poorly when interacting with gram-negative cell walls. This enzyme can be purchased for a price that is not excessively high and is produced from egg white preparations (Geciova et al., 2002). As previously noted, gram-negative bacteria, which include certain primary foodborne pathogens, are typically not amenable to lysozyme treatment. Accordingly, a combination of Triton X-100 or EDTA was created to broaden the application of lysozyme to Gram-negative bacteria (Salazar & Asenjo, 2007).

Additionally, various techniques have been developed to expand the uses of lysozyme to encompass Gram-negative bacteria. Among these are lysozyme denaturation and alteration

caused by covalent fatty acid or polysaccharide attachment. Enzymes involved in the breakdown of yeasts include protease, β-1, 3 glucanase (both lytic and non-lytic), β-1, 6 glucanase, mannanase, and chitinase. Proteases and glucanases are often required for the enzymatic digestion of yeasts. First, the mannans and wall proteins are released from the protein structure by the proteases, revealing the glucan surface; then, the β-1, 3 lytic glucanase attacks the interior wall and solubilizes the glucan (Hunter & Asenjo, 1987).

- **Methods of Enzymatic Cell Disruption**

Enzymatic cell disruption can be accomplished through several distinct methods, the most common of which are i) autolysis, ii) lytic enzyme and iii) phage lysis. Middleberg (1995) stated that autolysis and the use of foreign lytic enzymes are two alternate techniques that may find usefulness in industry.

i. Autolysis:

Autolysis is one technique for removing chemicals from yeast. Controlling autolysis is challenging since the underlying mechanism is not well understood. It is challenging to categorize this method, which involves modest chemical or thermal shocks that cause yeast to produce lytic enzymes. According to Harvey, McNeil, Berry, and White (1998), autolysis is a normal mechanism that halts the growth of microbial cells and arises from nutrient deficiency. Cell lysis is the result of its activity of the enzyme. To solubilize the cell's constituent parts within the cell, cell degradative enzymes are triggered during this phase (Tanguler & Erten, 2008).

Harvey et al. (1998) noted that this process can be either naturally occurring or induced, depending on whether nutrient contents are reduced or depleted or high temperatures are used. The lytic enzymes vary depending on the microbial strain since Gram-positive and Gram-negative bacteria, yeasts, and microalgae have different cell wall compositions (Salazar & Asenjo, 2007; Sierra, Dixon, & Wilken, 2017). Numerous microorganisms are capable of autolysis, or self-digestion. This method is frequently used to prepare yeast extracts and necessitates temperature and pH manipulation, even though several substances may speed up the preparation process.

The growth kinetics indicate that this activity takes during the decline or death phase of microbial cell growth. As was previously discussed, facultative and anaerobic bacteria can undergo cell autolysis due to a lack of nutrients. For the autolysis of 27 g/L suspensions of

Kluyveromyces marxianus, incubation at 35 and 50^{O}C for 15 h was sufficient, releasing 75% of the cell contents. Although bacterial autolysis is seen in many different kinds of bacteria, it is rarely found in industrial processes. Cold-shock autolysis in *Bacillus subtilis* produces autolysins and 80–90% disruption after 1 hour. Kitano, Tuomanen and Tomasz (1986) stated that *Escherichia coli* also exhibits autolysis, which may be initiated by trichloroacetic acid or a cell wall synthesis inhibitor (such as cephaloridine), but still with many autolysins, in a little slower process (about 18 h). After reaching the stationary phase of growth, it is best to use particular detergents or solvents to enhance bacterial and yeast autolysis (Breddam & Beenfeldt, 1991).

ii. Phage Lysis or phage-mediated lysis

Bacteriophages are viruses that infect bacteria. Some examples of bacteriophages include T4-phage, OX174, and ssRNA phage. Phage lysis is the process by which the bacteriophages enter and grow within the target bacterium. This mechanism ultimately results in cell lysis via the production of the enzymes endolysin and murein hydrolase. This approach is exclusively applicable to bacterial cells and requires the presence of suitable bacteriophages.The particular type of cell wall or membrane necessitating breakdown determines the appropriate enzyme to use.

iii. Lytic Enzymes

Enzymes include cellulase, lysozyme, and proteases are utilized for cell lysis. These enzymes can selectively degrade the cell walls or membranes of targeted cells. Lytic enzyme-mediated lysis is often employed on a small scale due of concerns over enzyme availability and cost. According to Salazar and Asenjo (2007), lytic enzymes can be categorized into two, including bacteriolytic enzymes and yeast-lysing enzymes

a) Bacteriolytic Enzymes

In the biotechnology sector, bacterial enzymes are widely utilized to disintegrate cells. These enzymes have several important uses, including spheroplasting for cell transformation and extracting sensitive nucleic acids from bacteria. The antimicrobial properties of bacteriolytic enzymes constitute the basis for other uses. Many attempts to liberate recombinant proteins from bacteria using lytic enzymes have proved successful. For example, a temperature-sensitive lytic system efficiently recovered recombinant proteins from

Escherichia coli. During batch culture, lytic enzymes helped recombinant *Clostridium perfringens* produce hyaluronidase more quickly. On its alone, lysozyme can lyse gram-positive bacteria. However, it is usually necessary to pre-treat Gram-negative cells with a cation-chelating agent (like EDTA) or a detergent (like Triton X-100) in order to remove their outer membranes. Bacteriolytic enzymes typically include lysozymes, autolysins, and endolysins. The group of enzymes that degrade bacterial peptidoglycan is known as murein hydrolases. Despite being a common enzyme, autolysins from *Streptococcus pneumonia*, *Bacillus subtilis*, and *Staphylococcus aureus* exhibit exceptional characterizations (Smith, Blackman, & Foster, 2000). Autolysins usually have a modular structure with an N-terminal signal peptide and a second domain containing the active site.

Additionally, the N- or C-terminal of the catalytic domain of these proteins is flanked by repetitive motifs. Last but not least, endolysins (or lysins) are lytic enzymes that, despite being phage-encoded enzymes, are functionally linked to autolysins. At the end of the phage reproduction cycle, they break down bacterial peptidoglycan, enabling the release of the viral offspring from the cell. Fischetti (2005) emphasized that new studies have shed information on the modular organization and three-dimensional structures of these hydrolases in addition to the diversity of these enzymes. β-N-acetylglucosaminidase, N-acetylmuramidase, endopeptidase, and amidase are all classifications of lysins. Most endolysins do not infiltrate the cytoplasmic membrane to target the peptidoglycan; rather, the holin enzyme, generated by the same phage lytic system, compromises the membrane to grant the lytic enzyme access to the peptidoglycan.

Yeast-lysing enzymes

Salazar and Asenjo (2007) conducted research indicating that the yeast cell wall consists of mannoprotein and fibrous β (1→3) glucans essential for its structure, flexibility, and mechanical integrity. The lytic protease disrupts yeast cells by adhering to the external mannoprotein layer, liberating wall proteins and mannans, and revealing the underlying glucan surface. The glucanase degrades the inner wall, expelling the plasma membrane as a protoplast. Various bacteria can produce extracellular yeast-lysing enzymes, including Cellulosimicrobium cellulans, Cytophaga sp., and Rhizoctonia sp.

2.4 Production of β-galactosidase

The production of crude or whole cell β-galactosidase is predominantly achieved through submerged fermentation. The majority of the β-galactosidase enzyme is synthesized intracellularly. Extracellular β-galactosidase synthesis has also been evidenced (Saqib et al., 2017; Vasiljevic & Jelen, 2001). Fed-batch culture resulted in superior enzyme synthesis on a lactose-based medium in a bioreactor compared to simple batch cultivation. Alazzeh, Ibrahim, Song, Shahbazi and AbuGhazaleh (2009) examined the development of β-galactosidase in six strains of *Lactobacillus reuteri* utilizing diverse protein and carbohydrate sources. The optimal sources for β-galactosidase induction are lactose for protein and yeast extract for carbohydrates. Manera et al. (2012) identified peptone as the primary nitrogen source for yeast species isolated from curd, utilized to synthesize β-galactosidase.

Furthermore, they found that as the concentration of lactose increased, the level of β-galactosidase also rose linearly. The approach proposed by Deng, Xu, Ji and Agyei (2020) facilitated the standard synthesis of crude β-galactosidase from microorganisms. The methodology was as follows: The bacterial cells were collected using centrifugation at 4000× g, 4 °C for 10 minutes. The samples were rinsed twice in ice-cold 50 mM potassium phosphate buffer at pH 7.0. Acid-washed glass beads (150–212 microns, Sigma-Aldrich, St. Louis, MO, USA) were incorporated into the bacterial suspension at a 1:3 volume ratio, comprising one-third of beads and two-thirds cell suspension. The mixture was vigorously vortexed for 2 minutes and then incubated in an ice bath for 2 minutes, repeating this procedure for three cycles. The crude enzyme was obtained following centrifugation at 12,000× g for 10 minutes at 4 °C.

2.5 Toxicity of Chemicals to Microbial Cells

Chemical toxicity to microbial cells can substantially affect their development, metabolism, and viability. Examples of these compounds are solvents and surfactants. Oxidative stress, denaturation of proteins, and membrane damage are the mechanisms of chemical toxicity. A few of the factors that contribute to this toxicity are: a) Solvent Polarity: Because polar solvents integrate differently into the lipid bilayer than non-polar solvents b) Hydrophobicity: As hydrophobic solvents build up in lipid membranes, they often cause more significant damage and are more hazardous. c) Concentration: Solvents at higher concentrations usually exhibit more pronounced toxicity. d) Microbial Species: The degree to

which different microorganisms can withstand solvents varies. For instance, whereas certain bacteria are more sensitive to high ethanol concentrations, others can withstand them. The toxicity of ionic and non-ionic detergents on β-galactosidase activity has been described by Guven and Bashan (1998). Additionally, the inhibition of β-galactosidases in assessing chemical toxicity has been described by Nweke and Okpokwasili (2011a, 2011b).

2.5.1 Chemical interactions in toxicology

Chemical interactions in toxicology refer to how two or more compounds affect each other's toxicological effects when they are present together. When compared to the effect observed when each drug is present separately, these interactions may alter the overall toxicity. For accurate assessment and management of chemical mixtures, it is essential to comprehend these interactions. According to Berenbaum (1985), the way that substances like medications, carcinogens, physiological stimuli, and environmental pollutants interact with one another is essential to the way that they produce their effects. In practically every area of biology and medicine, these interactions are extremely important and practically relevant. When many agents are combined, their interactions may result in an impact that is greater or less than what would be predicted given the dose-effect relationships of the individual agents (Berenbaum, 1985).

2.5.1.1 Types of chemical interactions in toxicology

i. Additive effects: The sum of the effects of two or more drugs taken separately equals the effect of those substances taken together.

ii. Synergistic effects: The combined effects of two or more substances are stronger than the sum of their individual actions. Berenbaum (1981) defined synergism as the situation where a chemical combination has a greater effect than expected.

iii. Antagonistic effects: When two or more chemicals work together, their total effect is smaller than the sum of their separate effects. Additionally, the author stated that antagonism occurs when the combined response is lower than anticipated.

iv. Potentiation: A chemical increases the toxicity of another chemical that does not itself have a toxic impact.

v. Inhibition: A chemical lessens or prevents the harmful effects of another chemical.

2.5.2 Models for predicting the toxicity of chemical mixtures

Predicting the toxicity of chemical mixtures necessitates using diverse models to consider the potential interaction among the compounds. These models are specifically designed to offer a more precise estimation of the combined effects by considering the particular toxicities of each chemical. Numerous primary models exist for this objective; however, three will be elaborated here. They encompass:

i. Concentration Addition (CA) Model

The concentration Addition (CA) Model is a scenario in which the compounds in the mixture have comparable mechanisms of action for a particular reaction and target the same site of action. CA, also known as dose addition, assumes that each chemical in the mixture contributes to the toxicity through a common site of action. This means the impact can be achieved by replacing one molecule with an equally hazardous chemical amount (Altenburger et al., 2000). Concentration addition has received significant recognition and has been suggested as the overall answer for analyzing the toxicity of mixtures, thanks to its sound pharmacological foundation (Berenbaum, 1985). The mathematical expression for concentration addition is given by equation 2.1 (Berenbaum, 1985; Nweke, Umeh, & Ohale, 2018).

$$EC_{x(mix)} = \left(\sum_{i=1}^{n} \frac{\pi_i}{EC_{xi}} \right)^{-1} \qquad (2.1)$$

Nweke, Ike and Ibegbulem (2016) utilized the method of concentration addition to evaluate the toxicity of quaternary mixtures of phenolic compounds and formulated glyphosate on the microbial population of river water. In the context of a CA (Concentration-Effect Analysis), a data point falling on the experimental line indicates additivity. Conversely, if the data point falls above the line, it suggests antagonism, while falling below the line suggests synergism.

ii. Indepedent Action (IA) Model

The Independent Action (IA) Model, often called Response Addition or Bliss Independence, is an alternative name for this concept. IA refers to a scenario in which the

chemicals present in a mixture exhibit distinct modes of action and operate independently. The overall effect is determined by considering the likelihood of each chemical producing a response independently. The equation for IA is provided in the following expression:

$$E\left(C_{mix}\right)=1-\prod_{i=1}^{n}\left[1-E\left(c_i\right)\right] \qquad (2.2)$$

Where *E*(*cmix*) represents the total effect or response (scaled from 0 to 1) of an *n*-component mixture, *ci* is the concentration of the *i*th component and *E*(*ci*) is the effect or response of the individual component.

iii. The Toxic Unit(TU) and Toxic Index(TI) model:

The Toxic Units model is commonly employed in ecotoxicology to quantify the relationship between the concentration of a specific component in a mixture and its toxicological acute endpoint. The Toxic Index (TI) of each mixture was determined by adding the toxic units for all the components. Antagonistic and synergistic interactions are indicated by TI > 1 and TI < 1, respectively. When TI = 1, there is no interaction, only additivity (Boillot & Perrodin, 2008). The mathematical expression for the TI equation is shown here.

$$\mathrm{TI}=\sum_{i=1}^{n}\frac{C_i}{EC_{50i}}=\quad\sum_{i=1}^{n}\frac{\pi_i EC_{50mix}}{EC_{50i}} \qquad (2.3)$$

Where EC_{50i} is the concentration of the *i*th component that elicited 50% inhibition of β-galactosidase activity when tested as an individual, *n* is the number of components in the mixture, C_i is the concentration of the *i*th component in the mixture at the EC_{50} of the mixture (EC_{50mix}), and, and π_i is the proportion of *i*th component in the mixture(Nweke, Umeh, & Ohale, 2018).

2.6 Chemical Permeabilization of the Microbial Cells

According to Panesar, Panesar, Singh and Bera (2007), chemical permeabilization is a technology that makes the cell structure porous to pass tiny molecules, such as substrates or products. To maximize the permeability of the cell wall and cellular membranes of microorganisms, it is essential to select the most favorable circumstances for permeabilization, such as temperature, pH, and concentration of the permeabilizing agent. The circumstances often rely on the enzymes, which are used to measure the level of

permeabilization within the cell. The effectiveness of permeabilization can be assessed by the activity of intracellular β-galactosidase and other enzymes. Hence, a crucial aspect of permeabilization involves the inhibition of enzyme leakage, as this would greatly diminish the efficiency of biocatalysts (Flores, Voget, and Ertola (1994). The choice of the permeabilization method is highly dependent on the composition of cell walls and membranes and their structure. Microbial cells (bacteria, fungi, notably yeast, and microalgae) are typically permeabilized using chemical permeabilizers or solvents, detergents, and surfactants. This is due to the unique properties possessed by each of these microbes. For example, i) in bacteria, the bacterial cell wall has several interconnected polymers. The composition may vary depending on whether the bacteria under investigation is Gram-positive or Gram-negative. Gomes et al. (2020) claimed that Gram-positive bacteria have lower lipids than Gram-negative bacteria. Consequently, they exhibit reduced resistance to chemical solvents. Furthermore, Gram-positive bacteria have more significant quantities of peptidoglycans (PGN) and other substances like teichoic acid, teichuronic acids, polyphosphates, and carbohydrates. Bacteria may synthesise a diverse array of biomolecules, including antigens, enzymes (Kovacic, Babic, Krauss, & Jaeger, 2019), and therapeutic proteins (Fabbri et al., 2018). The bulk of these chemicals are intracellular, allowing for the application of permeabilization methods, such as organic solvents and detergents. Despite the drawbacks of chemical lysis involving solvents, alkalis, or detergents—such as the need for a continuous supply, interaction with released biomolecules, and potential equipment damage—microbial cell permeabilization has garnered interest due to its lower energy requirements and scalability. ii) yeast, the yeast cell wall is composed of mannoproteins (mannose residues linked by α(1, 6) bonds with short oligosaccharide side chains), fibrous β(1, 3) glucans interspersed with β(1, 6) glucan branches, and chitin (polymers of N-acetylglucosamine). These components protect the cell from abrupt changes in the osmotic potential of its external environment (Luo et al., 2015; Salazar & Asenjo, 2007). Anwar, Muhammad, Awais and Akhtar (2017) discovered that glucans provide mechanical strength, structure, and elasticity to the internal region of the cell wall. A layer envelops the glucan fibrils of the glycoprotein, whereas the outside surface of the cell wall features a mannan mesh that limits water permeability. Salazar and Asenjo (2007) asserted that the layers of the yeast cell wall are interconnected via non-covalent contacts and covalent cross-links, particularly between the

mannoprotein layers and the glucan-chitin layers. The cytoplasm of yeast is abundant in several biomolecules, such as proteins and polysaccharides (Costa-Silva, Flores-Santos, Freire, Vitolo, & Pessoa-Jr, 2018). Unlike the bacterial cell wall, the yeast cell wall exhibits more excellent resistance to disruption owing to its larger size and distinctive composition of cell wall components. This complicates disruption. Liu, Zeng, Sun and Han (2013) proclaimed that achieving this objective necessitates the application of cell disruption techniques to dismantle the glucan layer.

So many scientists have shown that permeabilizing microbial cells with detergents or surfactants and organic solvents releases β-galactosidase from the microbial cells. Some of the chemicals permeabilizers that have been used to do this, including toluene (Kumari et al., 2011), ethanol (Panesar, Panesar, Singh, & Bera, 2007; de Faria et al., 2013; Panesar, 2008), Arvidson Sodium lauroyl sarcosinate(Sarcosyl) (Kippert, 1995), chloroform-SDS (Sodium Dodecyl Sulfate)(Miller, 1972), lysozyme-EDTA treatment (Prasad et al., 2013), Cetyltrimethylammonium bromide (CTAB)(Kaur, Panesar, Bera, & Singh, 2009) DMSO (Dimethylsulfoxide), etc. Also, Kumari et al. (2011) have reported some of the mixture of the permeabilizing agents like mixture of ethanol (50%, v/v) and acetone (30%, v/v), ethanol (50%, v/v) and isopropanol (40%, v/v), ethanol (50%, v/v) and n-butanol (10%, v/v) that have been used to permeabilize the microbial cells to release the β-galactosidase. Cell permeabilisation can be increased depending on the incubation period, temperature, concentrations of cells and solvents. Lactose in milk or whey can be hydrolyzed economically by the permeabilization process.

Yeast cells were employed to generate lactose-hydrolyzed milk by permeabilization technology (Panesar, Panesar, Singh, Kennedy, & Kumar, 2006). Under optimal conditions, the ethanol-permeabilized yeast cells achieved remarkable hydrolysis of milk lactose. Some researchers have described the steps for permeabilizing microbial cells for those who have used entire cells as a source of β-galactosidase manufacturing of dairy products. As an illustration, in accordance with Ustok et al. (2010) permeabilizing procedure, 10 ml of the harvested fermentation broth was centrifuged at 2800 g for 15 min at 4^{O}C. The pellet was then rinsed with 10 ml of 0.05 M Na-phosphate buffer (pH 7). The pellet was resuspended in 4.5 ml of the same buffer after 15 more min of centrifugation at 2800 g, and then it was vigorously vortexed. This solution was mixed with 100 mg of lysozyme (Sigma L 6876) for enzymatic

lysis, and it was then incubated at 37^{O}C for 15 min. After this time, it received 0.5 ml of a 4 M NaCl solution and another 50 minutes of incubation at 37^{O}C—the final step involved centrifuging the solutions at 2800 g for 15 min. The supernatant was then utilized to conduct the enzyme and protein assay.

Additionally, this method was also applied by de Faria et al.(2013), who utilized 50ml Erlenmeyer flasks that contained 5ml of the reactive solution made up of ethanol, 22 mg (dry weight) of *K. lactis* cells, and 0.1 M potassium phosphate buffer (pH 7.0) to perform chemical permeabilization. Flasks were incubated at a temperature and for a while on an orbital shaker at 150 rpm. After centrifuging the supernatant for five minutes at 2,000 rpm, the cells underwent two washes with the same buffer. To study the enzyme activity of the permeabilized cells, the final pellet was resuspended in 1 ml of buffer. Also, Trawcznska (2020) highlighted that a solution of Triton X-100, with a concentration of no more than 1%, has been used to permeabilize *S. cerevisiae* cells. Triton X-100 facilitates the passage of substances, such as substrates and products, through cell membranes by reducing their permeability barrier.

2.7 Substrates for β-galactosidase Assay

Compounds derived from galactopyranosides (Galactopyranoside derivative compounds) are often used as substrates in hydrolysis by enzymes containing β-galactosidase activity. Spectroscopy is an excellent tool for determining how concentrated the products of a reaction are. The following are the substrates:

2.7.1 *o*-Nitrophenyl β-D-galactopyranoside (*O*NPG)

Permease and β-galactosidase are two enzymes that microbes need to be able to ferment lactose. Permease enables lactose to penetrate the bacterial cell wall, which β-galactosidase subsequently digests into glucose and galactose. The bacteria can then metabolize the glucose and galactose. Some organisms, meanwhile, do not have permease and show up as late or non-lactose-fermenters. It is thought that the *O*NPG test for lactose fermentation is susceptible. *O*-nitrophenyl-β-D-galactopyranoside (*O*NPG), also called 2-nitrophenyl-β-D-galactopyranoside or *O*rtho-Nitrophenyl-β-D-Galactopyranoside is an artificial and colourless substrate, included into this test and serves as the substrate for the β-galactosidase to determine the specific enzyme activity (Zanette, Mariano, Yukawa, Mendes, & Spier, 2019) which afterwards assists in the identification and differentiation of distinctive microorganisms. *O*-

nitrophenyl-β-D-galactopyranoside (*O*NPG) is a synthetic substrate structurally similar to lactose but differs because glucose is substituted with an o-nitrophenol group. It is a chemical analogue of the sugar lactose. The substrate, *O*-nitrophenyl-β-D-galactopyranoside (*O*NPG), can enter the bacterial cell without the aid of permease, unlike lactose. According to Kazemi, Khayati and Faezi-Ghasemi (2016), the *O*NPG test evaluates whether an organism contains the enzyme β-galactosidase. When measuring β-galactosidase activity using a colorimetric assay at 420 nm wavelength, *O*NPG is the preferred substrate (colorimetric and spectrophotometric substrate). This is due to its quick enzyme turnover rate, high stability in aqueous buffers, and relative ease of synthesizing or purchasing it from a vendor. The organism is obtained from a medium with a high lactose concentration and introduced into the *O*NPG broth as part of the broth testing technique. If the organism has β-galactosidase, the enzyme will split the β-galactoside link and release the yellow chemical called *o*-nitrophenol (*O*NP). This denotes a positive test. Spectroscopy makes it simple to determine the amount of o-nitrophenol derivative that the enzymatic process liberated. The cleavage of the β-anomeric C-O linkage between β-D-galactopyranose and aglycone, the hydrolysis reactivity of β-D-galactopyranosides to β-gal is entirely dependent upon the aglycone structure. In addition, though *O*NPG mimics lactose and is broken down by β-galactosidase, it cannot serve as an inducer for the lac operon. ONPG cannot be hydrolyzed without the help of another lactose analogue that can act as an inducer, such as isopropyl β-D-1-thiogalactopyranoside (IPTG).

2.7.2 *p*-Nitrophenyl-β-D-galactopyranoside (*p*NPG)

The *P*ara-Nitrophenyl- β -D-galactopyranoside (*p*NPG), sometimes known as 4-Nitrophenyl-β-D-galactopyranoside is also a chromogenic substrate for β-galactosidase. It is used to identify the presence of β-galactosidase by detecting lac Z activity. It performs the same task as o-Nitrophenyl- β-D-galactopyranoside when determining β galactosidase activity. The product liberated is p-nitrophenol.

2.7.3 Chlorophenol red-β-D-galactopyranoside (CPRG)

Lokur (2018) stated that CPRG is a chromogenic substrate utilized in the β-gal assay. Furthermore, the most often used substrate, o-nitrophenyl-β-D-galactopyranoside (*O*NPG), is ten times less sensitive than CPRG. Since CPRG is a galactose analogue, β-gal can catalyse the hydrolysis of this sugar. When CPRG is added to samples, β-gal transforms the yellow-orange CPRG substrate into the chlorophenol red, producing a dark red solution (Sicard et al.,

2014). The positive and negative test results (red and yellow) have a nice contrast that makes them simple to see on paper. Chlorophenol red-β-D-galactopyranoside (CPRG) is frequently used in colorimetric tests (Sicard et al., 2014).

2.7.4 4-methylumbelliferyl β-D-galactopyranoside (MUG)

This is a typical fluorogenic substrate. Assays using fluorogenic substrates are frequently employed to quantify the activity of the β-galactosidase enzyme. Fluorogenic methylumbelliferone (MU) substrates were assessed for the swift identification of faecal and total coliform bacteria (TC and FC) in potable water (Berg & Fiksdal, 1988). MUG is the most prevalent due to its swift turnover, despite several disadvantages concerning potential interference in the UV and blue regions, where the probe absorbs and emits light, respectively, and the requirement for an alkaline pH to optimize the fluorescence signal of the product (Gee et al., 1999).

2.7.5 X-Gal (5-Bromo-4-chloro-3-indolyl-β-galactopyranoside)

For the detection of β-galactosidase, this chromogenic substrate is the one that is utilized the most frequently (Kippert, 1995). According to the author, X-Gal is employed for in vivo detection of enzyme activity on plates since it readily enters the cells. X-Gal is, however, unsuitable for quantitative measurements(Rezaee, 2003). The substrate hydrolysis by the enzyme results in the formation of a precipitate with a dark blue color (the maximum absorbance wavelength is 615 nm). According to Kodaka, Ishikawa, Iwata, Kashitani, and Mizuochi (1995), X-Gal is mainly used for agarose-based techniques and histological analysis since it generates an insoluble result. This substrate was used to detect β-galactosidase since it can quickly enter the intact cell, unlike *O*NPG, which is used for quantitative analysis (Kippert, 1995; Rezaee, 2003).

2.7.6 Fluorescein mono-β-D–galactopyranoside (FMG)

The substrate for the fluorescence enzymatic assay was selected to be fluorescein mono-β-D-galactoside (FMG) as a result of the high fluorescence of its hydrolytic product (fluorescein) and its compatibility for hydrolysis by β-galactosidase (Huang, 1991). The author also mentioned that β-galactosidase hydrolyzes fluorescein mono-β-D-galactoside (FMG). FMG is a hydrolyzing intermediary for fluorescein di-β-D-galactoside (FDG). FMG has a relatively high absorption and is innately fluorescent at the wavelength required for detecting its hydrolytic product, fluorescein, despite being a suitable substrate for the enzyme.

2.8 β-galactosidase Assay in Microbial cells.

An assay for β-galactosidase in microbial cells is a method used in laboratories to quantify the amount of the enzyme β-galactosidase. According to Xavier et al. (2018), the main idea behind the β-galactosidase test is that the enzyme catalyzes the breakdown of β-galactosides, like lactose, into glucose and galactose. In the tests, a β-galactoside-containing substrate is introduced to a sample, and the formation of a product, typically a colored or fluorescent compound—is measured to indicate the enzyme's activity. The hydrolysis of *o*-nitrophenyl-β-D-galactoside (*o*NPG), a chromogenic substrate, can be used to measure the activity of β-galactosidase (Anisha, 2016). This assay is widely used in microbiology and molecular biology research for many purposes, such as detecting the stimulation of the lac operon in bacteria and analyzing gene expression.

2.8.1 Overview of β-galactosidase assay in microbial cells.

1. **Cell Preparation:** The target cells, such as yeast and bacteria, specifically *Escherichia coli* (*E. coli*) with the lacZ gene encoding β-galactosidase, are cultivated in a culture medium.
2. **Cell Lysis:** The cells are harvested and subsequently lysed to liberate their intracellular contents. These objectives can be accomplished via many techniques, including mechanical disruption, sonication, or chemical lysis.
3. **Assay Reagents:** The assay employs essential reagents, including:

a) Substrate: The often employed substrate is *o*-nitrophenyl-β-D-galactopyranoside (ONPG). This colorless molecule is enzymatically hydrolyzed by β-galactosidase, forming a yellow-colored product known as *o*-nitrophenol. The reaction is dependent upon the pH level.

b) Buffer: The appropriate pH is maintained for the enzymatic reaction by using a suitable buffer.

c) Stop Solution: A stop solution, commonly sodium hydroxide or carbonate, is added to bring the process to an end. This stabilizes the reaction products and halts the colour development.

d) Protease Inhibitors: A protease is applied to stop protein breakdown during the experiment.

3. **Enzyme Reaction:** The *O*NPG substrate and buffer are combined with the cell lysate or pure β-galactosidase enzyme at the proper pH and temperature. *O*NPG is hydrolyzed by β-galactosidase to produce galactose and o-nitrophenol.

4. Measurement: Using a spectrophotometer, the change in absorbance at a particular wavelength (often 420 nm) is tracked over time. The yellow colour develops during the reaction, and its intensity increases with increasing β-galactosidase activity.

5. Data analysis: The β-galactosidase activity is determined by measuring the absorbance change per unit of time or the rate of colour change. This activity can be expressed in several ways, including Miller Units, which normalize the activity in relation to protein concentration, cell density, or the amount of o-nitrophenol released throughout the experiment.

2.8.2 Types of β-galactosidase assay

β-galactosidase assays come in various forms, each intended for specific uses and investigative requirements. The intricacy, sensitivity, and information yielded by these assays differ. The following are a few typical types of β-galactosidase assays:

2.8.2.1 Colorimetric assay

The most popular and most straightforward kind of β-galactosidase assay is the colorimetric assay. To determine the presence of an enzyme, they rely on a colour change. The enzyme cleaves a colourless substrate (like *O*NPG) in this kind of assay to yield a coloured product (o-nitrophenol). A spectrophotometer may measure the colour change, and the rate at which colour develops is directly correlated with the enzyme activity. Basic assessments of enzyme activity frequently employ this test. Some examples of colorimetric assays include:

a) **The *O*NPG (*O*-Nitrophenyl-β-D-Galactopyranoside) assay**

This is a widely used technique for measuring the β-galactosidase enzyme activity (Miller, 1972). This assay is incredibly well-liked in molecular biology and microbiology labs for measuring the activity of β-galactosidase in microbial cells. The *O*NPG assay is predicated on β-galactosidase hydrolyzing *O*NPG to create galactose and o-nitrophenol. At 420 nm wavelength, the yellow o-nitrophenol product can be identified spectrophotometrically. The amount of β-galactosidase activity in the sample is directly correlated with the rate of o-nitrophenol formation. The following are the steps involved in the process: β-galactosidase is usually found in bacterial cultures, cell lysates, or

purified enzyme samples. To create a stock solution, dissolve *O*NPG in a buffer (Z-buffer is used frequently). Usually, the assay mixture's final *O*NPG concentration falls between 0.5 and 2 mM. After mixing the sample and *O*NPG solution, incubate the mixture for a predetermined time at a suitable temperature (often 30°C). Depending on the predicted β-galactosidase activity, the reaction time can change. To halt the reaction and increase pH, a strong base (such as sodium carbonate), which stabilizes the yellow o-nitrophenol result, is added. Lastly, a spectrophotometer was used to measure the absorbance of the reaction mixture at 420 nm—the amount of β-galactosidase activity in the sample increases with increased absorbance. Miller units, or the amount of o-nitrophenol released by the assay, are commonly used to express the β-galactosidase activity. Miller's units are computed using the formula: Miller Units = (A420 x 1,000) / (T x V x A600). V is the volume of culture used in the assay in millilitres; T is the reaction time in minutes; A600 is the optical density of the culture at 600 nm, representing the cell density; and A_{420} is the absorbance at 420 nm.

b) **X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) assay**

This is frequently employed in an assay for colorimetric β-galactosidase. β-galactosidase breaks down the chromogenic substrate, X-gal, to yield 5,5'-dibromo-4,4'-dichloro-indigo, a blue precipitate. The blue colour is very observable, rendering it a practical and extensively employed marker of β-galactosidase activity in a colorimetric experiment.
In molecular biology and microbiology, the X-gal assay is commonly employed to identify and visualise the expression of the lacZ gene, which encodes β-galactosidase, in bacterial colonies or cells. The enzymatic hydrolysis of X-Gal by β-galactosidase forms the basis of the X-Gal β-galactosidase assay. β-galactosidase breaks down X-Gal to form 5,5'-dibromo-4,4'-dichloro-indigo, a blue-colored substance. The β-galactosidase activity in the sample is directly correlated with the intensity of the blue colour. Rezaee (2003) devised a straightforward approach for detecting lacZ activity in yeast using X-Gal as the substrate. It was discovered that in yeast cell suspensions at pH 7.0, cell permeabilization with SDS/chloroform increases the X-Gal staining of ß-galactosidase. Before testing β-galactosidase with ONPG, cells were permeabilized using SDS/chloroform (Miller, 1972). However, X-Gal provides a quick and accurate test for ß-galactosidase in yeast cells.

2.8.2.2 Chemiluminescent β-galactosidase assay

Chemiluminescent β-galactosidase assays are biochemical assays that use chemiluminescent substrates to measure the β-galactosidase enzyme activity. Chemiluminescent β-galactosidase assay is a susceptible and frequently utilized method for measuring and identifying enzyme activity. They are commonly employed in both fundamental research and practical applications. The assay principle utilizes chemiluminescent substrates such as Galacton-Star and Emerald-Light, which emit light when enzymatically cleaved by beta-galactosidase. The measurement of this light emission is conducted using a luminometer. The strength of the chemiluminescent signal is directly proportional to the level of β-galactosidase activity in the sample. This enables the precise quantification of enzyme activity, which is vital in many applications such as gene expression investigations or high-throughput screening assays.

2.8.2.3 Fluorometric β-galactosidase assay

The fluorogenic substrate β-galactosidase assay is another name for the fluorometric β-galactosidase assay. Assays using fluorogenic substrates are frequently employed to quantify the activity of the β-galactosidase enzyme. These assays use a fluorogenic substrate that produces a fluorescent product when β-galactosidase hydrolyzes it. β-galactosidase activity can be measured sensitively and in real-time by utilizing a fluorometer or a fluorescence microplate reader to quantify the fluorescence. A fluorometer is used to measure the intensity of fluorescence. Compared to many β-galactosidase assays, fluorometric assays have the advantages of higher sensitivity and lower background (Huang, 1991). The idea behind β-galactosidase fluorogenic substrate assays is that the enzyme hydrolyzes a non-fluorescent substrate to provide a fluorescent result. When hydrolyzed, the fluorophore in the substrate of β-galactosidase becomes extremely luminous, but it is quenched in its non-hydrolyzed condition. The amount of β-galactosidase activity in the sample directly correlates with the rate of fluorescence production.

The assay involves several steps: first, the sample containing β-galactosidase is usually prepared as a pure enzyme, bacterial culture, or cell lysate. To prepare a working solution, the fluorogenic substrate was dissolved in a suitable buffer. The assay methodology or the needs of the experiment usually dictate the final concentration of the substrate in the assay mixture. After the sample was mixed with the fluorogenic substrate solution, the mixture was incubated at a suitable temperature (often 30°C) for a predetermined time. Depending on the

expected β-galactosidase activity, the incubation period may change. The reaction mixture fluorescence intensity was determined using a fluorometer or a fluorescence microplate reader. The fluorophore's unique emission and excitation wavelengths were utilized for detection. The relative fluorescence units (RFU) or arbitrary fluorescence units (AFU) are commonly used to express the β-galactosidase activity. These units can be calculated using standard fluorescence calibration curves with known fluorescent product concentrations. For fluorogenic substrates that exhibit notable inherent absorbance and fluorescence under the assay circumstances, a unique enzymatic assay method was created (Huang, 1991). Besides, a novel fluorimetric assay for β-galactosidase (β-gal) and faecal coliform bacteria has been reported by Sicard et al. (2014). It uses the long-wavelength dye chlorophenol red-β-Dgalactopyranoside (CPRG), commonly used in colorimetric assays. The unexpected formation of a significant fluorescent response from freed chlorophenol red (CPR) upon complexation with poly-L-arginine (pR) in solution is another novel characteristic of this new assay, according to the authors.

2.8.2.4 A single-step β-galactosidase assay

A single-step β-galactosidase assay, also known as a one-step β-galactosidase assay, is a simplified approach employed to identify and quantify the activity of the enzyme β-galactosidase. This approach is usually used to measure β-galactosidase activity swiftly, typically without requiring several reagent additions or extended incubation periods. In light of the challenges and discrepancies inherent in the conventional approach to quantifying LacZ activity, along with the utilization of hazardous agents for lysis (e.g., chloroform, toluene), an alternative methodology termed the single-step β-galactosidase assay has been established (Schaefer et al., 2016). This technique employed an alternative permeabilization method proposed through the application of a pop culture reagent, a chemical employed in protein purification (Thibodeau et al., 2004). The authors asserted that the pop culture reagent can penetrate the cell wall without harming the soluble proteins or influencing the optical density measurements. The β-galactosidase enzyme maintains stability for up to 18 hours. This alternate permeabilization approach addressed a limitation in accelerating the test, specifically the cell permeabilization process required for the *O*NPG substrate to penetrate the cell and engage with β-galactosidase. The transfer of cultures is crucial as permeabilization is often

achieved with chloroform/sodium dodecyl sulphate (SDS) or toluene (Miller, 1972), which may interfere with optical density measurements on standard microtiter plates.

2.8.3 Some existing methods for β-galactosidase activity assay in Microbial cells

In 1972, J. H. Miller introduced the Z-buffer, which contains chloroform and SDS to disrupt bacterial membranes, and used an aliquot of lysed cells to spectrophotometrically assess β-galactosidase activity through the formation of the chromophore o-nitrophenol (ONP) from the colourless substrate *o*-nitrophenyl-β-D-galactoside (*O*NPG) (Miller, 1972). This traditional method for measuring LacZ activity encounters challenges when applied to a large number of samples, is prone to variability, requires the use of toluene or a combination of chloroform and sodium dodecyl sulphate (SDS) for bacterial permeabilization, and is labour-intensive. Unfortunately, chloroform and toluene react with polystyrene, the principal material utilized in the manufacture of 96-well plates, leading to a deterioration of its optical clarity.

Numerous techniques for β-galactosidase assessment in bacteria have been recorded, with modifications to specific phases of the Miller method to accommodate plate readers (Arvidson, Youderian, Schneider, & Stormo, 1991; Griffith, & Wolf, 2002; Schneider, 1992; Thibodeau et al., 2004). Griffith and Wolf (2002) posited that these methodologies feature blocks are made of nonreactive polypropylene. Griffith and Wolf have presented a detailed strategy for doing single endpoint (non-kinetic) Miller experiments in a 96-well format using a high-throughput procedure. The specific activity values of β-galactosidase obtained are identical to those derived from the conventional single-tube approach of Miller. Consequently, results derived from this approach can be articulated in Miller units and readily compared to those documented in the literature. The 96-well format for enzyme-specific activity permeabilization and testing, along with 12-channel and repeater pipettors, facilitates the efficient processing of hundreds of samples within an 8-hour workday. To augment the quantity of samples amenable to facile assay, researchers have modified the Miller method to a 96-well format employing microplate readers, which includes an integrated system featuring a robotic pipetting apparatus and a microplate reader. This optimized process for quantifying β-galactosidase activity from many cultures offers several improvements over the conventional method outlined by Miller. A significant benefit of the abovementioned method is that an individual may process hundreds of samples (assayed twice and in triplicate) within

an 8-hour workday. Secondly, bacterial proliferation and cell permeabilization occur in compact 2.2-ml polypropylene block arrays, thus preventing the necessity for numerous culture tubes and the transition between culture tubes and microplates; furthermore, the doubling times achieved with the polypropylene blocks are comparable to those obtained with conventional glass culture tubes. The permeabilization achieved through aspiration in block arrays is as thorough as that obtained by the traditional approach of vortexing in culture tubes.

Consequently, the specific activity of β-galactosidase can be quantified in Miller units. Fourth, manipulations are facilitated by the utilization of multi-channel and repeater pipettors. The plate reader enables prompt and precise absorbance measurement. We have encountered difficulties using volatile organic solvents with multichannel pipets, as they quickly leak from the pipet tips, likely due to their low surface tension, and necessitate operation within chemical fume hoods.

Alternative approaches for cell disruption have been devised to leverage the high-throughput capabilities of microplate assays without the need for robotic assistance. Previous studies have suggested using infectious, high-titer bacteriophage for bacterial lysis (Arvidson et al., 1991; Bianco & Weinstock, 1994). However, this technique was undesirable since the purpose was to avert the possible introduction of phage contamination in the laboratory environment. Furthermore, Schupp, Travis, Price, and Shand (1995) delineated a method for the permeabilization of gram-negative bacterial cells employing the cationic cyclic polypeptide antibiotic, Polymyxin B in combination with Triton X-100. While permeabilization by this method is equally efficacious as that achieved with chloroform/SDS treatment, but polymyxin B addition was inappropriate.

Zhang and Bremer (1995) employed a mixture of the detergents cetyltrimethylammonium bromide (CTAB) and sodium deoxycholate to lyse *E. coli* cells while preserving LacZ function. The results were not compared to those obtained by lysis using chloroform. Toulouse, Häse and Steuber (2017) delineated a protocol for high-throughput β-galactosidase (LacZ) activity assessments in *Vibrio cholerae*, using detergent lysis (cetyltrimethylammonium bromide (CTAB) and sodium deoxycholate), which resulted in enhanced specific activities relative to lysates derived from the chloroform and SDS-inclusive "Z-buffer" proposed by Miller (1972). Thibodeau et al. (2004) suggested that the effort might be significantly diminished by modifying the Miller method for a 96-well plate format and

employing a kinetic enzyme assay rather than a single endpoint experiment. The authors have presented a description and validation of novel strategies for augmenting the proliferation of bacterial cells in 96-well plates. They have investigated the application of aqueous detergent solutions to enhance the release of β-galactosidase enzyme from cells.

Furthermore, they have employed kinetic assay data to juxtapose the acquired values with those derived from the traditional Miller method. The innovative high-throughput kinetic methodology we have established is distinguished by its rapidity, lessened labour demands relative to the original Miller method, and its capacity to support high-throughput applications requiring a substantial volume of β-galactosidase assays from bacterial cells. The authors propose an alternative method that employs a detergent-based solution to promote bacterial lysis and release β-galactosidase activity from cellular structures. Cells can be lysed directly in the culture medium utilizing aqueous solutions that eliminate the need for centrifugation. These solutions are compatible with the polystyrene used in most 96-well plates and may be effortlessly manipulated using conventional multichannel pipettes. They can be acquired from multiple sources, like PopCulture™ or Novagen, in Madison, WI, USA. The cells treated with PopCulture/lysozyme demonstrated similar or slightly increased levels of β-galactosidase activity relative to cells treated with chloroform/SDS.

Moreover, it is essential to highlight that β-galactosidase demonstrates exceptional stability in lysates produced using Popculture/lysozyme, as its enzymatic activity remains unchanged after an 18-hour incubation at room temperature. The lysis procedure in our research did not make excessive bubbles, a phenomenon noted in alternative detergent-based techniques (Griffith & Wolf, 2002; Miller, 1972). Nonetheless, employing a detergent-based solution (PopCulture reagent and lysozyme) to lyse bacteria and thereby release β-galactosidase activity from cells is expensive. Multiple factors of the Miller test procedure, including permeabilization duration, reaction time, and cell suspension concentration, significantly influenced the outcomes, and it could not monitor the β-galactosidase response in real-time. The permeabilization duration of cells was refined for a scanning technique using the Miller method to assess intracellular β-galactosidase activity in *E. coli* Tuner (DE3) expressing β-galactosidase in real-time (Li et al., 2012). Rather than applying sonic disruption to prepare cell extracts, the bacterial cells employed in the Miller method and its derivative colorimetric methods are subjected to surfactants and/or organic solvents, such as chloroform

and SDS, which partially compromise the cell membrane, facilitating the diffusion of small molecules like *o*NPG into the cell.

2.9 Applications of β-galactosidase

Applications of β-galactosidase are often divided into conventional and non-conventional categories. The former refers to those based on their hydrolytic activity (hydrolysis), which have been utilized for a long time in large-scale industrial processes. In contrast, the latter refers to those newer applications based on their transgalactosylation activity (Vera et al., 2020). Additionally, the creation of low-lactose and lactose-free milk and dairy products, as well as lactase tablets for those who are lactose intolerant, are the principal uses of β-galactosidase for lactose hydrolysis. The use of very high substrate concentrations, organic solvents, ionic liquids, or any other low media are examples of unconventional applications that employ a method that promotes transgalactosylation while suppressing hydrolysis. The use of non-aqueous media should be considered, though, as it may affect the stability and activity of the enzymes, significantly lower lactose solubility, require solvent separation from the product and most likely result in solvent recovery. Additionally, β-galactosidases derived from *Aspergillus oryzae* and *Bacillus circulans* demonstrated significant transgalactosylation activity, whereas the enzyme from *Kluyveromyces* displayed pronounced hydrolytic activity but comparatively diminished transgalactosylation activity (Guerrero et al., 2015). Interestingly, these two applications are grouped in some of the following applications below:

2.9.1 Biotechnological applications

According to Silanikove et al. (2015), lactose is a substance found in milk and milk products. Additionally, they stated that one of the roles of lactose is to promote the development of good bacteria, particularly *Bifidobacteria,* that live in the small intestine. The lactase enzyme must be active for lactose absorption, as excessive undigested lactose leads to flatulence, diarrhoea, and cramps. As stated by Sitanggang, Drews, and Kraume (2016), a significant segment of the global adult population experiences a deficiency in the lactase enzyme, leading to a condition termed lactose intolerance. Due to the absorbability of hydrolysed molecules in lactose maldigestion, enzymatic hydrolysis of lactose is a crucial biotechnological method (Nivetha & Mohanasrinivasan, 2017). The four principal types are primary, secondary, congenital, and developmental lactase deficiency. The most widespread type of lactase deficiency is primary lactase deficiency, also known as lactase non-persistence,

adult-type hypolactasia, or hereditary lactase deficiency. Lactase production diminishes with time in those afflicted with this condition. This decline typically commences at approximately two years of age. Nonetheless, it may commence at a later time. In cases of lactase deficiency, symptoms of lactose intolerance may not manifest until late adolescence or adulthood (about 20 years of age).

It occurs due to decreased β-galactosidase synthesis at the small intestine boundaries. Again, in the case of Secondary lactase deficiency, a small intestine injury leads to its insufficiency. An infection may harm the small intestine, a disease (such as coeliac disease, gastroenteritis, inflammatory bowel disease, diarrhoea, or cancer), or another issue like starvation, the lactose tolerance increases typically when the underlying reason is treated. At any age, it can happen. In the rare condition known as congenital lactase deficiency or alactasia, the small intestine produces little to no lactase enzyme from birth. This condition is brought on by parental gene inheritance (Saqib et al., 2017). The four lactase deficiency conditions are ranked in severity by this one. In premature babies, developmental lactase deficiency can happen. After birth, this condition often only lasts a short while.

The taste of lactose-hydrolyzed milk is enhanced by immobilizing β-galactosidase in liposomes. Cold-active β-galactosidase is utilized to produce lactose-free dairy products in the food industry. Following the lactose breakdown by β-galactosidase, the natural whey waste can be used as a cheap, readily available substrate for the culture of bacterial cells. Whey proteins are degraded and recovered by ultrafiltration to produce a variety of beneficial pharmaceutical compounds.

2.9.2 Food technology application

In dairy products such as frozen milk, ice cream, condensed milk, and whey spreads, the high quantity of lactose that is present combined with the low solubility of lactose induces lactose crystallization. This results in a gritty and filthy texture in the product. The application of β-galactosidase in the food industry can reduce lactose levels in dairy products. While simultaneously improving the creaminess, softness, digestibility, and sweetness of the products. This results in an overall improvement in the quality or value of the products (Neri et al., 2008). The β-galactosidase enzyme, which is found in whey and responsible for hydrolyzing lactose, can produce sweet syrup. The sugar in the sweet syrup can be used in

molasses, baking, ice cream, dairy desserts, soft drinks, sweets, and confectionery. It can also be used as a source of sugar in soft drinks.

2.9.3. Health application

Enzymatic hydrolysis of lactose can also be used to produce Galacto-oligosaccharides (GOS), which is another application of this process. These are indigestible substances that function as dietary fiber. They encourage the growth of *Bifidobacteria* in the intestine, which are necessary for the proper functioning of both the intestine and the liver.

Saqib et al. (2017) stated that GOS is produced from β-galactosidase through the transglycosylation activity occurring during lactose breakdown. This is crucial for the preservation of human health. GOS is a prebiotic component in food. The concentrations of oligosaccharides in GOS can vary from 1% to 45%, with specific values dependent on the total saccharide content and the enzyme source utilized. GOS are nondigestible prebiotics that can enhance human health by altering intestinal microbiota. They also promote the proliferation of advantageous intestinal bacteria, including species of *Lactobacillus* and *Bifidobacterium*. Due to the acidic conditions, GOS can be stored for an extended duration at room temperature and used across a diverse range of products without degradation. This is feasible due to the acidic environment. Due to lactose intolerance, about 75% of the global adult population cannot consume milk or dairy products.

Some typical tests used to diagnose lactose intolerance include the lactose intolerance test, the breath test and endoscopy. The lactose intolerance test measures blood sugar levels before and after a drink containing lactose. Suppose the blood sugar level rises above a crucial level. In that case, if the person is not lactose intolerant after consuming a beverage high in lactose, a breath test is conducted to check for the presence of H_2 gas, which is only produced during the fermentation of lactose (Kerber et al., 2007). The third test is an endoscopy, which entails passing a tube into the small intestine of the patient through the stomach. The tube allows medical professionals to examine the stomach lining and collect a tissue sample for biopsy. In milk and other dairy products, lactose hydrolysis is accomplished through an enzymatic or acidic process. Milder pH and temperature conditions are made possible by the application of enzymes. The acidic approach, according to Sinha, Radha, Prakash, and Kaul (2007), has several drawbacks, including protein denaturation, the formation of a brown-colored result, and the yield of unwanted by-products such as poisonous compounds like lysino-alanine.

Also, if the lactose that is present in the products is digested by an enzyme, β-galactosidase, into readily utilizable carbohydrates like glucose and galactose, this problem can be solved (Nivetha & Mohanasrinivasan, 2017).

CHAPTER THREE
MATERIALS AND METHODS

3.1 Materials

3.1.1 Reagents

The reagents were analytical grade chemicals purchased from Sigma-Aldrich St. Louis Mo USA. The reagents include solvents (chloroform, dimethylsulfoxide (DMSO), N-N- dimethyl formamide (DMF), ethanol, 1-butanol and 1-pentanol), surfactants (Cetyltrimethylammonuim bromide (CTAB), Triton X-100, sodium Deoxycholate(SDC), sodium dodecyl sulfate (SDS), sodium lauryl sarcosinate (Sarcosyl), 1-cetylpyridinium chloride monohydrate (CPC), EDTA (Chelator), Sodium carbonate, *o*-Nitrophenyl-β-D-galactopyranoside (*o*NPG), *o*-Nitrophenol, mercaptoethanol.

3.1.2 Sample collection

The *Nunu* (locally fermented cow milk) sample was bought from a local vendor at the Ama-Hausa market in Owerri, Imo state. Also, a stool sample was collected aseptically using a sterile swap stick.

3.2 Methods

3.2.1 Isolation of test organisms

The *E. coli* was isolated from the stool sample using Eosin Methylene Blue (EMB) agar and stocked on Nutrient agar slant. Also, the yeast cells were isolated from the *nunu* sample using SDA and enriched in Sabouraud Dextrose broth supplemented with 200mg/l streptomycin for 48 hours at room temperature. After that, yeast colonies were purified on SDA using the streak plate method. Pure colonies were stocked in SDA agar slant.

3.2.2 Screen test for β-Galactosidase Production

The yeast and bacterial isolates were cultivated for 48 h at 30°C in a culture medium containing (g/l): casamino acid, 5.0; peptone, 5.0; yeast extract, 3.0; lactose, 5; Ammonium sulphate, 2.0; KH_2PO_4, 1.0, as modified from Panesar et al. (2007). After incubation, 1 ml of culture was transferred into a test tube. Cells were permeabilized by adding 0.1 ml of 0.1% SDS solution for 10 min. After that, 0.2 ml of 0.2% ONPG was added to the cell suspension and incubated at 30°C for 30 min. Yellow color production indicates a positive test.

3.2.3 Morphological and biochemical characterization of the isolates

The method of Barrow and Feltham (2003) was used to carry out the morphological and biochemical characterizations of the bacterial cell. Some of the tests done are as follows: colonial morphology, Gram reaction test, motility test, Oxygen utilization test, catalase test, oxidase, urease test, methyl red test, Voges Proskauer (VP), Citrate utilization, Urease, Hydrogen sulfide (H_2S), glucose test, Lactose Fermentation test, growth and color on Media (MacConkey agar, Eosin Methylene Blue (EMB), chromogenic Urinary Tract Infection (UTI) agar (Sisco Research Laboratories PVT. LTD., Mumbai, India), chromogenic Salmonella agar) (Sisco Research Laboratories PVT. LTD., Mumbai, India). Also, the morphological and biochemical characterizations of yeast cell were done. Some tests performed on the yeast cell were morphology test, fermentation and assimilation tests of the following sugars (glucose, galactose, sucrose, raffinose and lactose) and assimilation of cellobiose test.

3.2.4 Molecular identification of the yeast isolate

The method of Horsfall, Stanley and Ogugbue (2024)**,** with slight modification, was adapted to perform the molecular identification of the yeast isolate.

3.2.4.1 Extraction of DNA

Extraction was done using a Zymo Research (ZR) fungal/bacterial DNA mini-prep extraction kit supplied by Inqaba, South Africa. Heavy growth of the pure culture of the suspected isolate was suspended in 200 microliters of isotonic buffer in a ZR Bashing Bead lysis tubes, and 750 microliters of lysis solution were added to the tube. The tubes were secured in a bead beater fitted with a 2ml tube holder assembly and processed at maximum speed for 5 min. The ZR bashing bead lysis tubes were centrifuged at 10,000x*g* for 1 min.

Four hundred (400) microliters of supernatant were transferred to a Zymo-Spin IV spin Filter (orange top) in a collection tube and centrifuged at 7000 x*g* for 1 minute. One thousand two hundred (1200) microliters of fungal/bacterial DNA binding buffer were added to the filtrate in the collection tubes, bringing the final volume to 1600 microliters. Eight hundred (800) microliter was then transferred to a Zymo-Spin IIC column in a collection tube and centrifuged at 10,000x*g* for 1 min; the flow through was discarded from the collection tube. The remaining volume was transferred to the same Zymo-spin and spun. Two hundred (200) microliter of the DNA Pre-Wash buffer was added to the Zymo-spin IIC in a new collection

tube and spun at 10,000x*g* for 1 min, followed by the addition of 500 microliter of fungal/bacterial DNA Wash Buffer and centrifuged at 10,000x*g* for 1 minute.

The Zymo-Spin IIC column was transferred to a clean 1.5 microliter centrifuge tube, and 100 microliter of DNA elution buffer was added to the column matrix and centrifuged at 10,000x*g* microliter for 30 secs to elute the DNA. The ultra-pure DNA was then stored at -20 degrees for other downstream reaction.

3.2.4.2 DNA quantification

The extracted genomic DNA was quantified using the Nanodrop 1000 spectrophotometer. The equipment software was launched by double-clicking on the Nanodrop icon. The equipment was initialized with 2 µl of sterile distilled water and blanked using normal saline. Two microlitres of the extracted DNA were loaded onto the lower pedestal; the upper pedestal was brought down to contact with the extracted DNA on the lower pedestal. The DNA concentration was measured by clicking on the "measure" button.

3.2.4.3 The Internal Transcribed Spacer (ITS) Region Amplification

The ITS region of the isolate was amplified using the ITS1F: 5'-CTTGGTCATTTAGAGGAAGTAA-3' and ITS4: 5'- TCCTCCGCTTATTGATATGC-3, primers on a ABI 9700 Applied Biosystems thermal cycler at a final volume of 30 microlitres for 35 cycles. The PCR mix included the X2 Dream Taq Master mix supplied by Inqaba, South Africa (Taq polymerase, DNTPs, $MgCl_2$), the primers at a concentration of 0.4µM and the extracted DNA as template. The PCR conditions were as follows: Initial denaturation, 95ºC for 5 minutes; denaturation, 95ºC for 30 secs; annealing, 53ºC for 30 seconds; extension, 72ºC for 30 secs for 35 cycles and final extension, 72ºC for 5 min.

3.2.4.4 PCR Product Analysis using agarose gel electrophoresis

The PCR product was analyzed by running an agarose gel electrophoresis. The product was resolved on a 1% agarose gel at 120V for 15 min. The amplified DNA bands were visualized on a blue light transilluminator after staining the gel with a DNA-specific dye. The size of the PCR product bands was compared with a DNA ladder.

3.2.4.5 DNA Sequencing and DNA Sequencing Analysis

Sequencing of PCR-amplified DNA was done using the BigDye Terminator kit on a 3510 ABI sequencer by Inqaba Biotechnological, Pretoria, South Africa. The sequencing was done at a final volume of 10µl. The components included 0.25 µl BigDye® terminator v1.1/v3.1,

2.25µl of 5 x BigDye sequencing buffer, 10µM Primer PCR primer, and 2-10ng PCR template per 100bp. The sequencing conditions were as follows: 32 cycles of 96°C for 10s, 55°C for 5s and 60°C for 4min. Obtained sequences were edited using the bioinformatics algorithm Trace edit. Similar sequences were downloaded from the National Center for Biotechnology Information (NCBI) database using the Basic Local Alignment Search Tool for Nucleotides (BLASTN).

3.2.4.6 Phylogenetic analysis

These DNA sequences were aligned using ClustalX. The evolutionary history was inferred using the Neighbor-Joining method in MEGA 6 (Saitou & Nei, 1987; Tamura, Stecher, Peterson, Filipski, & Kumar, 2013). The bootstrap consensus tree inferred from 500 replicates(Felsenstein, 1985) was taken to represent the evolutionary history of the taxa analyzed. The evolutionary distances were computed using the Jukes-Cantor method(Jukes & Cantor, 1969).

3.2.5 Preparation of 2-nitrophenol stock solutions

Stock solution (600mg/l) of 2-nitrophenol (2-NP) was prepared by dissolving 0.03g of 2-NP in 50ml of solution. Then, the stock solution was diluted in 1 in 10 to obtain 60mg/l 2-NP working stock solution.

3.2.6 Determination of wavelength of maximum absorption (λmax) by 2-nitrophenol

Three millilitres (3ml) of 10mg/l aqueous solution of 2-NP was prepared from 60mg/l working solution. The solution contained 0.6ml of Z-buffer, 1.0ml of 1M Na_2CO_3 solutions, 0.5ml of the 60mg/l 2-NP solution, and 0.9ml of distilled water. The absorbance of the solution was measured over a range of wavelengths (340-520nm) in a UV-visible spectrophotometer. Finally, the wavelength of maximum absorption was determined**.**

3.2.7 The preparation of calibration curve for 2-nitrophenol determination

Graded concentrations of 2-NP solution (2-28mg/l) in buffer were prepared in 3ml final volume using the protocol in Table 3.1 below. After putting all the components according to the protocol in the test tubes, the content of each tube was mixed. The absorbances at 420nm were measured. The data obtained were fitted to a linear model using Sigma plot 12.

3.2.8 Standardization of inoculum

The McFarland turbidity standards (obtained by combining appropriate volumes of 1% barium chloride and 1% H_2SO_4) were used to standardize the approximate number of cells in

a liquid suspension by comparing the turbidity of the bacterial and yeast suspension with that of the McFarland standard.

3.2.9 Production of crude β-galactosidase from microbial cells

The cells were grown for 48 h at 30°C in a culture medium containing (g/l): casamino acid, 5.0; peptone, 5.0; yeast extract, 3.0; lactose, 5; Ammonium sulfate, 2.0; KH_2PO_4, 1.0, as modified from Panesar et al. (2007). The cells were harvested by centrifugation and washed twice in 50 mM Z buffer (pH of 7.0). The bacterial cells were suspended in a Z-buffer to a cell density of $1.17x10^9$ cells/ml (A_{600} = 0.6), while the yeast cells were suspended to a cell density of A_{600} = 1. The cell suspension was cooled under ice. The cells were then broken by blending in the presence of glass beads. The mixture was filtered through a 0.45µm membrane filter to obtain the crude enzyme.

3.2.10 β-Galactosidase activity inhibition assays with crude (cell-free) enzymes

3.2.10.1 β-Galactosidase activity inhibition by individual solvents, surfactants and EDTA

Inhibitions of the activity of cell-free β-galactosidase from *E. coli* and *Kluyveromyces marxianus* by individual solvents and surfactants were determined using *o*-nitrophenyl-β-D-galactopyranoside (*o*NPG) as chromogenic substrate. The inhibition assay was done in a 2-ml reaction mixture contained in triplicate 15-ml screw cap culture tubes following the protocols shown in Tables 3.2 to 3.6. After adding other components in each tube, the reaction was initiated by adding 0.2 ml of Z-buffered 0.2% *o*NPG. The reaction mixtures were shaken to mix and incubated at 30°C for 30 min. After incubation, the reaction was halted by adding 1 ml of 1M Na_2CO_3 aqueous solution. The absorbance of the 2-nitrophenol (2-NP) solution produced in each tube was measured at 420nm in a spectrophotometer.

Table 3.1: Protocol for preparation of graded concentration of 2-nitrophenol for calibration of spectrophotometer

Tube number	Blank	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Vol. of Z-buffer (ml)	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6
Vol. of distilled water (ml)	1.4	1.3	1.2	1.1	1.0	0.9	0.8	0.7	0.6	0.5	0.4	0.3	0.2	0.1	0.0
Vol. of 60 mg/l 2NP solution (ml)	0.0	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9	1.0	1.1	1.2	1.3	1.4
Vol. of 1M Na_2CO_3 solution (ml)	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Concentration of 2-NP (mg/l)	0	2	4	6	8	10	12	14	16	18	20	22	24	26	28

Table 3.2: Protocol for the preparation of graded concentration of individual solvents and solvent mixtures for inhibition assay with crude β-galactosidase from *Escherichia coli* and *Kluyveromyces marxianus*.

Tube number	Blank	1	2	3	4	5	6	7	8	9	10	11	12
Vol. of Z-buffer (ml)	0.4	-	-	-	-	-	-	-	-	-	-	-	-
Vol. of crude enzyme (ml)	0.0	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.	0.
Vol. of 100% solvent/solvent mixture (ml)	0.0	0.0	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	1.0	1.	1.
Vol. of distilled water (ml)	1.4	1.4	1.3	1.2	1.1	1.0	0.9	0.8	0.7	0.6	0.4	0.	0.
Vol. of Z-buffered 0.2% oNPG (ml)	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.	0.
Concentration of solvent (%)	0	0	5	10	15	20	25	30	35	40	50	60	70

Table 3.3: Protocol for the preparation of graded concentration of surfactants (Triton X-100, SDC, Sarcosyl, Tween-20, SDS and Tween-80) for inhibition assay with crude β-galactosidase from *Escherichia coli* and *Kluyveromyces marxianus.*

Standard surfactant solution	**0.2% surfactant solution**						**2% surfactant solution**					**10% surfactant solution**				
Tube number	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
Distilled water (ml)	1.4	1.2	1.0	0.8	0.6	0.4	1.2	1.0	0.8	0.6	0.4	1.1	1.0	0.9	0.8	0.6
Surfactant solution (ml)	0.0	0.2	0.4	0.6	0.8	1.0	0.2	0.4	0.6	0.8	1.0	0.3	0.4	0.5	0.6	0.8
Crude enzyme in Z-buffer (ml)	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4
Z-buffered 0.2% oNPG (ml)	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
Conc. of surfactant (%)	0.0	0.02	0.04	0.06	0.08	0.1	0.2	0.4	0.6	0.8	1.0	1.5	2.0	2.5	3.0	4.0

Blank contains 0.4 ml Z-buffer, 1.4 ml distilled water and 0.2 ml Z-buffered 0.2% oNPG

Table 3.4: Protocol for the preparation of graded concentration of (CTAB) for inhibition assay with crude β-galactosidase from *Escherichia coli* and *Kluyveromyces marxianus.*

Standard CTAB solution	**0.02% CTAB solution**						**0.2% CTAB solution**							
Tube number	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Distilled water (ml)	1.4	1.2	1.0	0.8	0.6	0.4	1.28	1.26	1.24	1.22	1.20	1.0	0.8	0.6
Surfactant solution (ml)	0.0	0.2	0.4	0.6	0.8	1.0	0.12	0.14	0.16	0.18	0.2	0.4	0.6	0.8
Crude enzyme in Z-buffer (ml)	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4
Z-buffered 0.2% oNPG (ml)	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
Conc. of surfactant (%)	0.00	0.002	0.004	0.006	0.008	0.01	0.012	0.014	0016	0.018	0.02	0.04	0.06	0.08

Blank contains 0.4 ml Z-buffer, 1.4 ml distilled water, and 0.2 ml Z-buffered 0.2% oNPG

Table 3.5: Protocol for the preparation of graded concentration of (CPC) for inhibition assay with crude β-galactosidase from *Escherichia coli* and *Kluyveromyces marxianus*.

Standard CPC solution	**0.002% CPC solution**						**0.02% CPC solution**						
Tube number	1	2	3	4	5	6	7	8	9	10	11	12	13
Distilled water (ml)	1.4	1.2	1.0	0.8	0.6	0.4	1.28	1.26	1.24	1.22	1.20	1.0	0.8
CPC solution (ml)	0.0	0.2	0.4	0.6	0.8	1.0	0.12	0.14	0.16	0.18	0.2	0.4	0.6
Crude enzyme (ml)	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4
0.2% oNPG (ml)	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
Conc. of CPC (%)	0.00	0.0002	0.0004	0.0006	0.0008	0,001	0.0012	0.0014	0.0016	0.0018	0.002	0.003	0.004

Blank contains 0.4 ml Z-buffer, 1.4 ml distilled water, and 0.2 ml Z-buffered 0.2% *o*NPG

Table 3.6: Protocol for the preparation of graded concentration of EDTA for inhibition assay with crude β-galactosidase from *Escherichia coli* and *Kluyveromyces marxianus*.

Standard EDTA solution	**0.02% surfactant solution**						**0.2% surfactant solution**					**2% EDTA solution**				
Tube number	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
Distilled water (ml)	1.4	1.2	1.0	0.8	0.6	0.4	1.2	1.0	0.8	0.6	0.4	1.25	1.2	1.0	0.8	0.6
EDTA solution (ml)	0.0	0.2	0.4	0.6	0.8	1.0	0.2	0.4	0.6	0.8	1.0	0.15	0.2	0.4	0.6	0.8
Crude enzyme (ml)	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4
0.2% *o*NPG (ml)	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
Conc. of EDTA (%)	0.0	0.002	0.004	0.006	0.008	0.01	0.02	0.04	0.06	0.08	0.1	0.15	0.2	0.4	0.6	0.8

Blank contains 0.4 ml Z-buffer, 1.4 ml distilled water, and 0.2 ml Z-buffered 0.2% *o*NPG

3.2.10.2 β-Galactosidase activity inhibition by binary mixtures of water-miscible and water-immiscible solvents

Water-miscible solvents (ethanol, dimethylsulfoxide and N, N-dimethylformamide) and water-immiscible solvents (*n*-butanol, *n*-pentanol and chloroform) were combined in two binary mixture ratios (9:1 and 7:3), given a total of 18 binary mixtures of solvents (2x3x3). Inhibitions of the activity of cell-free β-galactosidase from *E. coli* and *Kluyveromyces marxianus* by binary mixtures of solvents were determined according to the procedure described for individual solvents in section 3.2.10.1 using the protocols shown in Tables 3.2. While keeping the mixture ratio constant, the total concentration of the mixture was varied to obtain a complete concentration-response relationship of the mixture.

3.2.10.3 β-Galactosidase activity inhibition by binary mixtures of water-miscible solvents

Binary mixtures of water-miscible solvents (ethanol + dimethylsulfoxide and ethanol + N, N-dimethylformamide) were combined in four binary mixture ratios (9:1, 8:2, 7:3 and 6:4), given a total of 8 binary mixtures of solvents (2 x 4). Inhibitions of the activity of cell-free β-galactosidase from *E. coli* and *Kluyveromyces marxianus* by the binary mixtures of solvents were determined according to the procedure described for individual solvents in section 3.2.10.1 using the protocols shown in Tables 3.2. Each binary mixture was prepared and added to the reaction mixture as a single solvent. While keeping the mixture ratio constant, the total concentration of the mixture was varied to obtain a complete concentration-response relationship of the mixture.

3.2.10.4 Computation of enzyme activities and relative β-galactosidase activity

In each assay, the enzyme activity unit (U) is the amount of enzyme that liberated one µmole of 2-NP per minute under the assay condition. It is computed as shown in Eq. (3.1).

$$\text{Enzyme activity (U/ml)} = \frac{A_{420} \times V_R}{m \times M \times T \times V_E} \qquad (3.1)$$

Where A_{420} is the absorbance of the product (2-NP) formed in the reaction mixture following enzyme activity, V_R is the volume (ml) of the reaction mixture, *m* is the slope of the calibration curve for 2-NP determination, M is the molar mass (g) of 2-NP, T is the incubation time (min), and V_E is the volume (ml) of the crude enzyme preparation in the reaction mixture.

The relative β-galactosidase activity was computed as shown in Eq. 3.2.

$$\beta\text{-Galactosidase activity}\,(\%) = \frac{A_{Test}}{A_{Control}} \times 100 \qquad (3.2)$$

Where A_{Control} is the enzyme activity in the control and A_{Test} is the enzyme activity in the tests.

3.2.11 Data Analysis

3.2.11.1 Determination of effective concentrations (ED_K)

The concentration-response relationship of the individual surfactant, solvent or mixtures was fitted with a 4-parameter logistic function (Eq. 3.3) or the Gormpertz model (Eq. 3.4).

$$y = c + \frac{d-c}{1+\left(\frac{x}{a}\right)^b} \qquad (3.3)$$

In the logistic model, *y* is the response, *x* is the concentration of the inhibitor, *d* represents the β-galactosidase activity of the untreated control, *a* is the concentration of β-galactosidase inhibitor at half *d-c* (ED_{50}), *c* is the response at infinite *x* and *b* is the relative slope around ED_{50}.

]

$$y = c + (d-c)\exp\left(-\left(\frac{x}{a}\right)^b\right) \qquad (3.4)$$

In the Gormpertz model, *y* is the response, *x* is the concentration of the inhibitor, *d* represents the β-galactosidase activity of the untreated control, *c* is the response at infinite *x*, *b* determines the steepness of the curve, and *a* determines the placement of the curve on the concentration scale.

To obtain any arbitrary effective concentration (ED_K) with the logistic function for *K*% inhibition of β-galactosidase activity, Eq. (3.5) was solved for a^b to obtain Eq. (3.6).

$$c + \frac{100-\mathrm{K}}{100}(d-c) = c + \frac{d-c}{1+\left(\frac{ED_{\mathrm{K}}}{a}\right)^b} \qquad (3.5)$$

$$a^b = {ED_K}^b \frac{100-\mathrm{K}}{100} \tag{3.6}$$

Substituting a^b into Eq. (3.2) resulted in a logistic concentration-response model (Eq. 3.7) to incorporate any effective concentration.

$$y = c + \frac{d-c}{1+\frac{100}{100-\mathrm{K}}\left(\frac{x}{ED_{\mathrm{K}}}\right)^b} \tag{3.7}$$

To obtain any arbitrary effective concentration (ED_K) with Gormpertz function for K% inhibition of β-galactosidase activity, Eq. (3.8) was solved for a^b to obtain Eq. (3.9).

$$c + \frac{100-\mathrm{K}}{100}(d-c) = c + (d-c)\exp\left(-\left(\frac{x}{a}\right)^b\right) \tag{3.8}$$

$$a^b = -\frac{{ED_{\mathrm{K}}}^b}{\ln\left(\frac{100-\mathrm{K}}{100}\right)} \tag{3.9}$$

Substituting a^b into Eq. (3.4) resulted in a sigmoid concentration-response model (Eq. 3.10) for incorporation of any effective concentration.

$$y = c + (d-c)\exp\left[\left(\frac{x}{ED_{\mathrm{K}}}\right)^b \ln\left(\frac{100-\mathrm{K}}{100}\right)\right] \tag{3.10}$$

Multistep dose-response relationships are described with the bilogistic model (Eq. 3.11)

$$y = \left[c + \frac{d-c}{1+\left(\frac{x}{a}\right)^{b}} \right] - \left[e + \frac{c-e}{1+\left(\frac{x}{f}\right)^{g}} \right] \qquad (3.11)$$

$$b > 0; g < 0$$

Where d is the upper asymptote representing the response of the control (y at x=0) in the first curve, e is the lower asymptote representing the response at an infinite dose in the second curve, c is the lower asymptote of the first curve, a is the dose at the center of the first curve, b is the slope of the first curve at a, f is the dose at the center of the second curve, and g is the slope of the second curve at f.

To obtain ED_{50}, Eq. 3.12 was solved for a^b to obtain Eq. 3.13.

$$e + \frac{100-K}{100}(d-e) = \left[c + \frac{d-c}{1+\left(\frac{ED_K}{a}\right)^{b}} \right] - \left[e + \frac{c-e}{1+\left(\frac{ED_K}{f}\right)^{g}} \right] \qquad (3.12)$$

$$b > 0; g < 0$$

$$a^b = \left\{ \left[\frac{100\left(1+\left(\frac{ED_K}{f}\right)^{g}\right)(d-c)}{100e\left(1+\left(\frac{ED_K}{f}\right)^{g}\right) + (100-K)(d-e)\left(1+\left(\frac{ED_K}{f}\right)^{g}\right) - 100(c-e)\left(\frac{ED_K}{f}\right)^{g}} - 1 \right] \frac{1}{{ED_K}^{b}} \right\}^{-1} \qquad (3.13)$$

$$b > 0; g < 0$$

By substituting Eq. 3.13 into Eq. 3.11, a model that incorporates any arbitrary effective dose (ED_K) was obtained. The ED_{50} (for K=50) values were estimated by curve-fitting concentration-response data into the respective model using Table Curve 2D v5.01.

3.2.11.2 Estimation of No-Observed-Effect-Concentrations (NOEC)

The NOEC is the highest concentration of surfactant and solvent at which no inhibition of β-galactosidase activity was observed. Theoretically, the β-galactosidase activity at this concentration would not be statistically different from that of the control. This means that the β-galactosidase activity at NOEC will overlap the control values. Based on this information, we adopted a simple novel approach to estimate NOEC using the coefficient of variation (CV) of the control β-galactosidase activity as a benchmark. The CV is a statistical measure that

expresses the relative variability of a set of data points in relation to their mean. To express CV as a percentage, the standard deviation (SD) of the control values was divided by the mean and then multiplied by 100, as shown in Eq. (3.12).

Thus, if the CV is taken as *K*, the concentration of the inhibitor that inhibited β-galactosidase activity by CV percent (ED_{CV}) was taken to be NOEC and was computed by fitting the concentration-response data into any of the reparameterized sigmoid models (Eq. 3.7 or Eq. 3.10) using TableCurve 2D v. 5.01. During curve fitting, the mean value of the control was used as the upper asymptote (*d*), and the effect at infinite concentration (*c*) was fixed at zero. When the response was reported as relative β-galactosidase activity, *d* was fixed at 100%.

3.2.11.3 Prediction of inhibitory effects of binary mixtures of solvents

The inhibitory effects of the binary mixtures of water-miscible solvents on the activities of crude β-galactosidase from *Escherichia coli* and *Kluyveromyces marxianus* were predicted from the inhibitory effects of the individual solvents if the relative composition of each component is quantitatively known, using concentration addition (CA) model. The CA model can be written as in Eq. (3.13) (Berenbaum, 1985):

$$EC_{x(mix)} = \left(\sum_{i=1}^{n} \frac{\pi_i}{EC_{xi}} \right)^{-1} \qquad (3.13)$$

Where $ECx_{(mix)}$ is the total concentration of the mixture that elicited *x*% effect, EC_{xi} is the concentration of *i*th component that gave *x* effect when tested as an individual, *n* is the number of components, π_i is the proportion of *i*th component in the mixture. Using equation 3.13, the inhibitory effects of the mixtures were predicted as described elsewhere (Altenburger et al., 2000; Backhaus et al., 2000; Nweke et al., 2018). The total concentration of each mixture that elicited 1 – 99% relative β-galactosidase activities was calculated in steps of 1%. The resulting 99 concentration/response pairs were plotted as a line chart, which visualised the predicted dose-response curve. In the first step, the EC_x values for 1 – 99% enzyme activity were calculated for each component from the logistic or Gormpertz dose-response model that fitted the individual dose-response data. In the second step, the EC_x values were substituted in equation 3.13 to obtain the 1 – 99% $ECx_{(mix)}$ values for each mixture. These calculations were done with Microsoft Excel 2013, and the data obtained were plotted using Sigmaplot 10.

3.2.11.4 Computation of the toxic index (TI)

To evaluate the interactive effect of the mixtures on the β-galactosidases, the Toxic Index (TI) of each mixture was calculated as the sum of toxic units for all the components of the mixture, as shown in Eq. (3.14).

$$\mathrm{TI} = \sum_{i=1}^{n} \frac{C_i}{EC_{50i}} = \sum_{i=1}^{n} \frac{\pi_i EC_{50mix}}{EC_{50i}} \qquad (3.14)$$

Where C_i is the concentration of the *i*th component in the mixture at the EC_{50} of the mixture (EC_{50mix}), and EC_{50i} is the concentration of the *i*th component that elicited 50% inhibition of β-galactosidase activity when tested as an individual, *n* is the number of components in the mixture and π_i is the proportion of *i*th component in the mixture. Antagonistic and synergistic interactions are denoted by TI > 1 and TI < 1, respectively, while there is no interaction (additivity) when TI = 1 (Boillot & Perrodin, 2008).

3.2.12 Permeabilization of cells and β-galactosidase activity assay

Activities of β-galactosidase in *Escherichia coli* and *Kluyveromyces marxianus* whole cells were determined following permeabilization of cells in reaction mixtures containing varying concentrations of individual solvents and surfactants as well as 9:1 mixtures of ethanol with chloroform, pentanol, butanol, dimethylsulfoxide (DMSO) and N, N-dimethylformamide (NNF). In the batches of experiments conducted, similar concentration ranges were used in the crude enzyme assays and the permeabilization assays. The essence of the assay is to determine the concentration or range of concentrations of each solvent, solvent mixture and individual surfactant that would be suitable for permeabilization of *Escherichia coli* and *Kluyveromyces marxianus* cells for β-galactosidase activity assay. The cells were grown for 48 hours at 30^{o}C in a culture medium containing (g/l): casamino acid, 5.0; peptone, 5.0; yeast extract, 3.0; lactose, 5; Ammonium sulfate, 2.0; KH_2PO_4, 1.0, as modified from Panesar et al. (2007). The cells were harvested by centrifugation and washed twice in 50 mM Z buffer (pH of 7.0). *Escherichia coli* cells were suspended in a Z-buffer to a cell density of 1.17×10^9 cells/ml ($A_{600} = 0.6$), while the *Kluyveromyces marxianus* cells were suspended to a cell density of $A_{600} = 1$. The suspensions were used in the permeabilization and β-galactosidase activity assays.

The cell permeabilization assay was done in a 2-ml reaction mixture contained in triplicate 15-ml screw cap culture tubes following the protocols shown in Tables 3.7 to 3.11. *Escherichia coli* and *Kluyveromyces marxianus* cells were exposed to varying concentrations of solvents and surfactants in a reaction mixture containing 0.4 ml of cell suspension and requisite volumes of distilled water, solvent or surfactant solution. The reaction mixtures were incubated at 30^{o}C for 20 min. After that, 0.2 ml of Z-buffered 0.2% oNPG solution was added into each tube and further incubated at 30^{o}C for 30 min. After incubation, the reaction was halted by adding 0.8 ml of 1M Na_2CO_3 aqueous solution. The absorbance of the 2-nitrophenol (2-NP) solution produced in each tube was measured at 420nm in a spectrophotometer against blank preparation containing cells but not enzyme substrate. The β-galactosidase activity in each tube was calculated as stated in section 3.11.4.

Table 3.7: Protocol for the preparation of graded concentration of individual solvents and solvent mixtures for permeabilization of *Escherichia coli* and *Kluyveromyces marxianus* cells during β-galactosidase assay.

Tube number	Blank	1	2	3	4	5	6	7	8	9	10	11	12
Vol. of distilled water (ml)	1.6	1.6	1.5	1.4	1.3	1.2	1.1	1.0	0.9	0.8	0.6	0.4	0.2
Vol. of 100% solvent/solvent mixture (ml)	0.0	0.0	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	1.0	1.2	1.4
Vol. of Z-buffered cells (ml)	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4
	After addition of the cells, incubate tubes for 20 min at 30^{o}C												
Vol. of Z-buffered 0.2% oNPG (ml)	0.0	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
Vol. of Z-buffer (ml)	0.2	-	-	-	-	-	-	-	-	-	-	-	-
	After addition of the enzyme substrate, incubate tubes for 30 min at 30^{o}C												
Concentration of solvent (%)	0	0	5	10	15	20	25	30	35	40	50	60	70

After 30 minutes of incubation, 0.8 ml of 1M Na_2CO_3 solution was added into each tube to stop the reaction.

Table 3.8: Protocol for the preparation of graded concentration of surfactants (Triton X-100, SDC, Sarkosyl, Tween-20, SDS and Tween-80) for cell permeabilization during β-galactosidase activity assay in *Escherchia coli* and *Kluyveromyces marxianus*.

Standard surfactant solution	**0.2% surfactant solution**						**2% surfactant solution**					**10% surfactant solution**				
Tube number	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
Distilled water (ml)	1.6	1.4	1.2	1.0	0.8	0.6	1.4	1.2	1.0	0.8	0.6	1.3	1.2	1.1	1.0	0.8
Surfactant solution (ml)	0.0	0.2	0.4	0.6	0.8	1.0	0.2	0.4	0.6	0.8	1.0	0.3	0.4	0.5	0.6	0.8
Z-buffered cells (ml)	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4
	Incubate tubes at 30°C for 20 min						Incubate tubes					Incubate tubes				
Z-buffered 0.2% oNPG (ml)	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
	Incubate tubes at 30°C for 30 min															
Conc. of surfactant (%)	0.0	0.02	0.04	0.06	0.08	0.1	0.2	0.4	0.6	0.8	1.0	1.5	2.0	2.5	3.0	4.0

Blank contains, 1.6 ml distilled water and 0.4 ml Z-buffered cell suspension. After incubation for 20 min, 0.2 ml of Z-buffered 0.2% oNPG was also added in the blank.

After 30 min incubation, 0.8 ml of 1M Na_2CO_3 solution was added into each tube to stop the reaction

Table 3.9: Protocol for the preparation of graded concentration of (CTAB) for cell permeabilization during β-galactosidase activity assay in *Escherichia coli* and *Kluyveromyces marxianus*.

Standard CTAB solution	**0.02% CTAB solution**						**0.2% CTAB solution**							
Tube number	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Distilled water (ml)	1.6	1.4	1.2	1.0	0.8	0.6	1.48	1.46	1.44	1.42	1.40	1.2	1.0	0.8
Surfactant solution (ml)	0.0	0.2	0.4	0.6	0.8	1.0	0.12	0.14	0.16	0.18	0.2	0.4	0.6	0.8
Z-buffered cells (ml)	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4
	After that, incubate tubes at 30°C for 20 min.						After that, incubate tubes at 30°C for 20 min.							
Z-buffered 0.2% oNPG (ml)	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
	After that, incubate tubes at 30°C for 30 min.						After that, incubate tubes at 30°C for 30 min.							
Conc. of surfactant (%)	0.00	0.002	0.004	0.006	0.008	0.01	0.012	0.014	0016	0.018	0.02	0.04	0.06	0.08

Blank contains: 1.6 ml distilled water and 0.4 ml Z-buffered cell suspension. After incubation for 20 min, 0.2 ml of Z-buffered 0.2% oNPG was also added in the blank.

After 30 min incubation, 0.8 ml of 1M Na_2CO_3 solution was added into each tube to stop the reaction

Table 3.10: Protocol for the preparation of graded concentration of (CPC) for cell permeabilization during β-galactosidase activity assay in *Escherichia coli* and *Kluyveromyces marxianus*.

Standard CPC solution	**0.002% CPC solution**						**0.02% CPC solution**						
Tube number	1	2	3	4	5	6	7	8	9	10	11	12	13
Distilled water (ml)	1.6	1.4	1.2	1.0	0.8	0.6	1.48	1.46	1.44	1.42	1.40	1.2	1.0
CPC solution (ml)	0.0	0.2	0.4	0.6	0.8	1.0	0.12	0.14	0.16	0.18	0.2	0.4	0.6
Cell suspension (ml)	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4
	Thereafter, incubate tubes at 30°C for 20 min						After that, incubate tubes at 30°C for 20 min						
0.2% oNPG (ml)	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
	Thereafter, incubate tubes at 30°C for 30 min						Thereafter, incubate tubes at 30°C for 30 min						
Conc. of CPC (%)	0.00	0.0002	0.0004	0.0006	0.0008	0,001	0.0012	0.0014	0.0016	0.0018	0.002	0.003	0.004

Blank contains: 1.6 ml distilled water and 0.4 ml Z-buffered cell suspension. After incubation for 20 min, 0.2 ml of Z-buffered 0.2% oNPG was also added in the blank.

After 30 min incubation, 0.8 ml of 1M Na_2CO_3 solution was added into each tube to stop the reaction

Table 3.11: Protocol for the preparation of graded concentration of SDS for cell permeabilization during β-galactosidase activity assay in *Escherchia coli* and *Kluyveromyces marxianus.*

Standard surfactant solution	**0.02% SDS solution**						**0.2% SDS solution**					**2% SDS**	
Tube number	1	2	3	4	5	6	7	8	9	10	11	12	13
Distilled water (ml)	1.6	1.4	1.2	1.0	0.8	0.6	1.4	1.2	1.0	0.8	0.6	1.3	1.2
Surfactant solution (ml)	0.0	0.2	0.4	0.6	0.8	1.0	0.2	0.4	0.6	0.8	1.0	0.3	0.4
Z-buffered cells (ml)	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4
	Incubate tubes at 30°C for 20 min						Incubate tubes					Incubate	
Z-buffered 0.2% oNPG (ml)	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
	Incubate tubes at 30°C for 30 min												
Conc. of surfactant (%)	0.0	0.002	0.004	0.006	0.008	0.01	0.02	0.04	0.06	0.08	0.1	0.2	0.4

Blank contains 1.6 ml distilled water and 0.4 ml Z-buffered cell suspension. After incubation for 20 min, 0.2 ml of Z-buffered 0.2% oNPG was also added in the blank.

After 30 minutes of incubation, 0.8 ml of 1M Na_2CO_3 solution was added into each tube to stop the reaction.

3.2.13 β-galactosidase activity assay after permeabilization of *Escherichia coli* and *Kluyveromyces marxianus* cells by selected concentrations of solvents and surfactants

In another batch of experiments, activities of β-galactosidase in *Escherichia coli* and *Kluyveromyces marxianus* whole cells were determined following permeabilization of cells in reaction mixtures containing selected concentrations of ethanol and surfactants as well as 9:1 mixtures of ethanol with chloroform, pentanol and butanol, In these experiments, the range of concentrations of each solvent, solvent mixture and individual surfactant that indicated high permeabilization of *Escherichia coli* and *Kluyveromyces marxianus* cells were selected as outlined in Table 3.11. The assay was done as described in section 3.2.12

Table 3.12 Selected promising concentrations of solvents and surfactants for cell permeabilization and β-galactosidase activity assay

Solvent, surfactant and solvent mixture	**Selected concentrations (%)**
Escherichia coli	
Ethanol	15, 20, 25
Ethanol + Chloroform (9:1)	5, 10, 15
Ethanol + Pentanol (9:1)	5, 10, 15
Ethanol + Butanol (9:1)	10, 15, 20
Sarcosyl	0.04, 0.06, 0.08, 0.10
Sodium deoxycholate (SDC)	0.04, 0.06, 0.08, 0.1
Sodium dodecyl sulphate (SDS)	0.008, 0.01, 0.02
CPC	0.0004, 0.0006, 0.0008, 0.001
Kluyveromyces marxianus	
Ethanol	25, 30, 35
Ethanol + Chloroform (9:1)	10, 15, 20, 25
Ethanol + Pentanol (9:1)	10, 15, 20, 25
Ethanol + Butanol (9:1)	20, 25, 30
Sarcosyl	0.2, 0.4, 0.6, 0,8
Sodium deoxycholate (SDC)	0.2, 0.4, 0.6, 0.8
CTAB	0.004, 0.006, 0.008
CPC	0.0006, 0,0008, 0.001, 0.0012, 0.0014

3.2.14 Effect of mercaptoethanol and EDTA on β-galactosidase activity in *Escherichia coli* and *Kluyveromyces marxianus* cells permeabilized with solvents and surfactants

The influence of mercaptoethanol and EDTA on β-galactosidase activity in *Escherichia coli* and *Kluyveromyces marxianus* cells permeabilized with solvents (ethanol, 9:1 mixture of ethanol with chloroform, pentanol and butanol) and surfactants (Sarcosyl, SDC, SDS, CPC and CTAB). The reaction mixture containing selected concentrations of solvents and surfactants was supplemented with the sub-inhibitory concentration of mercaptoethanol (0.1%) or EDTA (0.002% for *Escherichia coli* and 0.0002% for *Kluyveromyces marxianus*) in separate tubes. The β-galactosidase activities were determined as described in section 3.2.12.

3.2.15 Comparison of Methods for permeabilization of *Escherichia coli* for β-galactosidase Activity Assay

The standard assay described by Miller (1972) for quantification of β-galactosidase activity in bacterial cells was compared with optimized conditions obtained from our experiments, involving the spectrophotometric determination of yellow-coloured *o*-nitrophenol (ONP) as the hydrolytic product of β-galactosidase activity on the colourless substrate, *o*-nitrophenyl-β-D-galactoside (*O*NPG). By the Miller method, 0.2 ml of *Escherichia coli* cell suspension in Z-buffer (A600 = 0.6) was diluted with 0.8 ml of Z-buffer. The cells were permeabilized by adding 100 µl chloroform and 50 µl 0.1% SDS (sodium dodecyl sulfate). The tubes were equilibrated for 20 min at 30^{o}C. The reaction was started by adding 0.2 mL substrate, *o*-nitrophenyl-β-D-galactoside (*O*NPG; 4 mg/mL). The enzymatic reaction was stopped after 30min by adding 0.5 mL 1M Na_2CO_3 aqueous solution. The absorbance of the yellow-coloured ONP was measured over a blank containing all components except the enzyme substrate. Miller's SDS-chloroform method was compared with other permeabilization agents, as shown in Table 3.13.

Table 3.13 Comparison of permeabilization treatments for β-galactosidase activity assay in *Escherichia coli*

S/N	Solvent, surfactant and solvent mixture	Selected concentrations (%)
1	SDS + Chloroform (Miller, 1972)	≈ 0.005 SDS + 8.7 Chloroform
2	Ethanol	25
3	Ethanol + Chloroform (9:1)	5
4	Ethanol + Pentanol (9:1)	15
5	Ethanol + Butanol (9:1)	20
6	SDS	0.02
7	Triton X-100	0.02
8	Tween 20	0.02
9	Sodium Deoxycholate (SDC)	0.02
10	Sarcosyl	0.1, 0.2,
11	CTAB	0.002
12	1-Cetylpyridinium chloride (CPC)	0.0008
13	CTAB+SDC	0.064 (CTAB)+0.032 (SDC)

3.2.16 Comparison of methods for permeabilization *Kluyveromyces marxianus* for β-galactosidase activity assay

The standard permeabilization and β-galactosidase activity assay described by Kippert (1995) for yeasts was compared with optimized conditions obtained from our experiments, involving the spectrophotometric determination of yellow-coloured *o*-nitrophenol (ONP) as the hydrolytic product of β-galactosidase activity on the colourless substrate, *o*-nitrophenyl-β-D-galactoside (ONPG). By Kippert's method, 0.2 ml of *Kluyveromyces marxianus* cells suspension in Z-buffer (A600 = 0.4) was diluted with 0.4 ml of distilled water in a culture tube. Then,1.2 ml of Z-buffer containing 0.2% sarcosyl was added, leading to a 0.133% sarcosyl final concentration. The tubes were mixed and incubated for 20 min at 30^{o}C. The reaction was started by adding 0.45 mL substrate, *o*-nitrophenyl-β-D-galactoside (ONPG; 4 mg/mL). The enzymatic reaction was stopped after 30min by adding 1.2 mL of 1.5M Na_2CO_3 aqueous solution. The absorbance of the yellow-coloured ONP was measured over a blank containing all components except the enzyme substrate. Kippert's sarcosyl method was compared with other permeabilization agents, as shown in Table 3.14.

Table 3.14 Comparison of permeabilization treatments for β-galactosidase activity assay in *Kluyveromyces marxianus*

S/N	Solvent, surfactant and solvent mixture	Selected concentrations (%)
1	Sarcosyl	0.133 (Kippert, 1995)
2	Sarcosyl	0.15
3	Sarcosyl	0.2
4	CPC	0.0008
5	Sodium Deoxycholate (SDC)	0.4
6	CTAB	0.004
7	CTAB + CPC	0.004 (CTAB) + 0.0002 (CPC)
8	CTAB + CPC	0.004 (CTAB) + 0.0004 (CPC)
9	CTAB + CPC	0.004 (CTAB) + 0.0006 (CPC)
10	CTAB + CPC	0.004 (CTAB) + 0.0008 (CPC)
11	Ethanol + Chloroform (9:1)	15
12	Ethanol + Pentanol (9:1)	15
13	Ethanol + Butanol (9:1)	25

3.2.17 Statistical Analysis

Quantitative data are presented as means ± standard deviation. One-way ANOVA implemented in IBM SPSS 20 was used to test for significant differences among treatments. A P-value of < 0.05 was regarded as statistically significant.

CHAPTER FOUR

RESULTS AND DISCUSSION

4.1 Results

4.1.1 Colonial Morphology and Biochemical Characteristics of Bacterial and Yeast Isolates

The colonial morphology and biochemical characterization of the bacterial isolate are shown in Table 4.1. Also, Table 4.2 shows the colonial morphological and biochemical characterization of a yeast isolate. The bacteria was identified as *Escherichia coli*, while the yeast was identified as *Kluyveromyces* sp.

Table 4.1: Colonial morphology and biochemical characteristics of bacterial isolate

Morphological and Biochemical characteristics	**Results**
Shape, Elevation and edge	Round, Raised and Entire, respectively
Pigmentation	Cream
Gram reaction	Gram-negative rods
Motility	Positive (+ ve)
Oxygen utilization	Facultative
Catalase	Positive (+ ve)
Oxidase	Negative (- ve)
Indole	Positive (+ ve)
Methyl red	Positive (+ ve)
Voges Proskauer (VP)	Negative (- ve)
Citrate utilization	Negative (- ve)
Urease	Negative (- ve)
Acid and gas from glucose	Positive (+ ve)
Hydrogen sulfide (H_2S)	Negative (- ve)
Lactose Fermentation	Positive (+ ve)
Growth/color on MacConkey	Positive (+ ve) / Pink
Color on Eosin Methylene Blue (EMB)	Greenish metallic sheen
Color on chromogenic Urinary Tract Infection (UTI) agar	Pink
Color on chromogenic Salmonella agar	Blue
Probable isolate	*Escherichia coli*

Table 4.2 Colonial morphology and Biochemical characteristics of the yeast isolate

Morphological and Biochemical Characterization	Results
Shape and edge	Circular and smooth edge
Size	Small/medium
Texture	Smooth or slightly rough texture
Color	Cream-color
Elevation	Slightly raised above the surface of the agar
Surface	Moist and glistening
Opacity	Opaque
Sugar fermentation	
Glucose	positive
Galactose	positive
Lactose	positive
Raffinose	positive
Sucrose	positive
Sugar assimilation	
Glucose	positive
galactose	positive
Lactose	positive
raffinose	positive
sucrose	positive
cellobiose	positive
Enzyme production	
Beta-galactosidase	positive
Probable isolate	*Kluyveromyces* sp.

4.1.2 Agarose gel electrophoresis showing the amplified ITS and Phylogenetic Analysis

Figure 4.1 shows the Agarose gel electrophoresis of the amplified ITS. Lane 2 represents the amplified 650bp, while lane L represents the 100bp DNA ladder. The phylogenetic tree showing the evolutionary distance between the yeast isolates is shown in Figure 4.2. The obtained ITS sequence from the isolates produced an exact match during the mega blast search for highly similar sequences from the NCBI non-redundant nucleotide (nr/nt) database. The ITS of the isolates showed a percentage similarity to other species at 100%. The evolutionary distances computed using the Jukes-Cantor method were in agreement with the phylogenetic placement of the ITS of the isolates within the *Kluyveromyces* sp and revealed a close relatedness to *Kluyveromyces marxianus.*

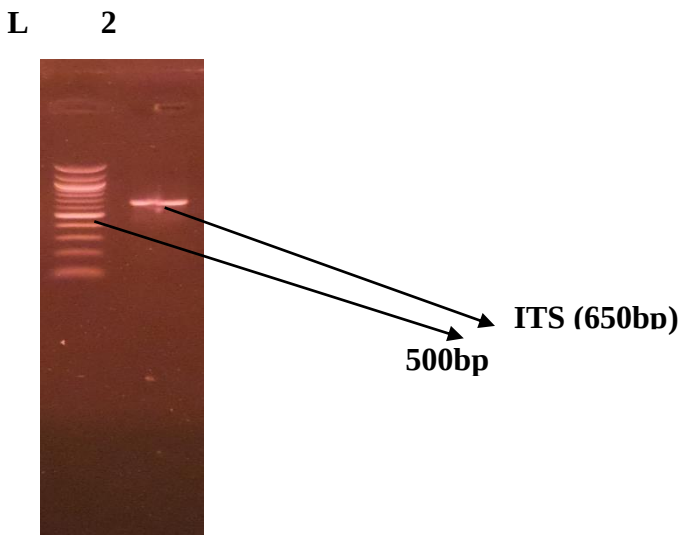

Figure 4.1 Agarose gel electrophoresis showing the amplified ITS.

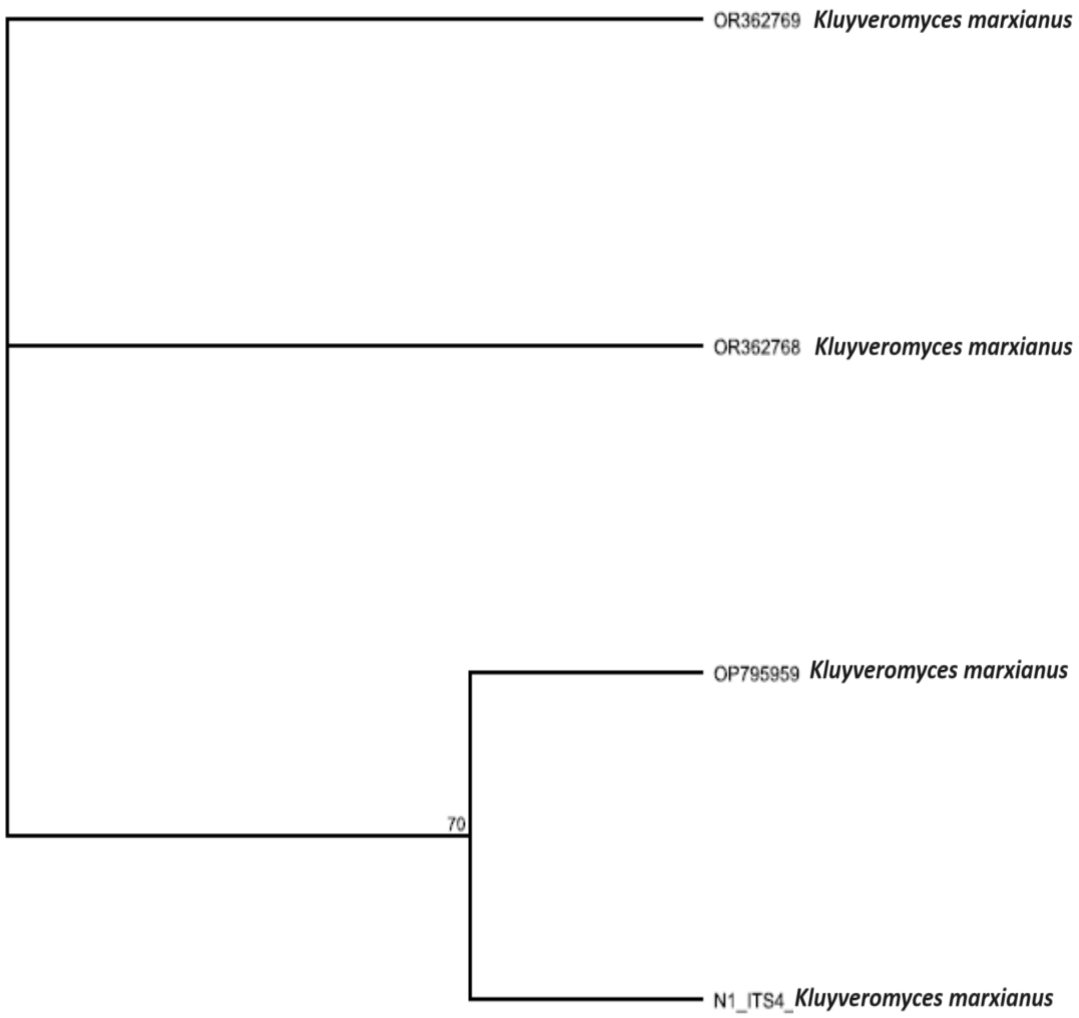

Figure 4.2 Phylogenetic tree showing the evolutionary distance between the yeast isolates.

4.1.3 Adsorption spectrum and calibration curve for determination of 2-nitrophenol

Figure 4.3a shows the absorption spectra of 2-nitrophenol. The wavelength of maximum absorption was 420 nm. The absorption spectrum has a single peak between 340 nm and 520 nm wavelengths. The calibration curve for the spectrophotometric determination of 2-nitrophenol is shown in Figure 4.3b. The R^2 value was 0.9999, indicating a reliable concentration-absorbance relationship.

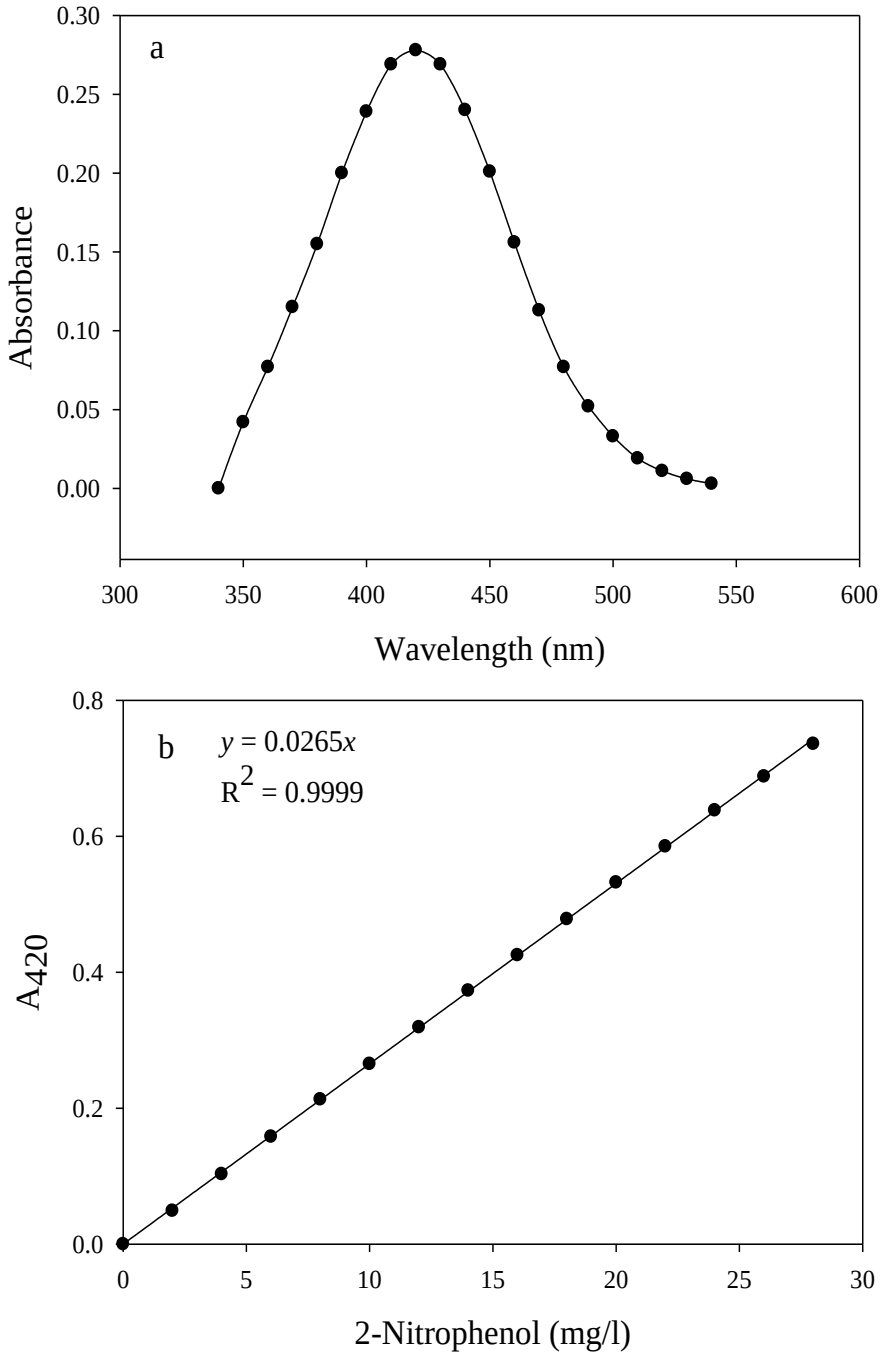

Figure 4.3 Absorption spectra of 2-nitrophenol and calibration curve for spectrophotometric determination of 2-nitrophenol.

4.1.4 Inhibition of the activities of cell-free β-galactosidase from *Escherichia coli* and *Kluyveromyces marxianus* by water-miscible solvents

The inhibition of the activities of cell-free β-galactosidase from *Escherichia coli* and *Kluyveromyces marxianus* by water-miscible solvents, ethanol, dimethyl sulfoxide (DMSO) and N, N-dimethylformamide (DMF) are shown in Figures 4.4 and 4.5. The observed concentration-response data were well-described by the Gormpertz model. There was stimulation of the activity of cell-free β-galactosidase from *Escherichia coli* by 5%, 10% and 15% ethanol. At concentrations greater than 15%, ethanol progressively inhibited β-galactosidase activity until complete inhibition occurred at 50% ethanol (Fig. 4.4). DMSO and DMF progressively inhibited *Escherichia coli* cell-free β-galactosidase from 5% until complete inhibition occurred at 50% DMSO and 40% DMF.

As shown in Fig. 4.5, ethanol, DMSO and DMF were inhibitory to the activity of cell-free β-galactosidase from *Kluyveromyces marxianus*. All the water-miscible solvents progressively inhibited the enzyme activity from 5% until total inhibition occurred at 50% ethanol, 35% DMF and 50% DMSO. Ethanol exhibited a two-step enzyme inhibition pattern that the bilogistic concentration-response model described. The concentration-response curves for DMSO and DMF were monotonic and describable with the Gormpertz concentration-response model.

The EC_{50} and NOEC values for the solvents and their statistical associations among *Escherichia coli* and *Kluyveromyces marxianus* are shown in Table 4.3. The EC_{50} of the solvents for *Escherichia coli* were significantly different from each other ($P < 0.05$). In the case of *Kluyveromyces marxianus*, The EC_{50} of DMSO and DMF were not significantly different from each other ($P > 0.05$) and were both significantly different from the EC_{50} of ethanol. Unlike the NOEC of the solvents for *Escherichia coli*, the NOEC values of the solvents for *Kluyveromyces marxianus* were not significantly different from each other.

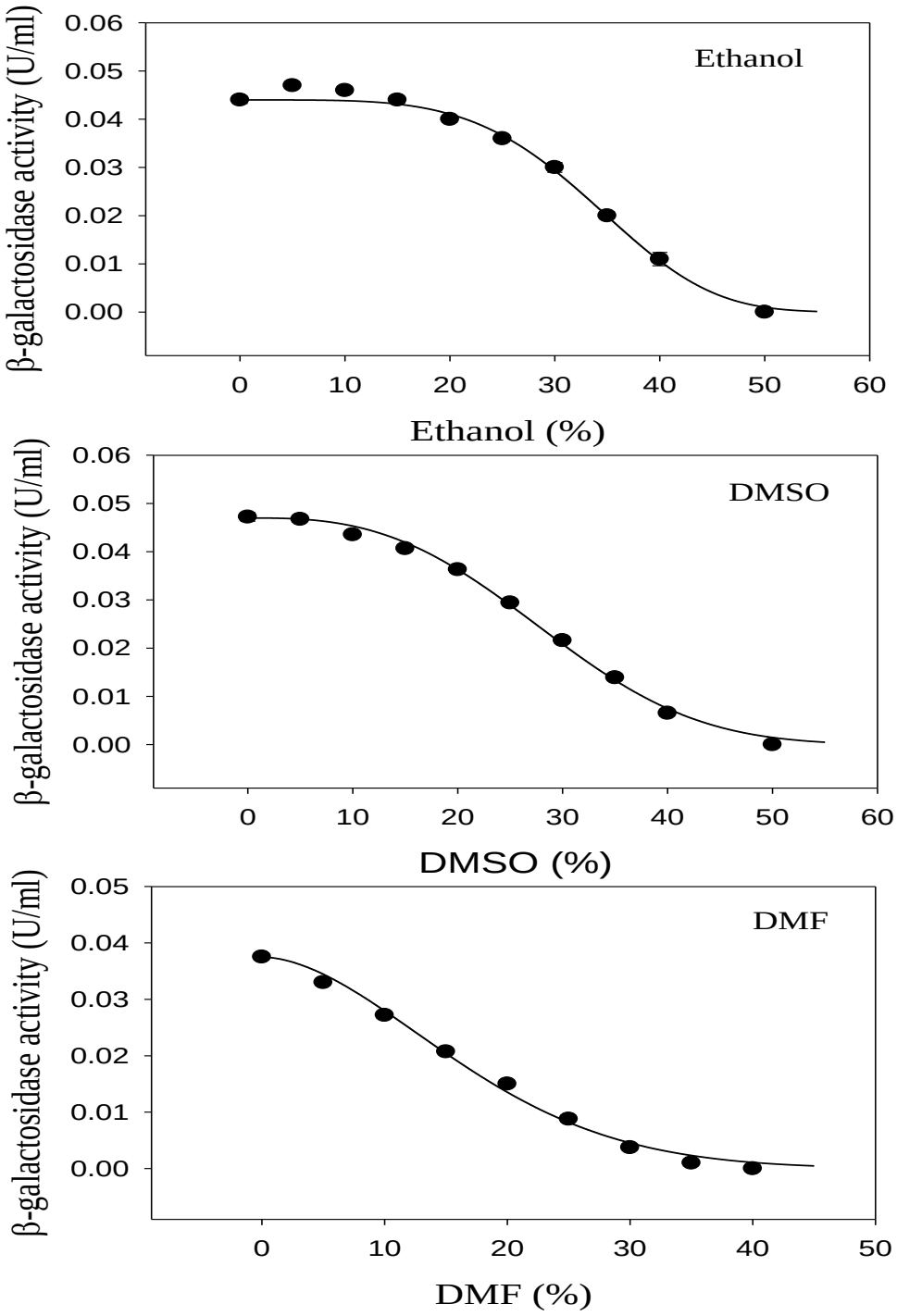

Figure 4.4 Inhibition of the activities of cell-free β-galactosidase from *Escherichia coli* by ethanol, DMSO and DMF. The solid line represents the Gormpertz model fit to the observed data.

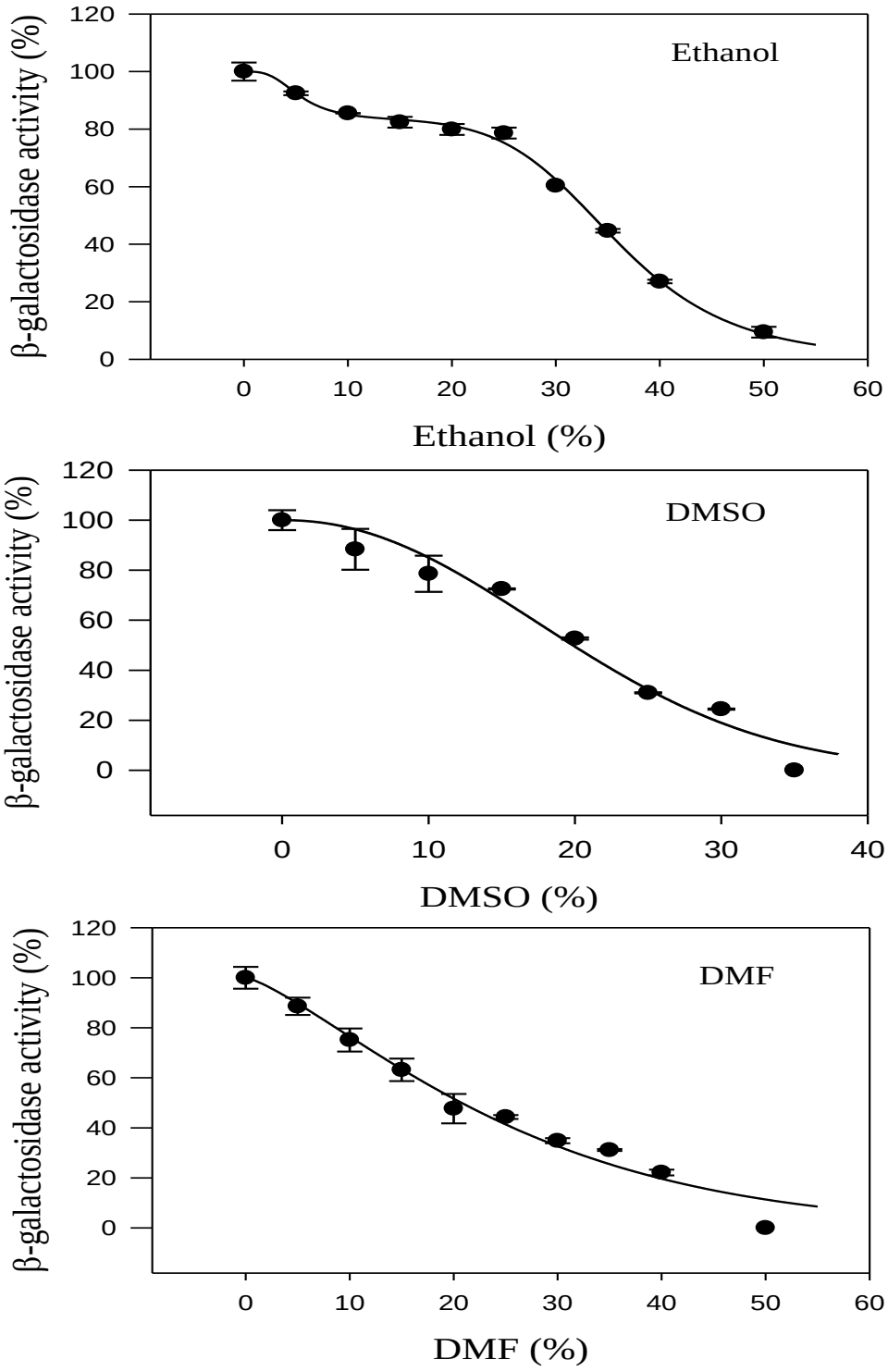

Figure 4.5 Inhibition of the activities of cell-free β-galactosidase from *Kluyveromyces marxianus* by ethanol, DMSO and DMF. The solid line represents the Gormpertz model fit to the observed data.

Table 4.3 The threshold inhibitory concentrations of water-miscible solvents against β-galactosidase from *Escherichia coli* and *Kluyveromyces marxianus*

Solvents	EC_{50} (%)	NOEC (%)
Escherichia coli		
Ethanol	33.922 ± 0.539^{a}	14.439 ± 1.270^{a}
DMSO	28.344 ± 0.386^{b}	7.939 ± 0.584^{b}
DMF	16.143 ± 0.481^{c}	0.398 ± 0.107^{c}
Kluyveromyces marxianus		
Ethanol	33.405 ± 0.445^{a}	$3.368 + 1.567^{a}$
DMSO	19.856 ± 1.216^{b}	1.414 ± 0.710^{a}
DMF	20.766 ± 1.285^{b}	2.514 ± 0.746^{a}

The data shown represents mean values and SD.

Within column for a particular organism, EC_{50} or NOEC values with the same subscript letter are not significantly different from each other ($P > 0.05$).

4.1.5 Inhibition of the activities of cell-free β-galactosidase from *Escherichia coli* by binary mixtures of water-miscible and water-immiscible solvents

Figure 4.6 shows the inhibition of the activities of cell-free β-galactosidase from *Escherichia coli* by binary mixtures of ethanol with water-immiscible solvents (chloroform, *n*-pentanol or butanol) in 9:1 and 7:3 ethanol-water immiscible solvent ratios. The mixtures exhibited progressive inhibition of β-galactosidase activity from 5% until total inhibition occurred at 35% of the 9:1 ethanol-chloroform mixture. In the other binary mixtures, total inhibition occurred at 40% or 50%. There was marginal stimulation of β-galactosidase activity by 5% of 9:1 ethanol-butanol mixture. All the concentration-response curves were described using the Gormpertz model. The EC_{50} and NOEC values and their statistical associations among the ethanol mixtures with pentanol, chloroform and butanol are shown in Table 4.4. EC_{50} values for 7:3 ethanol-pentanol and 7:3 ethanol-butanol were statistically the same ($P > 0.05$). The NOEC values for 9:1 ethanol-chloroform and 7:3 ethanol-chloroform mixtures are significantly different from other values. The ANOVA and Post Hoc Test Tables are shown in Appendix 3.0A.

Figure 4.7 shows the inhibition of the activities of cell-free β-galactosidase from *Escherichia coli* by binary mixtures of DMSO with water-immiscible solvents (chloroform, *n*-pentanol or butanol) in 9:1 and 7:3 ethanol: water-immiscible solvent ratios. As with ethanol, DMSO mixtures with chloroform, *n*-pentanol and butanol exhibited progressive inhibition of β-galactosidase activity from 5%. Total inhibition of enzyme activity occurred at 40% in all the mixtures except 9:1 ethanol: butanol, where total inhibition of β-galactosidase occurred at 50%. The 7:3 DMSO-pentanol mixture exhibited a 2-step concentration-response curve that was fitted with the bilogistic modification of the Gormpertz model. Concentration-response curves of other mixtures were describable with the single-step Gormpertz model. The EC_{50} and NOEC values and their statistical associations among the DMSO mixtures with pentanol, chloroform and butanol are shown in Table 4.4. The EC_{50} values for 7:3 DMSO-butanol were significantly different from others ($P < 0.05$). The NOEC values for 7:3 DMSO-chloroform were significantly different from others ($P < 0.05$). The ANOVA and Post Hoc Tests Tables are shown in Appendix 3.0B.

Figure 4.8 shows the inhibition of the activities of cell-free β-galactosidase from *Escherichia coli* by 9:1 and 7:3 binary mixtures DMF with chloroform, *n*-pentanol or butanol.

DMF mixtures with chloroform, *n*-pentanol or butanol exhibited progressive inhibition of β-galactosidase activity from 5%. Total inhibition of enzyme activity occurred at 40% of the 9:1 DMF-pentanol mixture. In all other mixtures, inhibition of β-galactosidase occurred at 25% or 30%. All concentration-response curves were describable with the single-step Gormpertz model. The EC_{50} and NOEC values and their statistical associations among the DMF mixtures with pentanol, chloroform and butanol are shown in Table 4.4. The EC_{50} values for 7:3 DMF-butanol, 9:1 DMF-pentanol and 9:1 DMF-butanol mixtures were statistically different from each other and from other DMF-pentanol and DMF-chloroform mixtures ($P < 0.05$). The NOEC value of the 7:3 DMF-pentanol mixture was significantly different from the NOEC values of other DMF mixtures with chloroform, butanol and pentanol (P, 0.05). The ANOVA and Post Hoc Tests Tables are shown in Appendix 3.0C.

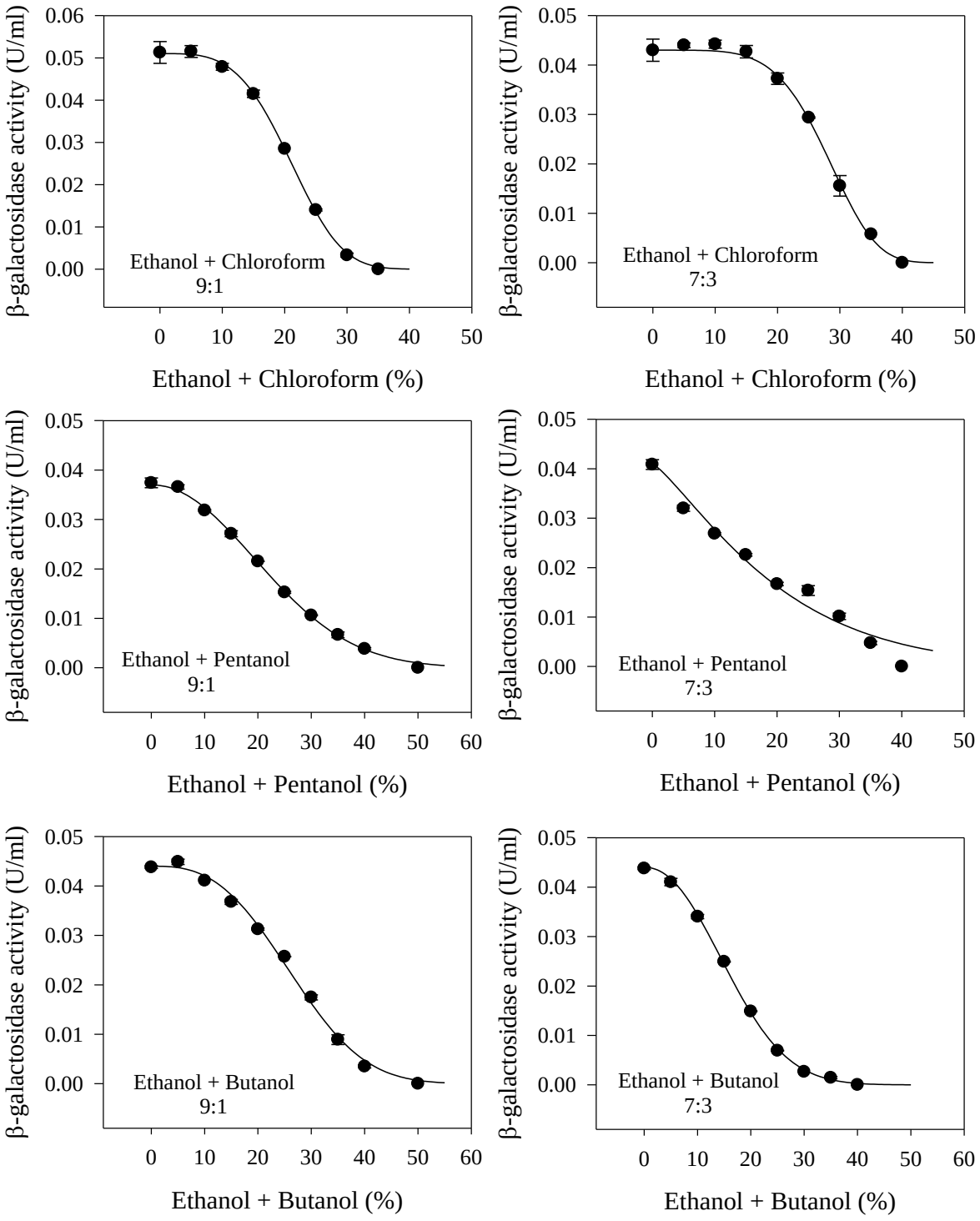

Fig. 4.6: Inhibition of the activities of cell-free β-galactosidase from *Escherichia coli* by binary mixtures of ethanol with chloroform, n-pentanol and butanol. The solid line represents the Gormpertz model fit to the observed data.

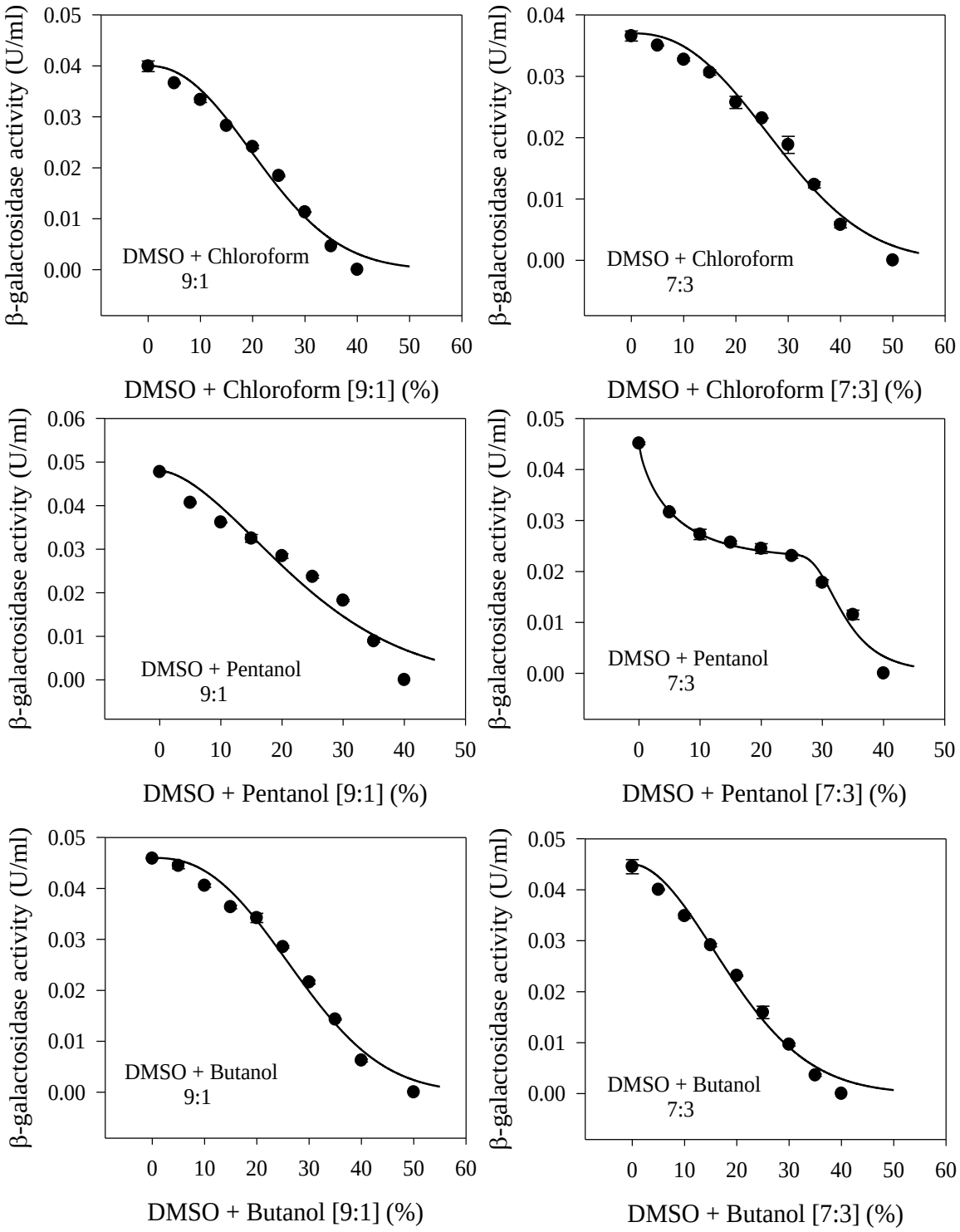

Fig. 4.7: Inhibition of the activities of cell-free β-galactosidase from *Escherichia coli* by binary mixtures of DMSO with chloroform, n-pentanol and butanol. The solid line represents the Gormpertz model fit to the observed data.

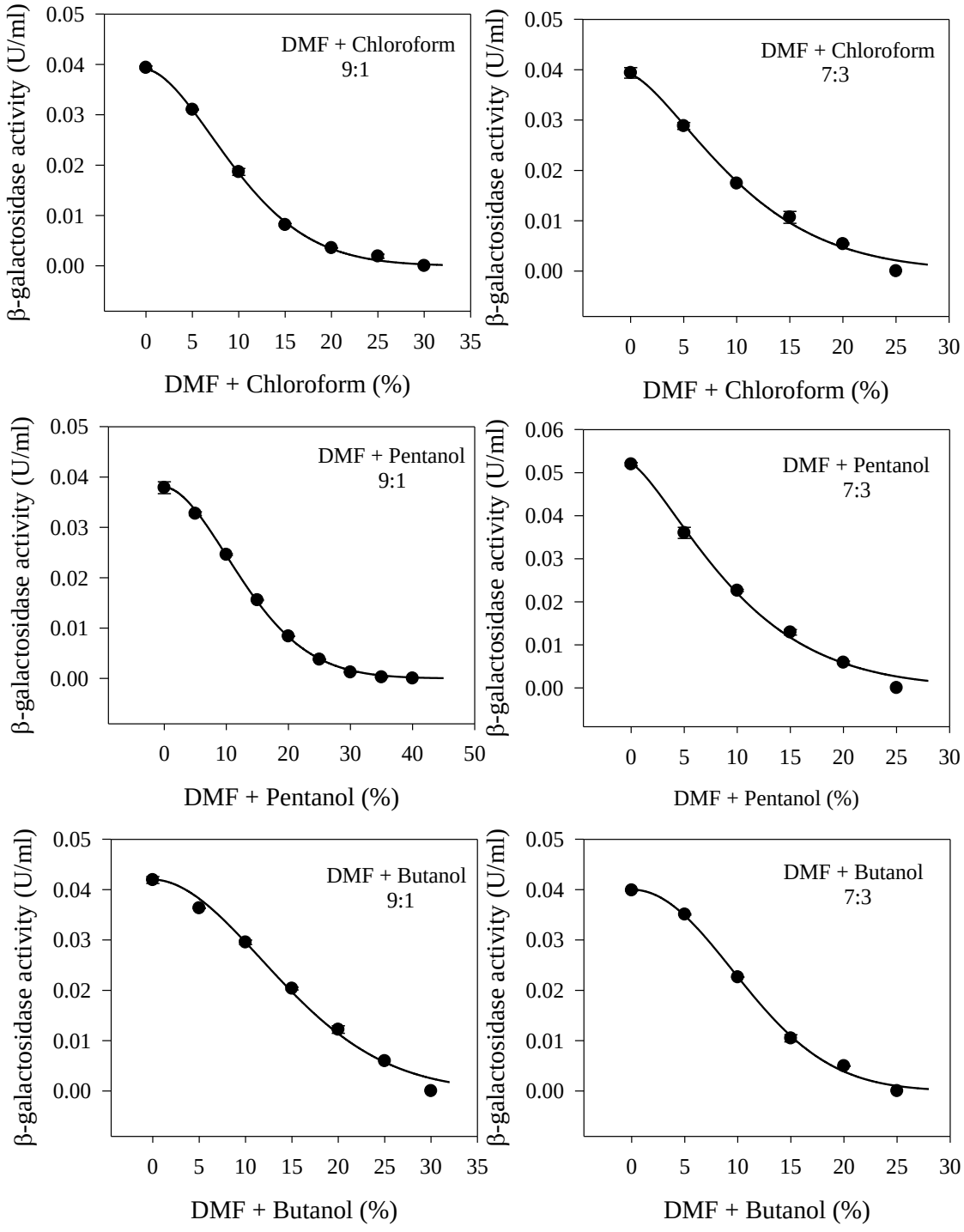

Fig. 4.8: Inhibition of the activities of cell-free β-galactosidase from *Escherichia coli* by binary mixtures DMF with chloroform, n-pentanol and butanol. The solid line represents the Gormpertz model fit to the observed data.

Table 4.4 The threshold inhibitory concentrations of binary mixtures of water-miscible and water-immiscible solvents against β-galactosidase from *Escherichia coli*

Solvents	EC50 (%)	NOEC (%)
Ethanol+Chloroform 9:1	20.946 ± 0.286^{b}	10.334 ± 0.527^{b}
Ethanol+Chloroform 7:3	27.983 ± 0.391^{c}	16.817 ± 0.859^{a}
Ethanol+Pentanol 9:1	22.343 ± 0.310^{d}	4.590 ± 0.311^{c}
Ethanol+Pentanol 7:3	15.839 ± 1.246^{a}	1.075 ± 0.460^{d}
Ethanol+Butanol 9:1	26.389 ± 0.436^{e}	4.920 ± 0.552^{c}
Ethanol+Butanol 7:3	16.145 ± 0.179^{a}	$1.835 \pm 0.132_{d}$
DMSO+Chloroform 9:1	22.240 ± 0.884^{b}	4.934 ± 0.970^{b}
DMSO+Chloroform 7:3	28.380 ± 0.882^{c}	6.983 ± 1.152^{c}
DMSO+Pentanol 9:1	21.713 ± 1.631^{b}	1.141 ± 0.679^{a}
DMSO+Pentanol 7:3	26.874 ± 1.876^{c}	ND
DMSO+Butanol 9:1	27.710 ± 0.824^{c}	0.667 ± 0.264^{a}
DMSO+Butanol 7:3	19.207 ± 0.770^{a}	3.693 ± 0.689^{b}
DMF+Chloroform 9:1	9.545 ± 0.190^{e}	0.694 ± 0.085^{b}
DMF+Chloroform 7:3	$9.166 \pm 0.497^{d,e}$	0.950 ± 0.249^{b}
DMF+Pentanol 9:1	12.860 ± 0.202^{b}	2.295 ± 0.160^{c}
DMF+Pentanol 7:3	8.517 ± 0.439^{d}	0.312 ± 0.103^{a}
DMF+Butanol 9:1	14.309 ± 0.521^{c}	1.972 ± 0.391^{c}
DMF+Butanol 7:3	11.028 ± 0.246^{a}	0.825 ± 0.132^{b}

The data shown represents mean values and SD.

Within the column for ethanol, DMSO, or DMF mixtures, EC_{50} or NOEC values with the same subscript letter are not significantly different from each other ($P > 0.05$).

4.1.6 Inhibition of the activities of cell-free β-galactosidase from *Kluyveromyces marxianus* by binary mixtures of water-miscible and water-immiscible solvents

Figure 4.9 shows the inhibition of the activities of cell-free β-galactosidase from *Kluyveromyces marxianus* by binary mixtures of ethanol with water-immiscible solvents. Ethanol-Butanol (9:1) did not inhibit enzyme activity at 5%, 10% and 15% concentration. Other mixtures exhibited progressive inhibition of β-galactosidase activity from 5%. Total inhibition occurred at 30% of ethanol-chloroform mixtures. In the other binary mixtures, total inhibition occurred at 50%. All the concentration-response curves were described using the single-step Gormpertz model. The EC_{50} and NOEC values and their statistical associations among the ethanol mixtures with pentanol, chloroform and butanol are shown in Table 4.5. The EC_{50} values for 9:1 ethanol-chloroform, 7:3 ethanol-chloroform, 7:3 ethanol-butanol and 9:1 ethanol-butanol mixtures were significantly different from each other and from ethanol-pentanol mixtures. The NOEC value for the 7:3 ethanol-chloroform mixture was statistically different from other NOEC values for ethanol-chloroform, ethanol-pentanol and ethanol-butanol mixtures. The ANOVA and Post Hoc Tests Tables are shown in Appendix 4.0 A.

Figure 4.10 shows the inhibition of the activities of cell-free β-galactosidase from *Kluyveromyces marxianus* by binary mixtures of DMSO with chloroform, *n*-pentanol or butanol. In all the mixtures, the activities of β-galactosidase were progressively inhibition from 5%. Total inhibition of enzyme activity occurred at 40% for all DMSO-Pentanol and DMSO-Chloroform. The DMSO-Butanol (9:1) and DMSO-Chloroform (9:1) mixtures were described with a single-step Gormpertz concentration-response model. Other mixtures exhibited a 2-step concentration-response pattern and were fitted with 2-step models. The EC_{50} and NOEC values and their statistical associations among the DMSO mixtures with pentanol, chloroform and butanol are shown in Table 4.5. The EC_{50} values for DMSO-chloroform and 7:3 DMSO-pentanol mixtures were not statistically different from each other ($P > 0.05$). Similarly, the EC_{50} values for 7.3 DMSO-chloroform, 7:3 DMSO-pentanol and 9:1 DMSO-butanol mixtures were not statistically different from each other ($P > 0.05$). The NOEC value for the 7:3 DMSO-pentanol mixture was significantly different from other values. The ANOVA and Post Hoc Tests Tables are shown in Appendix 4.0B.

Figure 4.11 shows the inhibition of the activities of cell-free β-galactosidase from *Kluyveromyces marxianus* by binary mixtures of DMF with chloroform, *n*-pentanol or

butanol. The mixtures exhibited progressive inhibition of β-galactosidase activity from 5%. Total inhibition of enzyme activity occurred at 60% of the 9:1 DMF-Pentanol mixture and 50% of the 7:3 DMF-Butanol mixture. Total inhibition of β-galactosidase activity occurred at 40% in other mixtures. 9:1 DMF-Butanol and both DMF-Pentanol mixtures were described with a 2-step concentration-response model, while other mixtures were described with the single-step Gormpertz model. The EC_{50} and NOEC values and their statistical associations among the mixtures are shown in Table 4.5. The EC50 values for 7:3 DMF-pentanol and both DMF-butanol mixtures are statistically different from each other ($P < 0.05$). The NOEC values for 9:1 DMF-pentanol, 7:3 DMF-chloroform and 7:3 DMF-butanol mixtures were statistically different from each other ($P < 0.05$). The ANOVA and Post Hoc Tests Tables are shown in Appendix 4.0C.

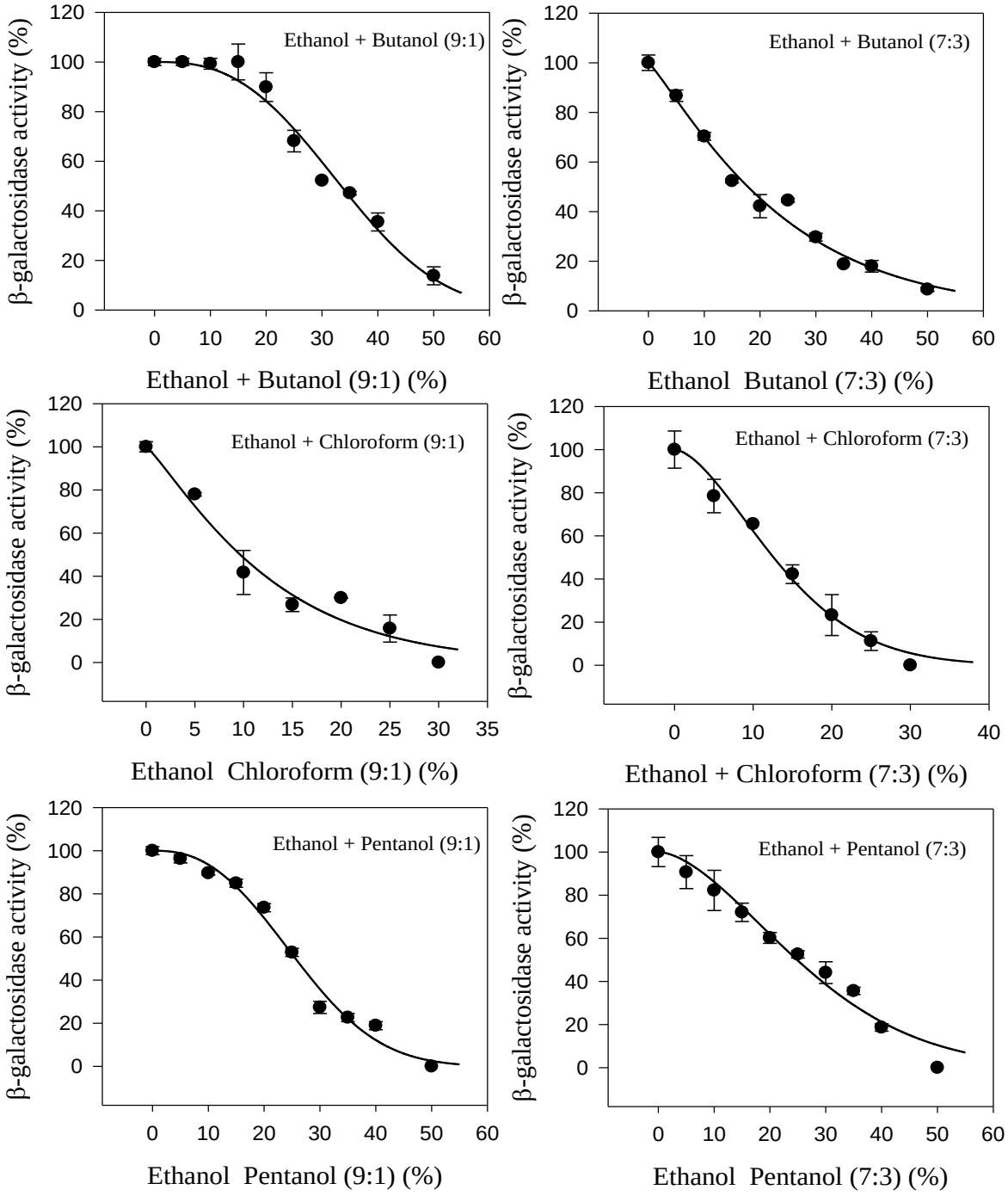

Fig. 4.9: Inhibition of the activities of cell-free β-galactosidase from *Kluyveromyces marxianus* by binary mixtures of ethanol with chloroform, n-pentanol and butanol. The solid line represents the Gormpertz model fit to the observed data.

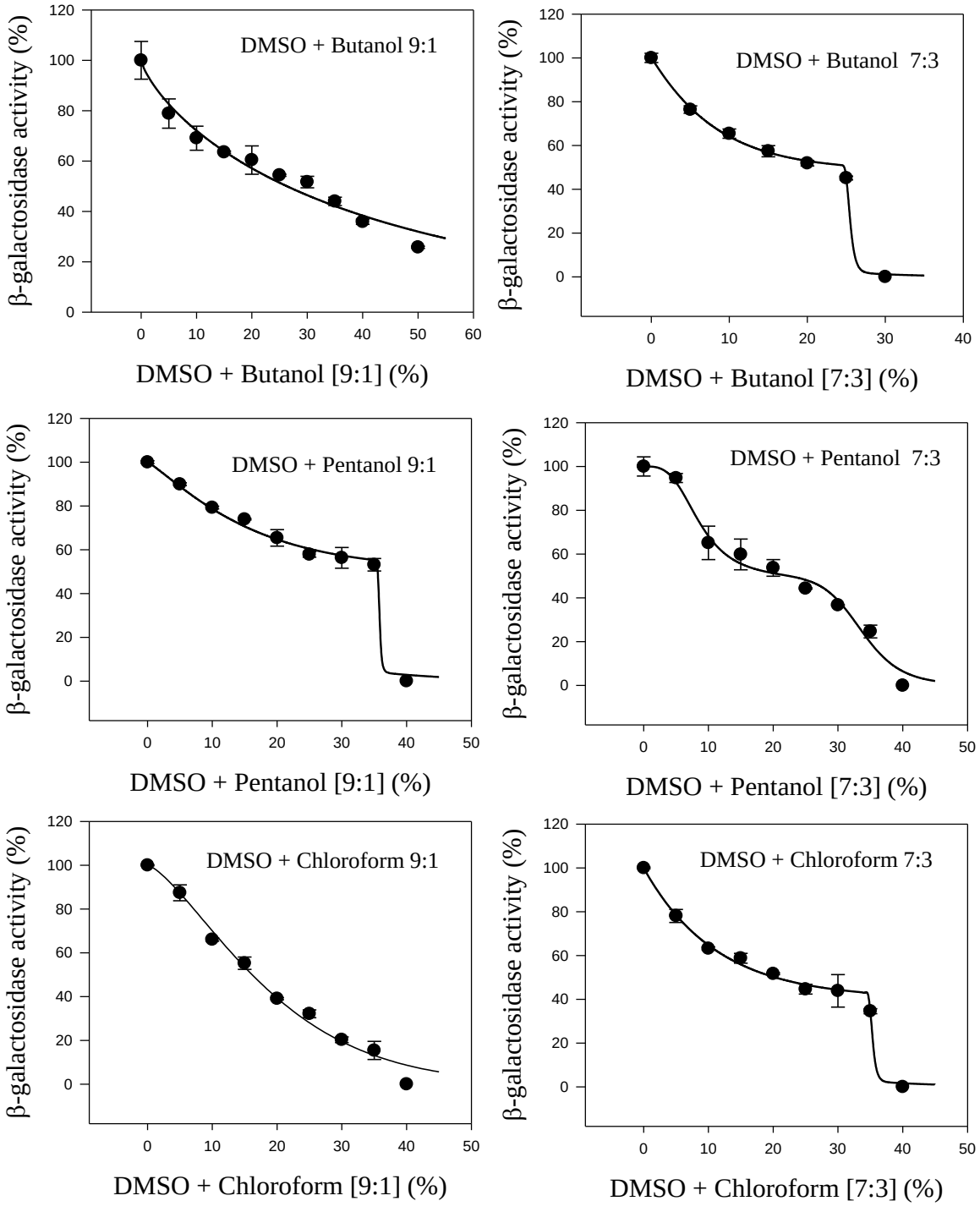

Fig. 4.10: Inhibition of the activities of cell-free β-galactosidase from *Kluyveromyces marxianusi* by binary mixtures of DMSO with chloroform, n-pentanol and butanol. The solid line represents the model fit to the observed data.

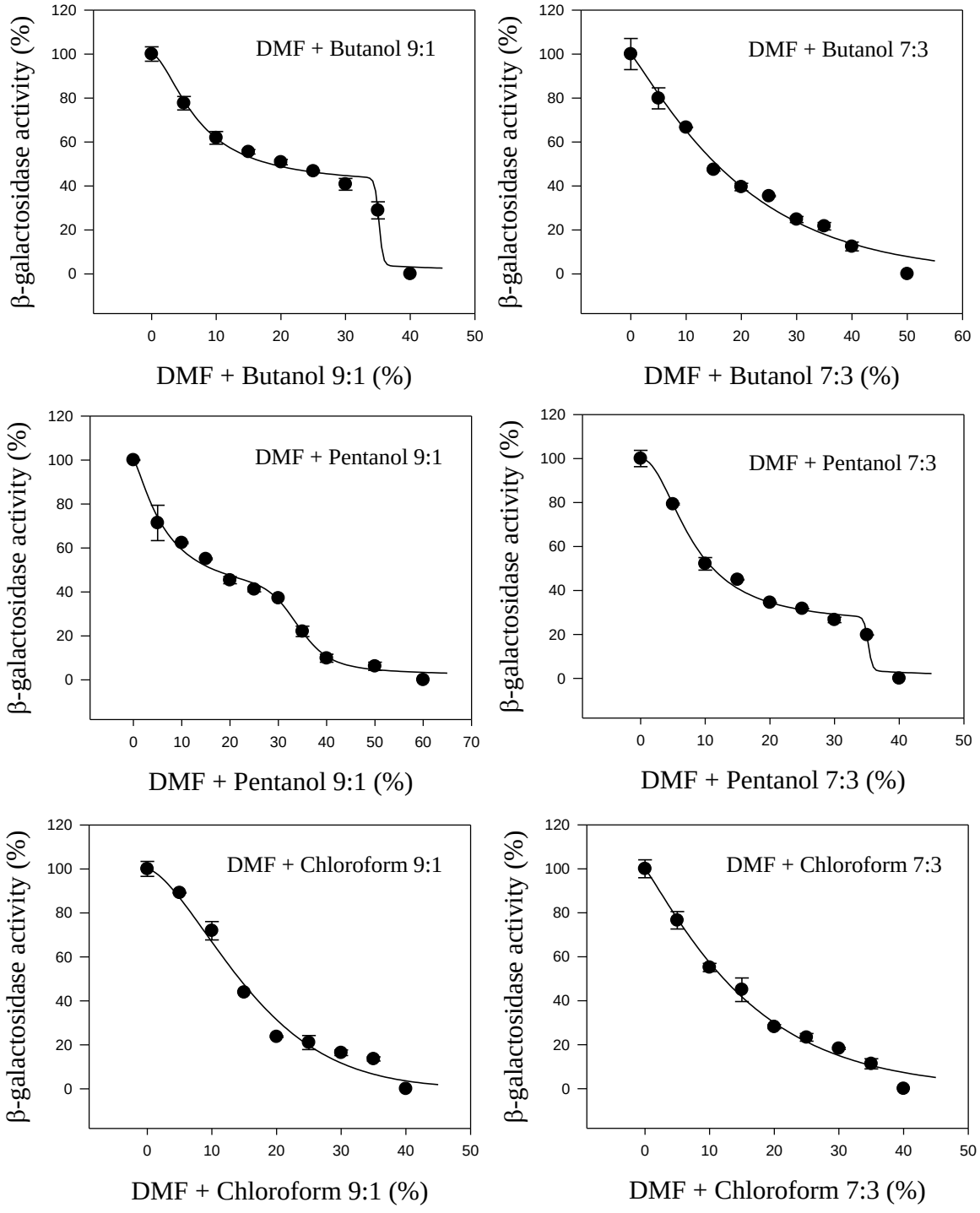

Fig. 4.11: Inhibition of the activities of cell-free β-galactosidase from *Kluyveromyces marxianus* by binary mixtures DMF with chloroform, n-pentanol and butanol. The solid line represents the model fit to the observed data.

Table 4.5 The threshold inhibitory concentrations of binary mixtures of water-miscible and water-immiscible solvents against β-galactosidase from *Kluyveromyces marxianus*

Solvents	EC50 (%)	NOEC (%)
Ethanol+Chloroform 9:1	9.648 ± 1.373^{a}	0.540 ± 0.399^{a}
Ethanol+Chloroform 7:3	12.599 ± 1.079^{b}	3.632 ± 1.021^{b}
Ethanol+Pentanol 9:1	25.615 ± 0.815^{d}	6.049 ± 1.029^{c}
Ethanol+Pentanol 7:3	24.859 ± 1.487^{d}	$6.307 \pm 1.498^{c,d}$
Ethanol+Butanol 9:1	33.422 ± 1.131^{e}	8.059 ± 1.131^{d}
Ethanol+Butanol 7:3	17.823 ± 0.966^{c}	1.211 ± 0.346^{a}
DMSO+Chloroform 9:1	16.156 ± 0.842^{a}	0.288 ± 0.124^{c}
DMSO+Chloroform 7:3	$20.130 \pm 3.225^{a,b}$	2.301 ± 1.062^{b}
DMSO+Pentanol 9:1	ND	ND
DMSO+Pentanol 7:3	$22.260 \pm 8.855^{a,b}$	4.213 ± 1.666^{a}
DMSO+Butanol 9:1	26.326 ± 2.049^{b}	$1.566 \pm 0.678^{b,c}$
DMSO+Butanol 7:3	ND	ND
DMF+Chloroform 9:1	$14.274 \pm 0.908^{b,c}$	1.986 ± 0.561^{c}
DMF+Chloroform 7:3	$12.183 \pm 0.822^{a,b}$	0.976 ± 0.294^{b}
DMF+Pentanol 9:1	$16.817 \pm 2.475^{c,d}$	0.081 ± 0.078^{a}
DMF+Pentanol 7:3	11.179 ± 0.751^{a}	$1.887 \pm 0.494^{b,c}$
DMF+Butanol 9:1	18.416 ± 2.467^{d}	$1.320 \pm 0.766^{b,c}$
DMF+Butanol 7:3	15.436 ± 0.990^{c}	2.019 ± 0.503^{c}

The data shown represents mean values and SD.

Within the column for ethanol, DMSO, or DMF mixtures, EC_{50} or NOEC values with the same subscript letter are not significantly different from each other ($P > 0.05$).

4.1.7 Inhibition of the activities of cell-free β-galactosidase from *Escherichia coli* by Ethanol-DMSO binary mixtures

Inhibitions of the activities of cell-free β-galactosidase from *Escherichia coli* by ethanol and DMSO in a different batch of experiments as individual components of ethanol-DMSO binary mixtures are shown in Figure 4.12. A similar trend of concentration-response relationship, as shown in Figure 4.4 for different experiments, was observed for the individual ethanol and DMSO. Ethanol stimulated β-galactosidase activity at 5% and 10%. Higher ethanol concentrations progressively inhibited the enzyme activity until total inhibition occurred at 50%. DMSO inhibited the enzyme activity progressively from 5% until total inhibition occurred at 50%.

Inhibitions of the activities of cell-free β-galactosidase from *Escherichia coli* by binary mixtures of ethanol and DMSO are shown in Figure 4.13. At low concentrations (5%, 10% and 15%), 9:1 and 8:2 ethanol-DMSO mixtures stimulated β-galactosidase activity. Similarly, 5% and 10% of 6:4 ethanol-DMSO mixture stimulated β-galactosidase activity. Minor stimulation of β-galactosidase activity occurred at 5% and 10% of 7:3 ethanol-DMSO mixture. At concentrations above 10% or 15%, as the case may be, β-galactosidase activity was progressively inhibited in all the mixture ratios. Total inhibition of β-galactosidase activity occurred at 50% in all ethanol-DMSO mixtures.

Table 4.6 shows the median inhibitory concentrations (EC_{50}) and No-Observable-Effect-Concentrations (NOEC) of ethanol-DMSO mixtures. The Toxic Index (TI) and combined effect of the mixtures on the activity of cell-free β-galactosidase from *Escherichia coli* are also shown in Table 4.6. The observed EC_{50} values for all the mixtures are not significantly different ($p<0.05$) from each other. Similarly, the CA-predicted EC_{50} values for all the mixtures are not significantly different ($p<0.05$) from each. The observed EC_{50} and CA-predicted EC_{50} for the 7:3 ethanol-DMSO mixture were not significantly different from each other ($P > 0.05$). The NOEC values for DMSO were significantly different from those of ethanol and all ethanol-DMSO mixtures. In all the mixtures, the TI values were marginally higher than one(1); thus, the combined effect was considered additive. The ANOVA and Post Hoc Tests Tables are shown in Appendix 5.0.

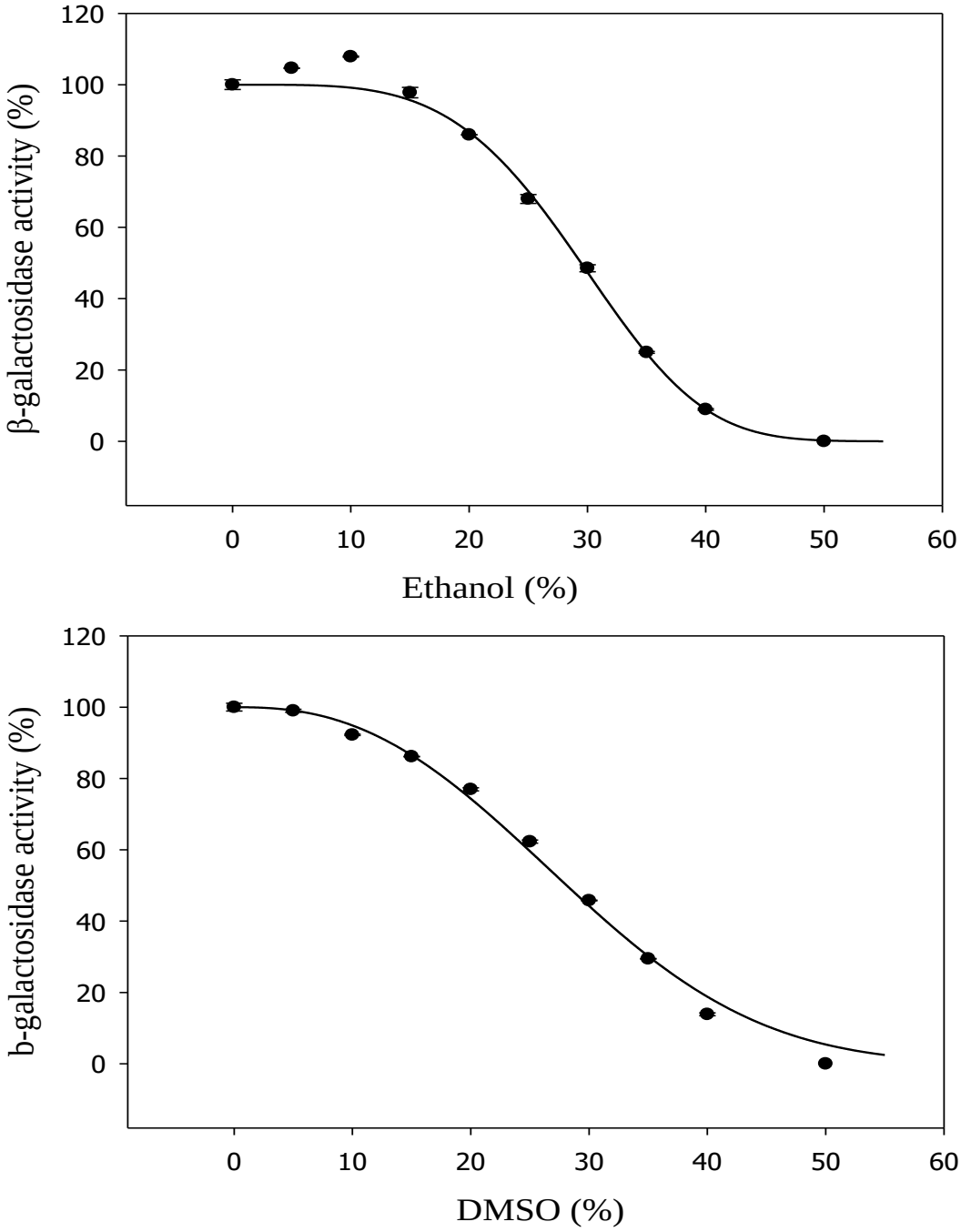

Figure 4.12 Inhibition of the activities of cell-free β-galactosidase from *Escherichia coli* by individual ethanol and DMSO in a different batch of experiment with binary mixtures of ethanol and DMSO. The solid line represents the Gormpertz model fit to the observed data.

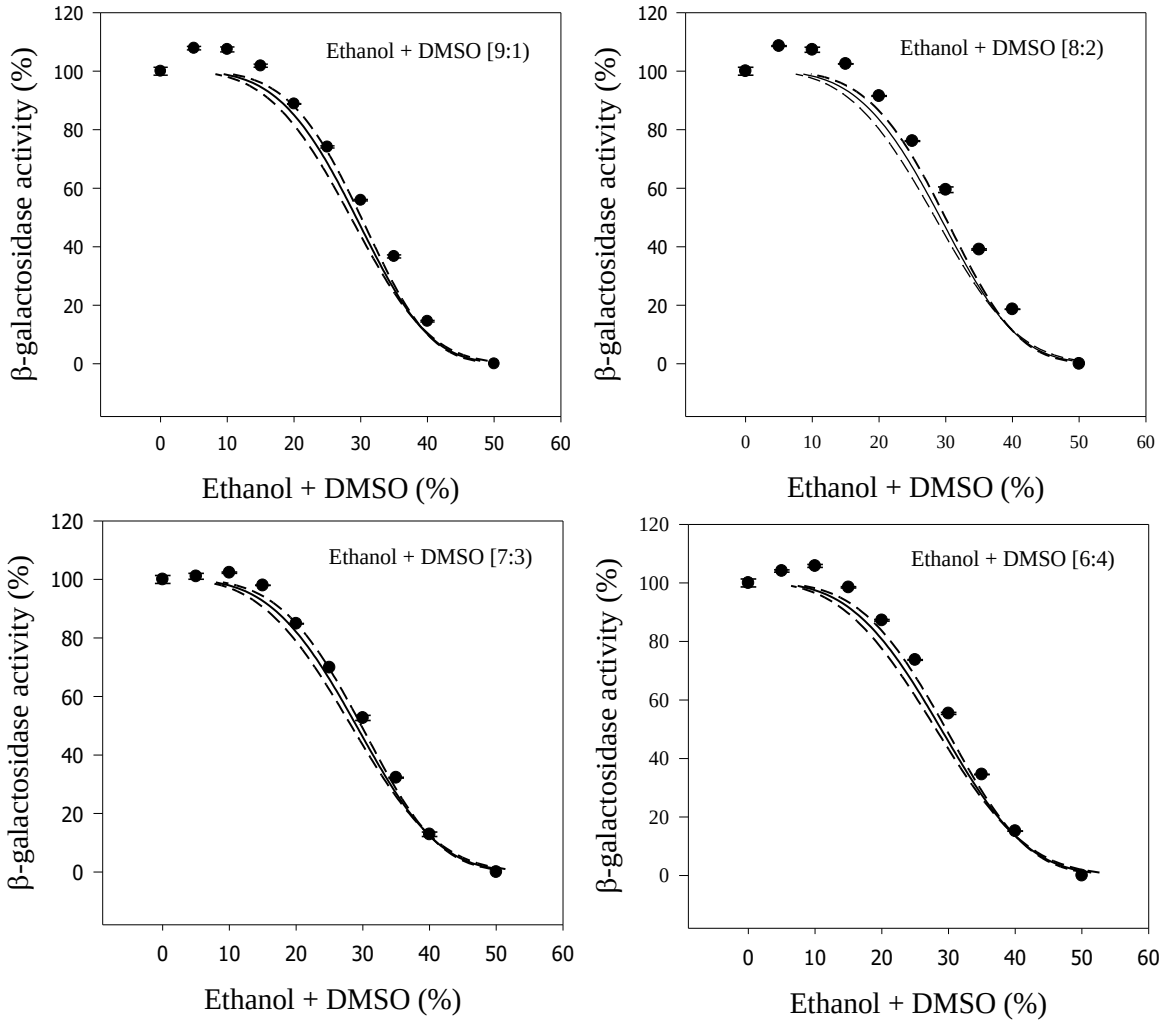

Figure 4.13 Inhibition of the activities of cell-free β-galactosidase from *Escherichia coli* by binary mixtures of ethanol and DMSO. The solid and dashed lines represent the mean and 95% confidence limit of CA model-predicted concentration-response relationships.

Table 4.6 Median inhibitory concentrations (*EC*50) of ethanol-DMSO mixtures, NOEC, toxic index and combined effect of ethanol-DMSO mixtures on cell-free β-galactosidase from *Escherichia coli*

Solvent/Solvent mixture	EC_{50} (%)		NOEC (%)	TI	Combined Effect
	Observed	CA-predicted			
Ethanol	29.246 ± 2.986[a,b]	-	11.265 ± 1.121[b]	-	-
DMSO	28.112 ± 0.471[a]	-	6.407 ± 0.564[a]	-	-
Ethanol: DMSO (9:1)	31.256 ± 0.642[b,c]	29.311 ± 0.772[a]	12.166 ± 1.453[b]	1.078 ± 0.079[a]	Additive
Ethanol: DMSO (8:2)	33.045 ± 1.883[c]	29.172 ± 0.767[a]	12.685 ± 1.158[b]	1.142 ± 0.032[a]	Additive
Ethanol: DMSO (7:3)	30.987 ±	29.034 ± 0.763[a*]	10.741 ± 0.660[b]	1.076 ± 0.046[a]	Additive
Ethanol: DMSO (6:4)	31.346 ± 0.345[b,c]	28.897 ± 0.758[a]	11.601 ± 0.940[b]	1.093 ± 0.062[a]	Additive

Values shown are Mean ± Standard Deviation.
Within a column, EC_{50} (observed and CA-predicted), NOEC or TI values with with same letter (asterik) are not significantly different from each other ($P < 0.5$).
Comparing observed and CA-predicted EC_{50}, values with asterisk are not statististically different from each other ($P > 0.05$)

4.1.8 Inhibition of the activities of cell-free β-galactosidase from *Escherichia coli* by Ethanol-DMF binary mixtures

Inhibitions of the activities of cell-free β-galactosidase from *Escherichia coli* by ethanol and DMF in a different batch of experiments as individual components of ethanol-DMF binary mixtures are shown in Figure 4.14. A similar trend of concentration-response relationship, as shown in Figures 4.4 and 4.12 for a different batch of experiment, was observed in this batch of experiment for the individual ethanol and DMF. At 5%, 10% and 15%, ethanol stimulated *Escherichia coli* β-galactosidase activity. Higher ethanol concentrations progressively inhibited the enzyme activity until total inhibition occurred at 50%. DMF inhibited the enzyme activity progressively from 5% until total inhibition occurred at 40%.

Inhibitions of the activities of cell-free β-galactosidase from *Escherichia coli* by binary mixtures of ethanol and DMF are shown in Figure 4.15. At low concentrations (5% and 10%), 9:1 ethanol-DMF mixture slightly stimulated β-galactosidase activity. In other mixtures, minor stimulation of β-galactosidase activity occurred at 5%. At concentrations above 5% or 10%, as the case may be, β-galactosidase activities were inhibited progressively until complete inhibition occurred at 50%

Table 4.7 shows the observed and CA-predicted median inhibitory concentrations (EC_{50}) and No-Observable-Effect-Concentrations (NOEC) of ethanol-DMF mixtures. The Toxic Index (TI) and combined effect of the mixtures on the activity of cell-free β-galactosidase from *Escherichia coli* are also shown in Table 4.7. The statistical associations of the EC_{50}, NOEC and TI values are also shown. Given a lower EC_{50} value, DMF is significantly more toxic than ethanol ($P < 0.05$). The observed EC_{50} values for the 7:3 ethanol-DMF mixture were not significantly different from the 6:4 and 8:2 ethanol-DMF mixtures. The EC_{50} values for the 9:1 ethanol-DMF mixture were significantly different from other mixtures ($P < 0.05$). The NOEC values for ethanol are statistically different from each other ($P < 0.05$). The NOEC value for the 9:1 ethanol-DMF mixture is statistically different from other ethanol-DMF mixtures. The TI values for 9:1 and 8:2 ethanol-DMF mixtures are not statistically different from each other ($P > 0.05$). Similarly, the TI values for 7:1 and 6:4 ethanol-DMF mixtures were not statistically different from each other ($P > 0.05$). In all the

mixtures, the TI values are marginally higher than one (1); thus, the combined effect was considered additive.

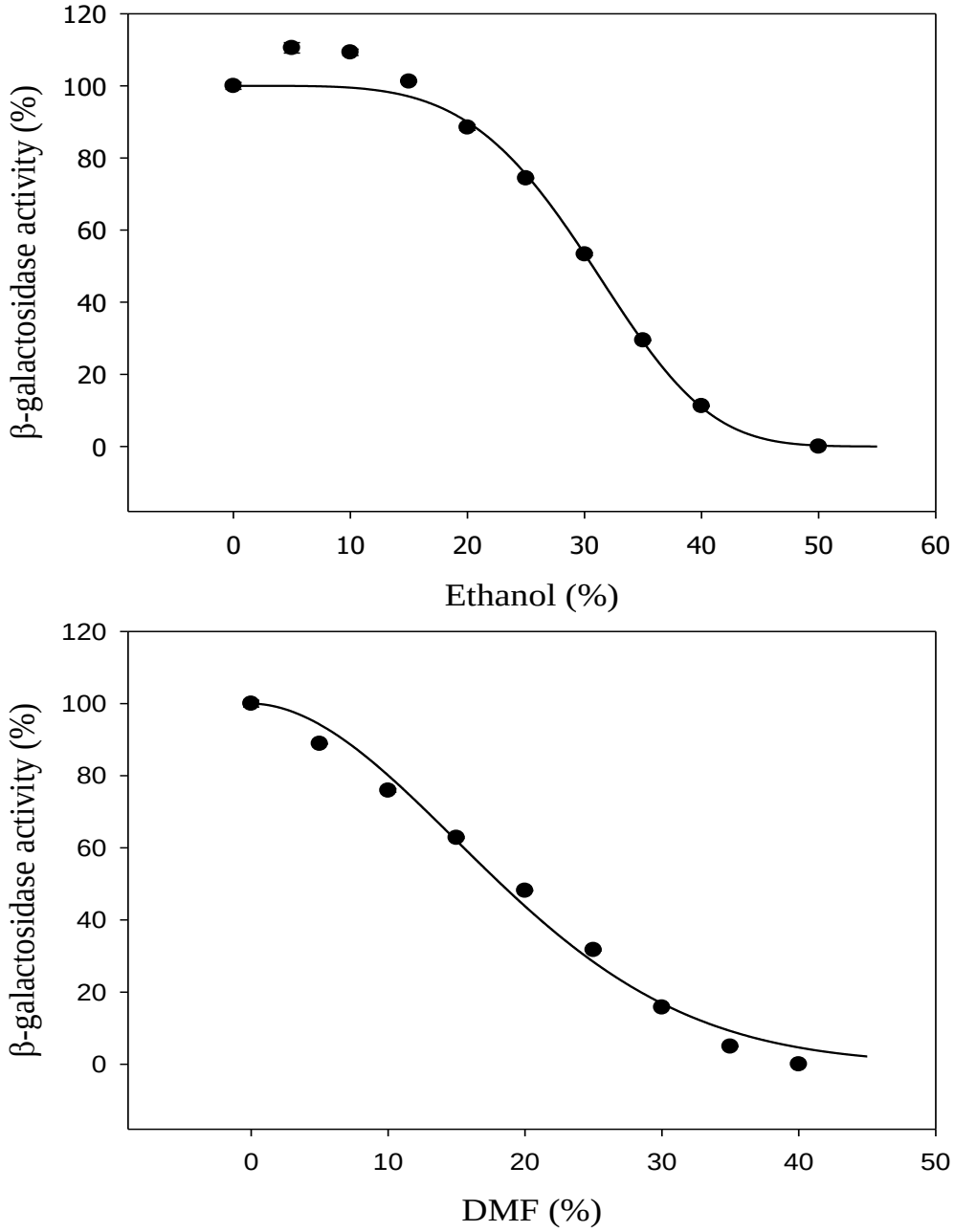

Figure 4.14 Inhibition of the activities of cell-free β-galactosidase from *Escherichia coli* by individual ethanol and DMF in a different batch of experiment with binary mixtures of ethanol and DMF. The solid line represents the Gormpertz model fit to the observed data.

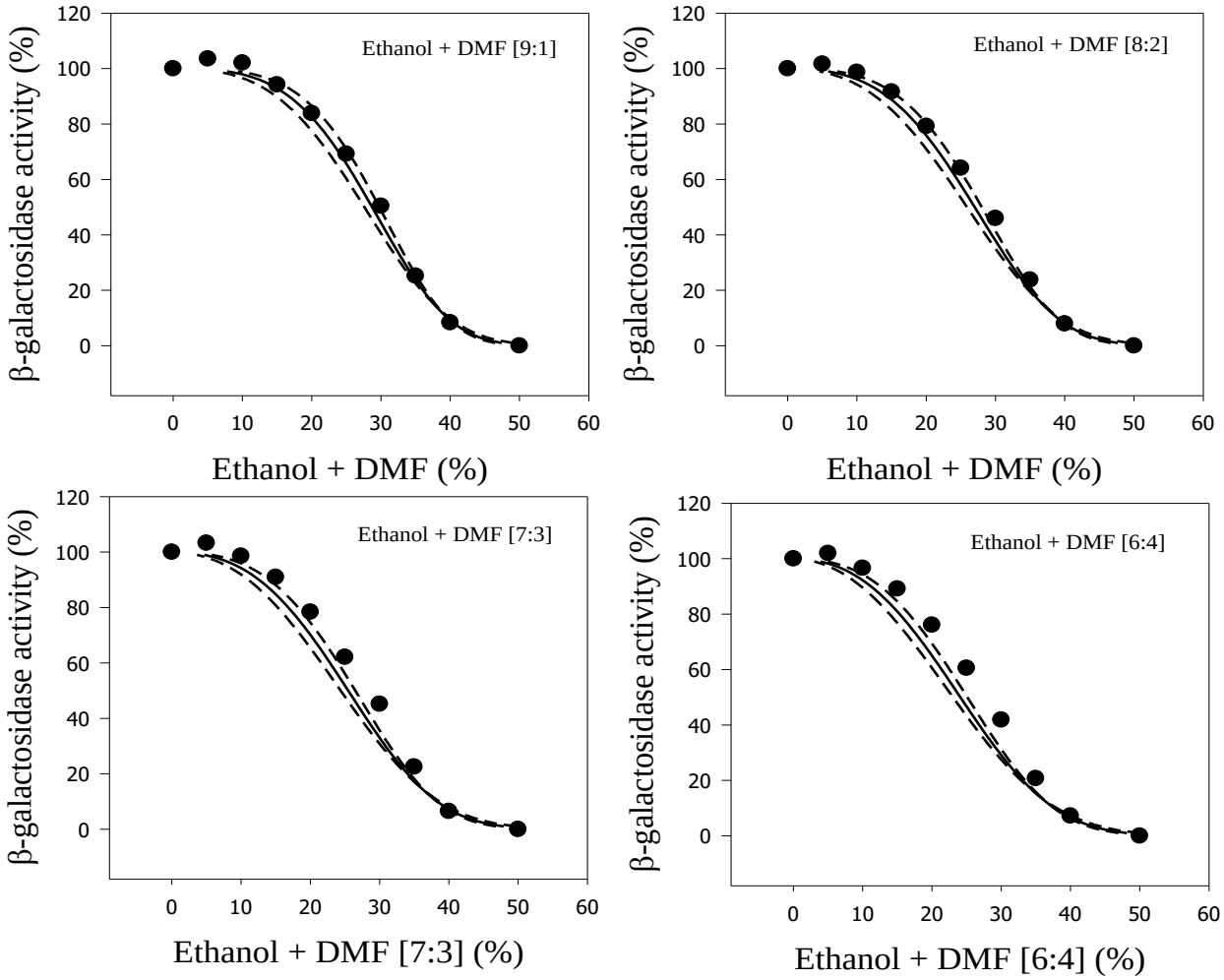

Figure 4.15 Inhibition of the activities of cell-free β-galactosidase from *Escherichia coli* by binary mixtures of ethanol and DMF. The solid and dashed lines represent the mean and 95% confidence limit of CA model-predicted concentration-response relationships.

Table 4.7: Median inhibitory concentrations (EC_{50}) of ethanol-DMF mixtures, NOEC, toxic index and combined effect of ethanol-DMF mixtures on cell-free β-galactosidase from *Escherichia coli*

Solvent/Solvent mixture	EC_{50} (%)		NOEC (%)	TI	Combined Effect
	Observed	CA-predicted			
Ethanol	30.687 ± 0.726a	-	11.556 ± 1.648[a]	-	-
DMF	18.244 ± 0.674[e]	-	1.897 ± 0.427[d]	-	-
Ethanol: DMF (9:1)	29.497 ± 0.329[d]*	28.703 ± 1.074[c]*	9.929 ± 0.670[c]	1.027 ± 0.015[a]	Additive
Ethanol: DMF (8:2)	28.395 ± 0.275[c]*	26.981 ± 1.066[b,c]*	8.237 ± 0.486[b]	1.052 ± 0.019[a]	Additive
Ethanol: DMF (7:3)	28.017 ± 0.346[b,c]	25.454 ± 1.053[a,b]	8.026 ± 0.604[b]	1.100 ± 0.018[b]	Additive
Ethanol: DMF (6:4)	27.368 ± 0.270[b]	24.091 ± 1.036[a]	7.283 ± 0.440[b]	1.136 ± 0.024[b]	Additive

Values shown are Mean ± Standard Deviation.
Within a column, EC_{50} (observed and CA-predicted), NOEC or TI values with with same letter (asterik) are not significantly different from each other ($P < 0.5$).
Comparing observed and CA-predicted EC_{50}, values with asterisk are not statististically different from each other ($P > 0.05$)

4.1.9 Inhibition of the activities of cell-free β-galactosidase from *Kluyveromyces marxianus* by Ethanol-DMSO binary mixtures

Inhibitions of the activities of cell-free β-galactosidase from *Kluyveromyces marxianus* by ethanol and DMSO in a batch of experiments as individual components of ethanol-DMSO binary mixtures are shown in Figure 4.16. A similar trend of concentration-response relationship, as shown in Figure 4.5 for different experiments, was observed for the individual ethanol and DMSO. Ethanol inhibited β-galactosidase activity from 5% until total inhibition occurred at 40%. Similarly, DMSO inhibited the enzyme activity progressively from 5% until total inhibition occurred at 50%.

Inhibitions of the activities of cell-free β-galactosidase from *Kluyveromyces marxianus* by ethanol-DMSO binary mixtures are shown in Figure 4.17. In all the mixtures, β-galactosidase activities were inhibited at 5%. Inhibition of enzyme activity continued progressively until total inhibition occurred at 50%

Table 4.8 shows the values and statistical associations of the observed and CA-predicted median inhibitory concentrations (EC_{50}), No-Observable-Effect-Concentrations (NOEC) and TI values for ethanol-DMSO mixtures. The observed EC_{50} values for ethanol, DMSO and all the ethanol-DMSO mixtures are not significantly different from each other ($P > 0.05$). Similarly, the CA-predicted EC_{50} values for all the ethanol-DMSO mixtures are not significantly different from each other ($P < 0.05$). In comparison, there was no statistical difference between observed EC_{50} and CA-predicted EC_{50} for all ethanol-DMSO ($P > 0.05$). There was no statistical difference among NOEC values for all ethanol-DMSO mixtures. The TI values of all the ethanol-DMSO mixtures were statistically equal to each other ($P > 0.05$). In all the mixtures, the TI values were marginally higher than one(1); thus, the combined effect was considered additive. The ANOVA and Post Hoc Tests Tables are shown in Appendix 7.0.

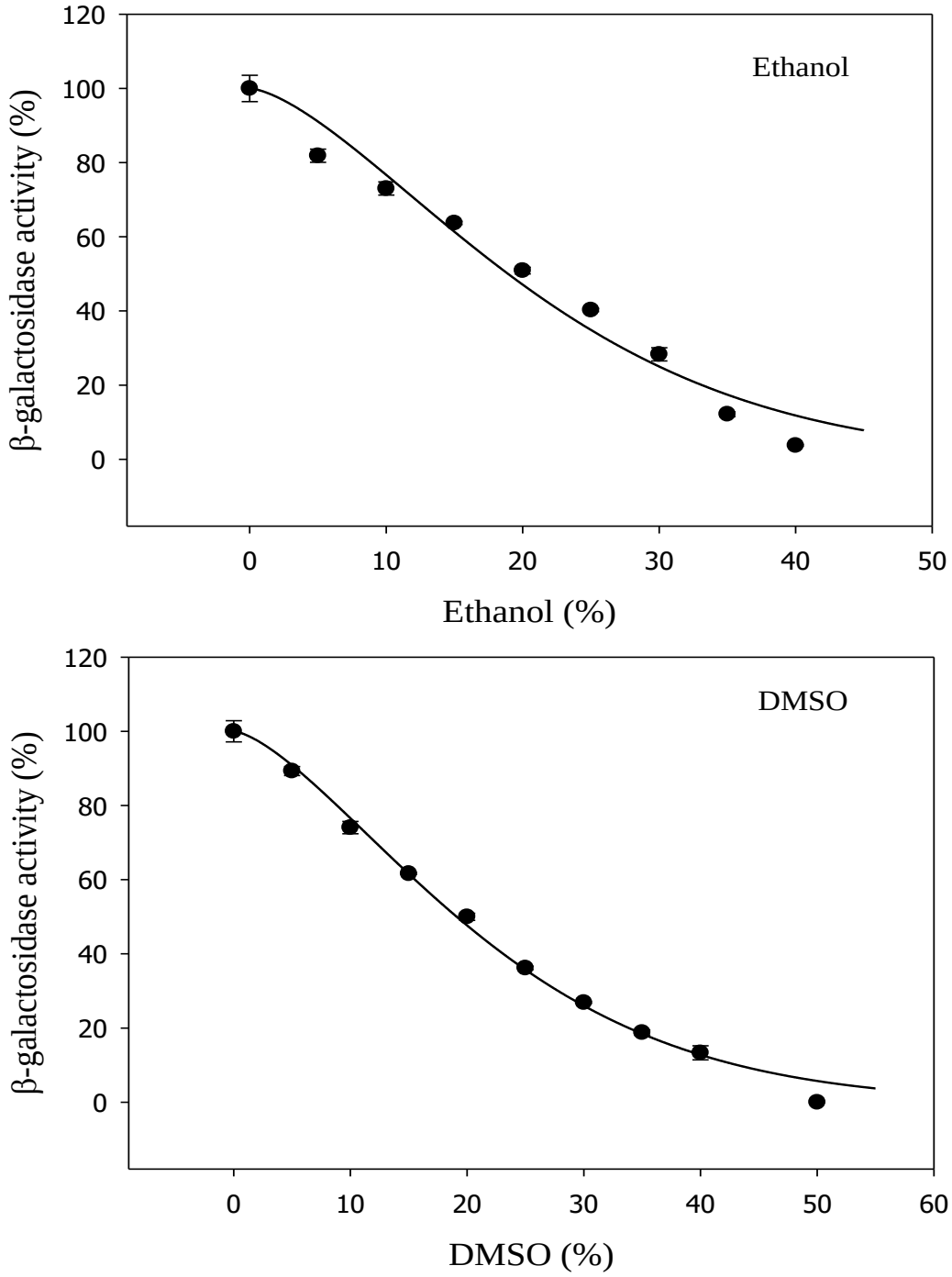

Figure 4.16 Inhibition of the activities of cell-free β-galactosidase from *Kluyveromyces marxianus* by individual ethanol and DMSO in a different batch of experiment with binary mixtures of ethanol and DMSO. The solid line represents the Gormpertz model fit to the observed data.

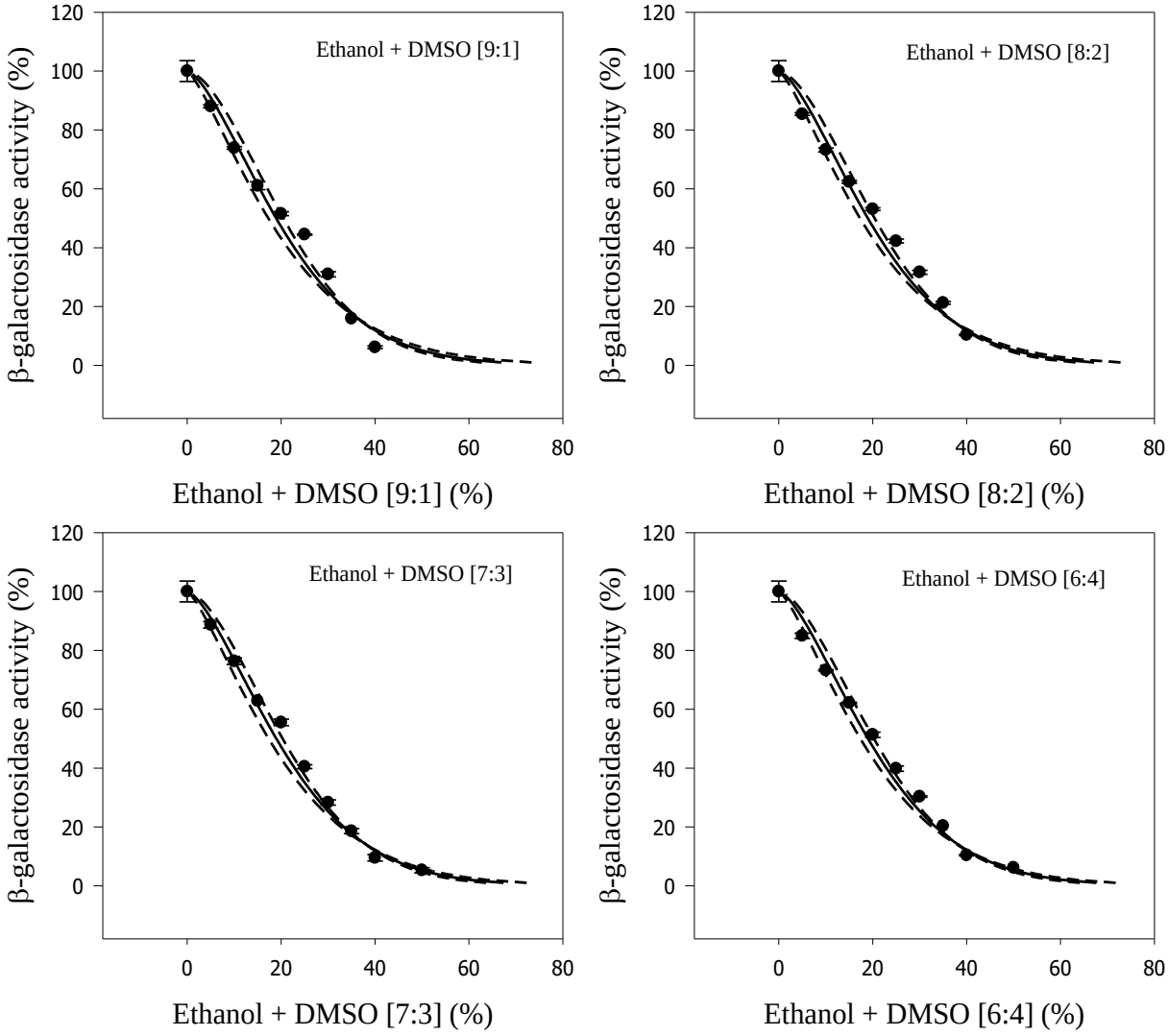

Figure 4.17 Inhibition of the activities of cell-free β-galactosidase from *Kluyveromyces marxianus* by binary mixtures of ethanol and DMSO. The solid and dashed lines represent the mean and 95% confidence limit of CA model-predicted concentration-response relationships.

Table 4.8: Median inhibitory concentrations (*EC*50) of ethanol-DMSO mixtures, NOEC, toxic index and combined effect of ethanol-DMSO mixtures on cell-free β-galactosidase from *Kluyveromyces marxianus*

Solvent/Solvent mixture	EC_{50} (%)		NOEC (%)	TI	Combined Effect
	Observed	CA-predicted			
Ethanol	18.930 ± 1.171[a]	-	2.651 ±0.796[a]	-	-
DMSO	19.114 ± 0.534[a]	-	2.215 ± 0.309[a]	-	-
Ethanol: DMSO (9:1)	19.662 ± 0.993[a*]	18.897 ± 1.604[a*]	2.659 ± 0.670[a]	1.038 ± 0.008[a]	Additive
Ethanol: DMSO (8:2)	19.823 ± 0.811[a*]	18.891 ± 1.551[a*]	2.222 ± 0.472[a]	1.046 ± 0.015[a]	Additive
Ethanol: DMSO (7:3)	20.153 ± 0.639[a*]	18.886 ± 1.497[a*]	2.953 ± 0.442[a]	1.062 ± 0.021[a]	Additive
Ethanol: DMSO (6:4)	19.350 ± 0.667[a*]	18.880 ± 1.444[a*]	2.235 ± 0.373[a]	1.018 ± 0.014[a]	Additive

Values shown are Mean ± Standard Deviation.
Within a column, EC_{50} (observed and CA-predicted), NOEC or TI values with with same letter (asterik) are not significantly different from each other ($P < 0.5$).
Comparing observed and CA-predicted EC_{50}, values with asterisk are not statistically different from each other ($P > 0.05$)

4.1.10 Inhibition of the activities of cell-free β-galactosidase from *Kluyveromyces marxianus* by Ethanol-DMF binary mixtures

Inhibitions of the activities of cell-free β-galactosidase from *Kluyveromyces marxianus* by ethanol and DMF in a different batch of experiments as individual components of ethanol-DMF binary mixtures are shown in Figure 4.18. Ethanol and DMF inhibited β-galactosidase activity from 5% until total inhibition occurred at 50%. The concentration-response of both ethanol and DMF could be described with the Gormpertz model.

Inhibitions of the activities of cell-free β-galactosidase from *Kluyveromyces marxianus* by ethanol-DMF binary mixtures are shown in Figure 4.19. In all the mixtures, β-galactosidase activities were inhibited at 5%. Inhibition of enzyme activity continued progressively with an increase in total concentrations.

Table 4.9 shows the values and statistical associations of the observed and CA-predicted EC_{50}, No-Observable-Effect-Concentrations (NOEC) and TI values for ethanol-DMF mixtures. The observed EC_{50} values for ethanol and DMF are significantly different from each other and all the ethanol-DMF mixtures ($P < 0.05$). In comparison, there was a significant difference between observed EC_{50} and CA-predicted EC_{50} for 9:1 and 8:2 ethanol-DMF mixtures ($P < 0.05$). On the other hand, there was no statistical difference between observed EC_{50} and CA-predicted EC_{50} for 7:3 and 6:4 ethanol-DMF mixtures ($P > 0.05$). There was no significant difference between NOEC values for DMF, 6:4 and 7:3 ethanol-DMF mixtures. NOEC values for ethanol and 8:2 ethanol-DMF mixture are significantly different from each other and from DMF and other ethanol-DMF mixtures. The TI values of 6:4 and 7: 3 ethanol-DMF mixtures were significantly different from each other and from other ethanol-DMF mixtures ($P < 0.05$). Similarly, the TI values of 9:1 and 8:2 ethanol-DMF mixtures were not significantly different from each other ($P > 0.05$). The ANOVA and Post Hoc Tests Tables are shown in Appendix 8.0.

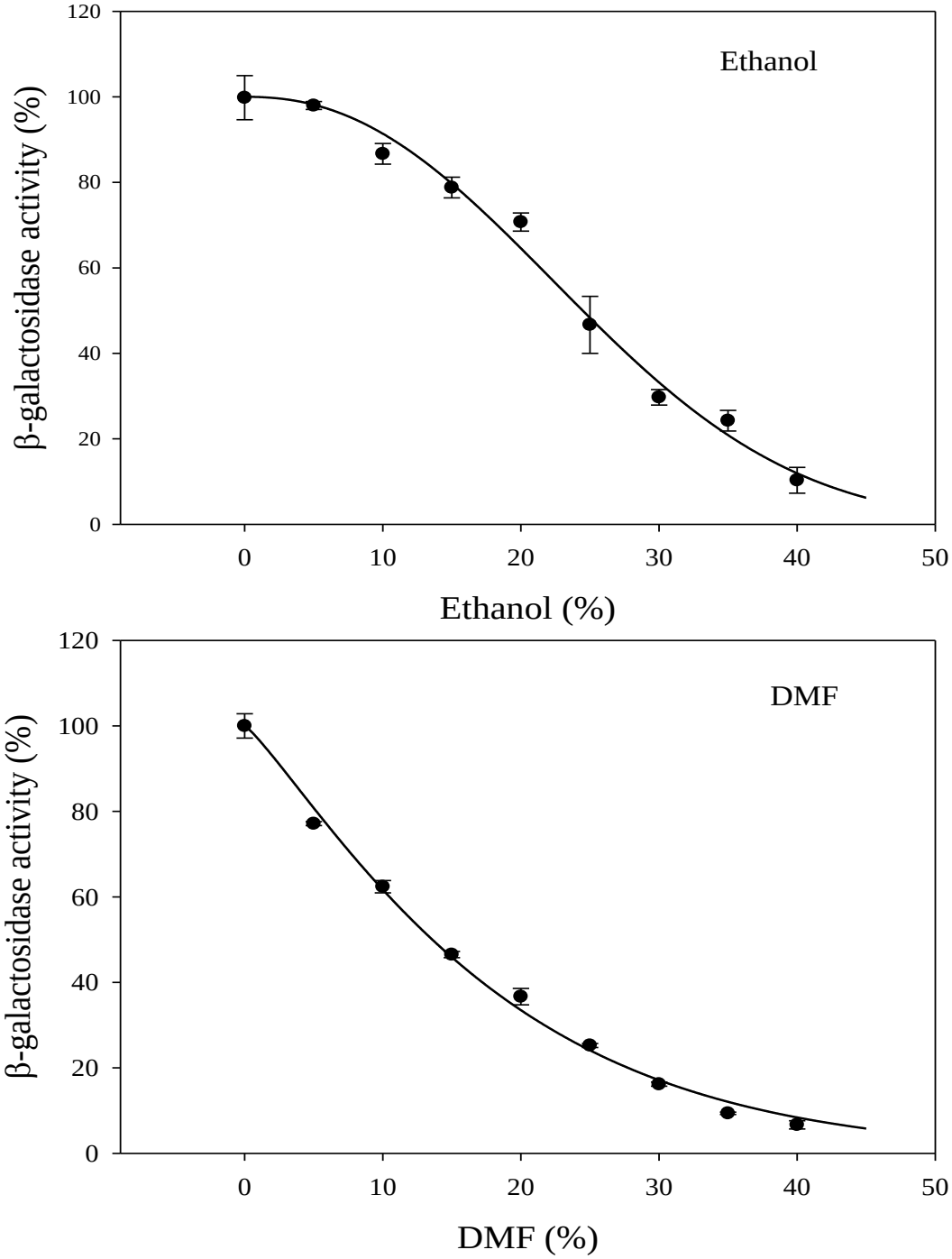

Figure 4.18 Inhibition of the activities of cell-free β-galactosidase from *Kluyveromyces marxianus* by individual ethanol and DMF in a different batch of experiments with binary mixtures of ethanol and DMF. The solid line represents the Gormpertz model fit to the observed data.

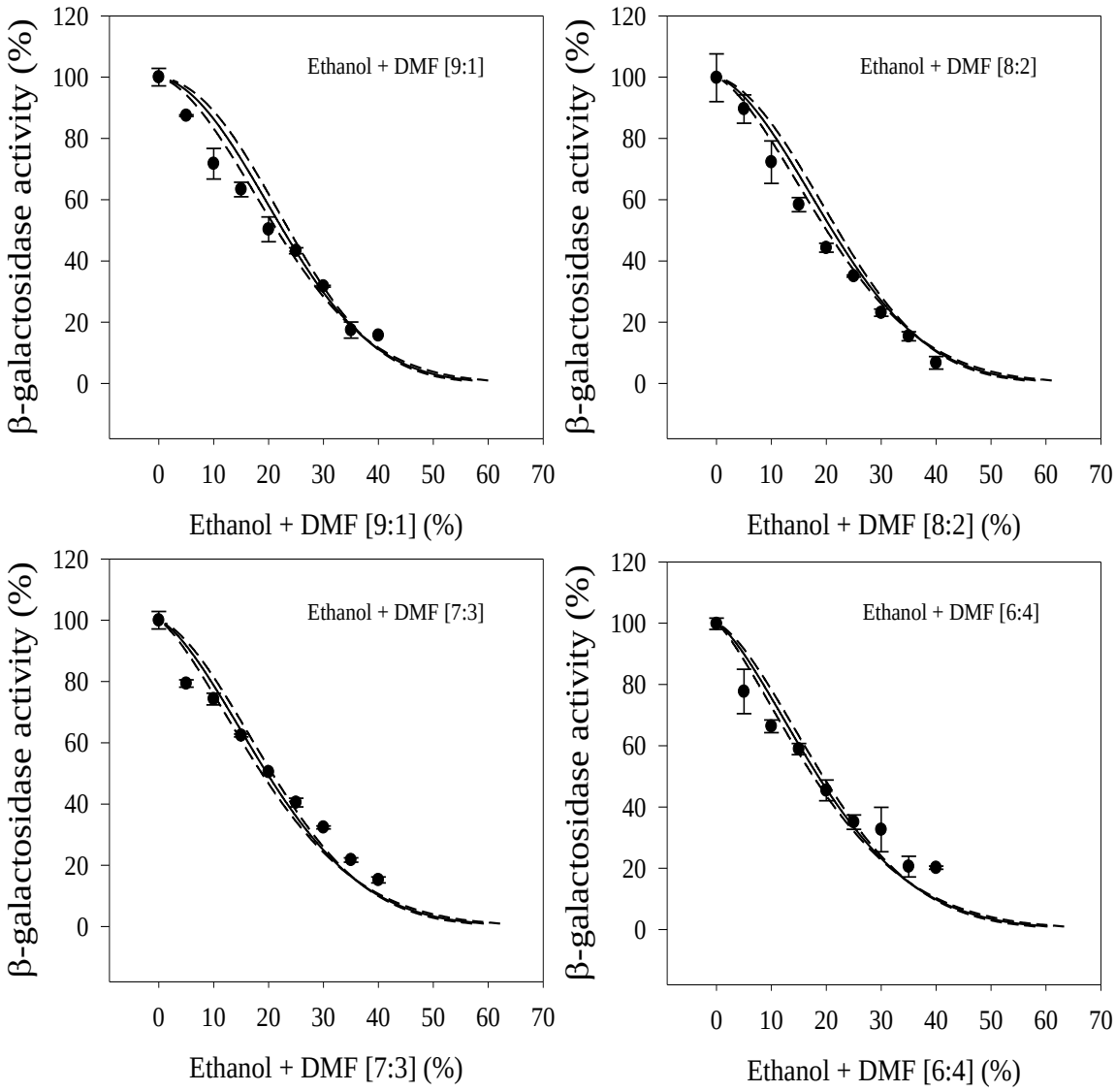

Figure 4.19 Inhibition of the activities of cell-free β-galactosidase from *Kluyveromyces marxianus* by binary mixtures of ethanol and DMF. The solid and dashed lines represent the mean and 95% confidence limit of CA model-predicted concentration-response relationships.

Table 4.9: Median inhibitory concentrations (EC_{50}) of ethanol-DMF mixtures, NOEC, toxic index and combined effect of ethanol-DMF mixtures on cell-free β-galactosidase from *Kluyveromyces marxianus*

Solvent/Solvent mixture	EC_{50} (%)		NOEC (%)	TI	Combined Effect
	Observed	CA-predicted			
Ethanol	24.475 ± 0.812^{e}	-	7.912 ± 1.081^{d}	-	-
DMF	13.587 ± 0.489^{a}	-	$0.920 \pm 0.161^{a,b}$	-	-
Ethanol: DMF (9:1)	19.717 ± 0.860^{d}	22.644 ± 1.099^{c}	1.779 ± 0.436^{b}	0.870 ± 0.009^{a}	Additive
Ethanol: DMF (8:2)	$17.819 \pm 0.788^{b,c}$	$21.081 \pm 1.017^{b,c}$	4.115 ± 0.663^{c}	0.845 ± 0.009^{a}	Additive
Ethanol: DMF (7:3)	$19.300 \pm 0.876^{c,d}$	$19.721 \pm 0.947^{a,b*}$	$1.381 \pm 0.366^{a,b*}$	0.978 ± 0.011^{c}	Additive
Ethanol: DMF (6:4)	17.170 ± 1.123^{b}	$18.525 \pm 0.886^{a*}$	$0.437 \pm 0.202^{a*}$	0.926 ± 0.028^{b}	Additive

Values shown are Mean ± Standard Deviation
Within a column, EC_{50} (observed and CA-predicted), NOEC or TI values with with same letter (asterik) are not significantly different from each other ($P < 0.5$).
Comparing observed and CA-predicted EC_{50}, values with asterisk are not statististically different from each other ($P > 0.05$)

4.1.11 Inhibition of the activities of cell-free β-galactosidase from *Escherichia coli* and *Kluyveromyces marxianus* by surfactants and EDTA

Inhibitions of the activities of cell-free β-galactosidase from *Escherichia coli* by surfactants and EDTA are shown in Figures 4.20 and 4.21. The inhibitions by SDS, CTAB, CPC and EDTA were concentration-dependent and could be described by the monophasic Gormpertz model (SDS, CTAB and EDTA) or biphasic Gormpertz model (CPC). Complete inhibition of β-galactosidase activity occurred at 0.003% CPC, 0.8% EDTA and 0.08% CTAB. At 4%, SDS inhibited β-galactosidase activity by 80%. Other surfactants were not inhibitory to β-galactosidase at concentrations up to 4% (SDC, Tween 20, Tween 80, Triton X-100) or 3% (Sarcosyl) (Fig. 4.21).

Inhibitions of the activities of cell-free β-galactosidase from *Kluyveromyces marxianus* by surfactants and EDTA are shown in Figures 4.22 and 4.23. Like the β-galactosidase of *Escherichia coli*, SDS, CTAB, CPC, and EDTA were inhibitory to the cell-free β-galactosidase from *Kluyveromyces marxianus* (Fig. 4.22). At the highest tested concentrations of 2% (SDS), 0.018% (CTAB and EDTA) and 0.0018% (CPC), *K. marxianus* β-galactosidase was inhibited by 85.3% (SDS), 97.9% (CPC), 98.3% (CTAB) and 96.4% (EDTA). Tween 20, Tween 80 and Triton X-100 were not inhibitory to *K. marxianus* β-galactosidase at concentrations up to 2% (Fig. 4.23). Although sarcosyl was mild, the activity of *K. marxianus* β-galactosidase was inhibited as the concentration of sarcosyl increased above 0.2%.

The median inhibitory concentrations (EC_{50}) and NOEC of EDTA and surfactants (CTAB, SDS and CPC) against the activity of cell-free β-galactosidase from *Escherichia coli* and *K. marxianus* are shown in Table 4.10. CPC with EC50 of 0.00138 ± 0.00002% (for E. coli β-galactosidase) and 0.0010 ± 0.00001% (for *K. marxianus* β-galactosidase) was most inhibitory to the enzymes.

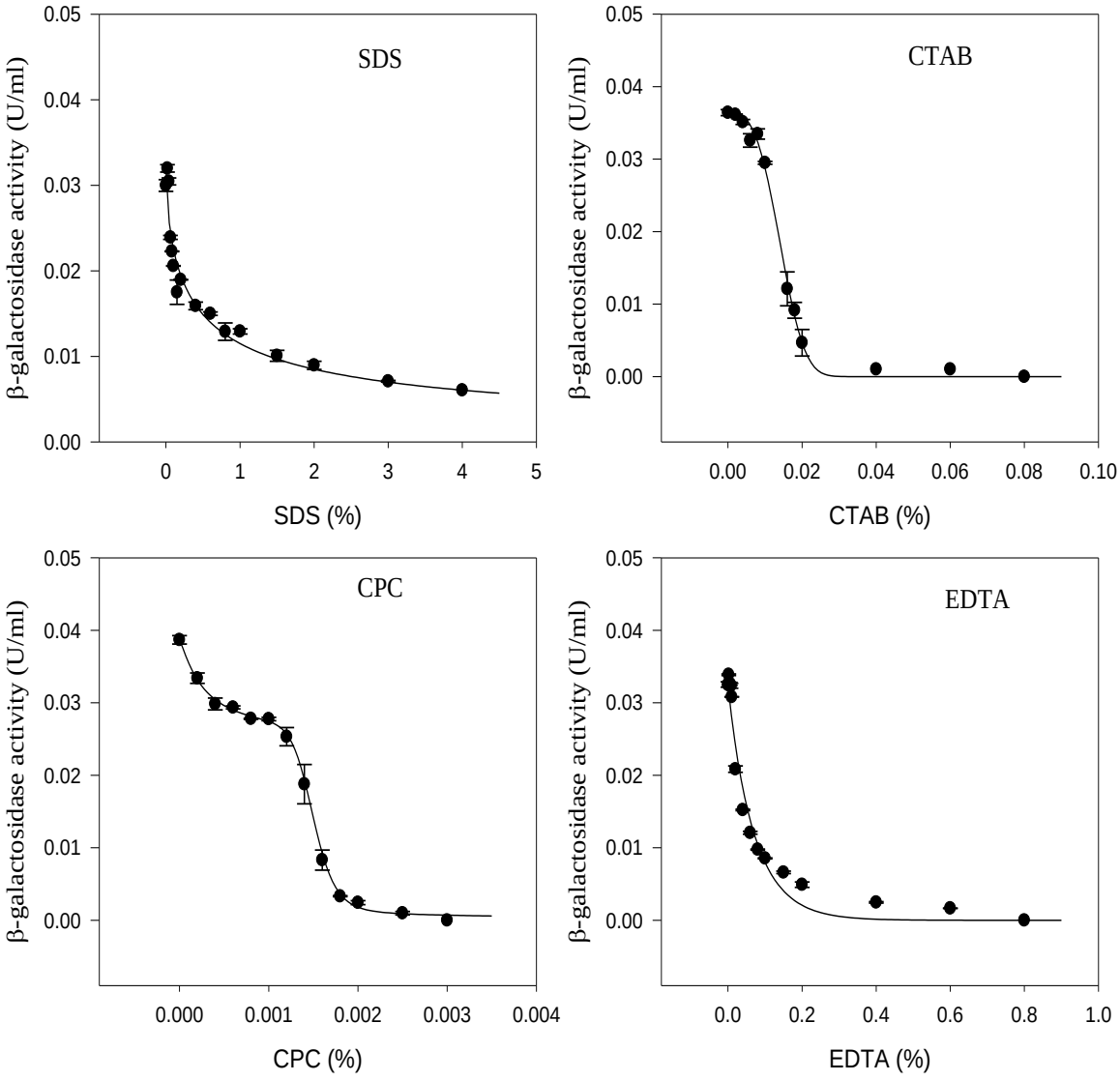

Figure 4.20 Inhibition of the activities of cell-free β-galactosidase from *Escherichia coli* by SDS, CTAB, CPC and EDTA. The solid line represents the Gormpertz model fit to the observed data.

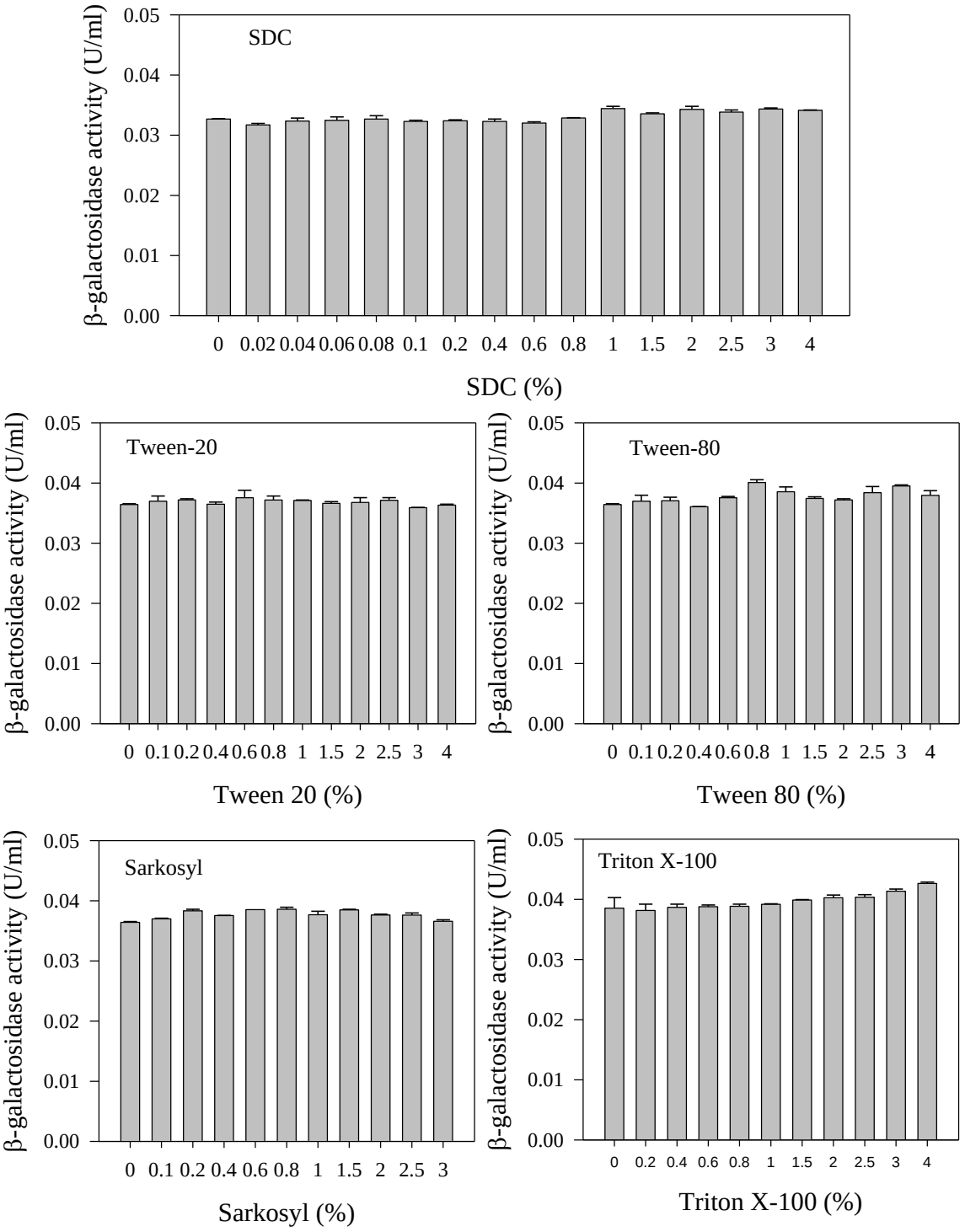

Figure 4.21 Inhibition of the activities of cell-free β-galactosidase from *Escherichia coli* by SDC, Tween-20, Tween-80, Sarcosyl and Triton X-100.

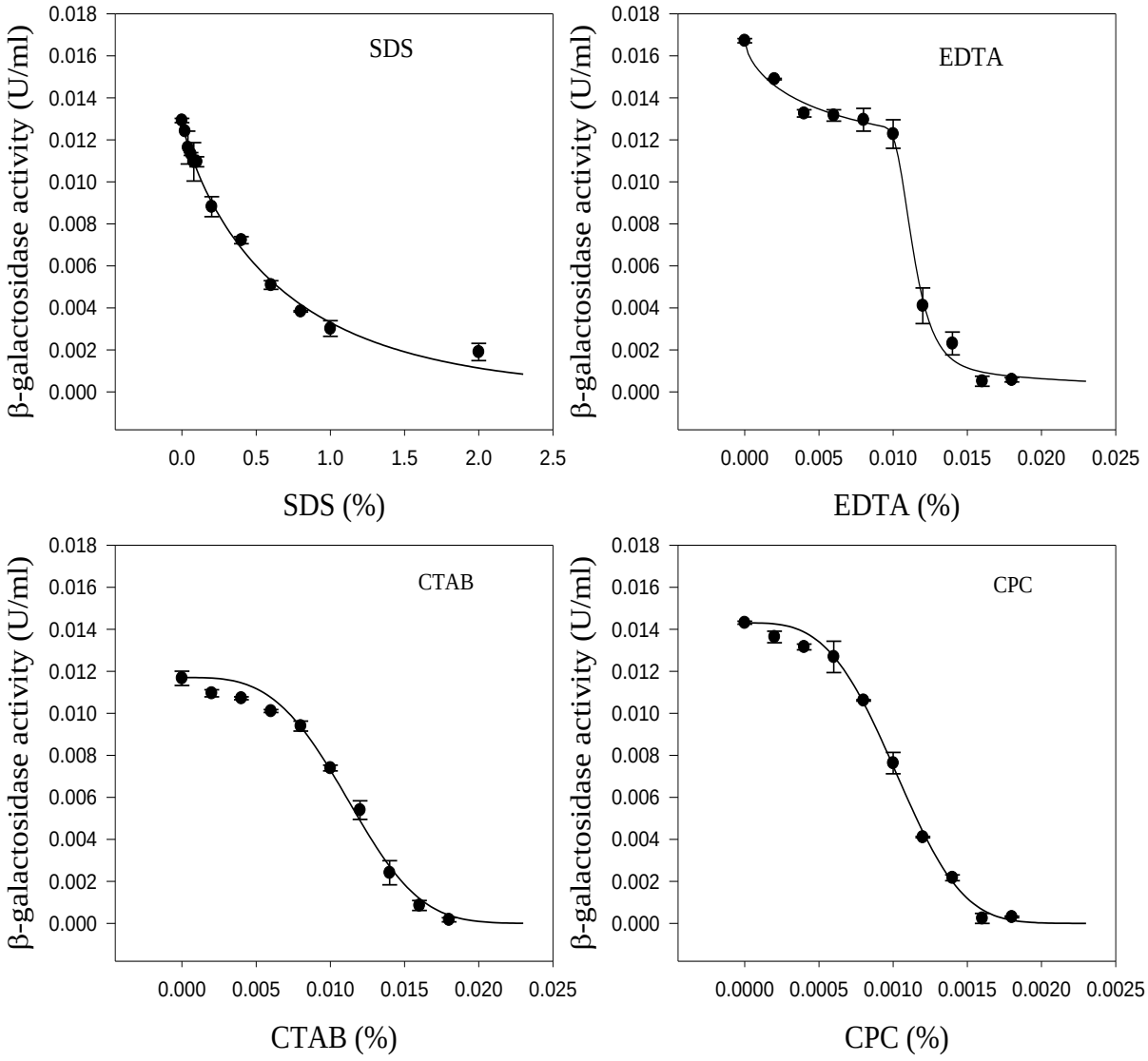

Figure 4.22 Inhibition of the activities of cell-free β-galactosidase from *Kluyveromyces marxianus* by SDS, CTAB, CPC and EDTA. The solid line represents Gormpertz, and the bilogistic model fits the observed data.

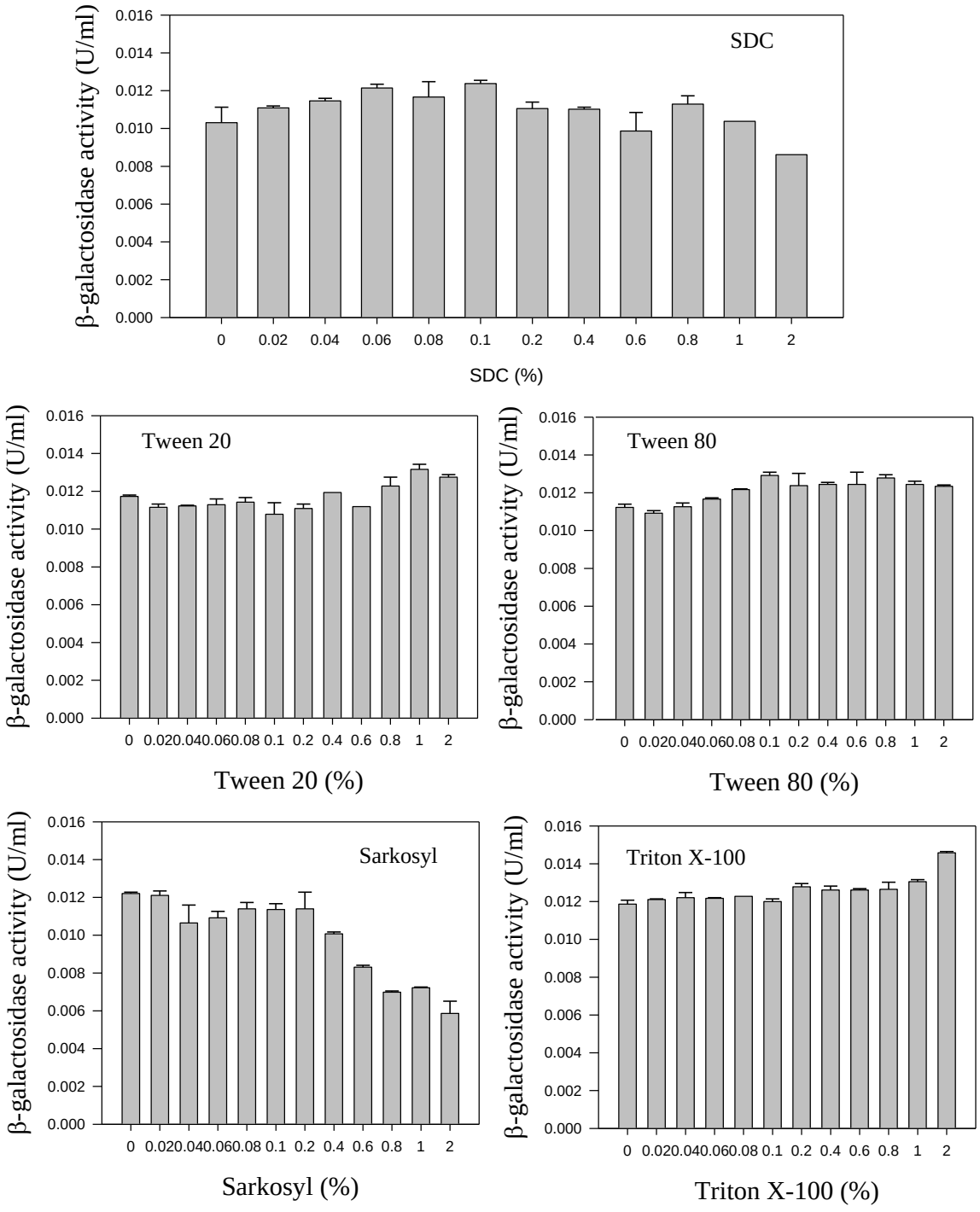

Figure 4.23 Inhibition of the activities of cell-free β-galactosidase from *Kluyveromyces marxianus* by SDC, Tween-20, Tween-80, Sarcosyl and Triton X-100.

Table 4.10 The median inhibitory concentrations (EC_{50}) and NOEC of EDTA and surfactants (CTAB, SDS and CPC) against the activity of cell-free β-galactosidase from *Escherichia coli* and *Kluyveromyces marxianus*

Solvents/EDTA	EC50 (%)	NOEC (%)
Escherichia coli		
CTAB	0.0141 ± 0.0003^{b}	0.004 ± 0.001^{b}
SDS	0.0032 ± 0.0020^{a}	ND
CPC	0.00138 ± 0.00002^{a}	$0.000019 \pm 0.000014^{a}$
EDTA	0.0483 ± 0.0054^{c}	0.0007 ± 0.0004^{a}
Kluyveromyces marxianus		
CTAB	0.0113 ± 0.0007^{a}	0.0043 ± 0.0009^{c}
SDS	0.445 ± 0.039^{b}	0.002 ± 0.001^{b}
CPC	0.0010 ± 0.00001^{a}	0.00023 ± 0.00005^{a}
EDTA	0.011 ± 0.001^{a}	ND

The data shown represents mean values and SD.

ND= Not Determined

Within column for a particular organism, EC_{50} or NOEC values with the same subscript letter are not significantly different from each other ($P > 0.05$).

4.1.12 Effects of solvents and solvent mixtures on cell permeabilization and β-galactosidase activity in *Escherichia coli* and *Kluyveromyces marxianus*

The influence of solvents and solvent mixtures on cell permeabilization for β-galactosidase activity assay in *E. coli* are shown in Figures 4.24 and 4.25. DMSO and DMF showed no evidence of cell permeabilization but inhibited the background β-galactosidase activity in *E. coli* even at 5% (Fig. 4.24). Ethanol showed evidence of cell permeabilization, given the increased β-galactosidase activity in *E. coli* as the concentration increased. The best concentration of ethanol ranged from 15% to 30% (Fig. 4.24). The solvent mixtures permeabilized *E. coli* cells. However, there was inhibition of β-galactosidase activity at concentrations above 20% (9.1 ethanol-pentanol mixture), 25% (9:1 ethanol-chloroform mixture), 35% (9:1 ethanol-DMSO mixture), 25% (9:1 ethanol-butanol mixture) and 35% (9:1% ethanol-DMF mixture) in *E. coli* (Fig. 4.25). The highest β-galactosidase activity was observed at 5% of 9;1 ethanol-chloroform mixture.

The influence of solvents and solvent mixtures on cell permeabilization for β-galactosidase activity assay in *K. marxianus* are shown in Figures 4.26 and 4.27. Unlike E. coli cells, *K. marxianus* cells did not have background β-galactosidase activity when no permeabilization agent was applied. DMSO and DMF were very poor cell permeabilization agents for *K. marxianus*. A low β-galactosidase activity of 0.046 ± 0.0015 U/ml and 0.056 ± 0.0008 U/ml was obtained at 40% DMSO and 40%DMF respectively. There was no enzyme activity in *K. marxianus* cells at ethanol concentrations ranging from 5% to 15%. A very low β-galactosidase activity (0.002 ± 0.00098 U/ml) was obtained at 20% ethanol. The best concentration of ethanol was 25%, which gave β-galactosidase activity of 0.128 ± 0.0006 U/ml. Ethanol inhibited β-galactosidase activity in *K. marxianus* cells as the concentration increased above 25% (Fig. 4.26). Solvent mixtures were equally promising as permeabilization agents for *K. marxianus* cells. Improved β-galactosidase activity was obtained with 9:1 mixtures of ethanol with chloroform, DMSO, pentanol or butanol. The best concentrations were 15% (9:1 ethanol-chloroform), 25% (9:1 ethanol-pentanol mixture), 20% and 25% (9:1 ethanol-butanol mixture) and 30% (ethanol-DMSO mixture). Ethanol-chloroform was the best mixture for *K. marxianus* permeabilization and in-situ β-galactosidase activity assay (Fig. 4.27).

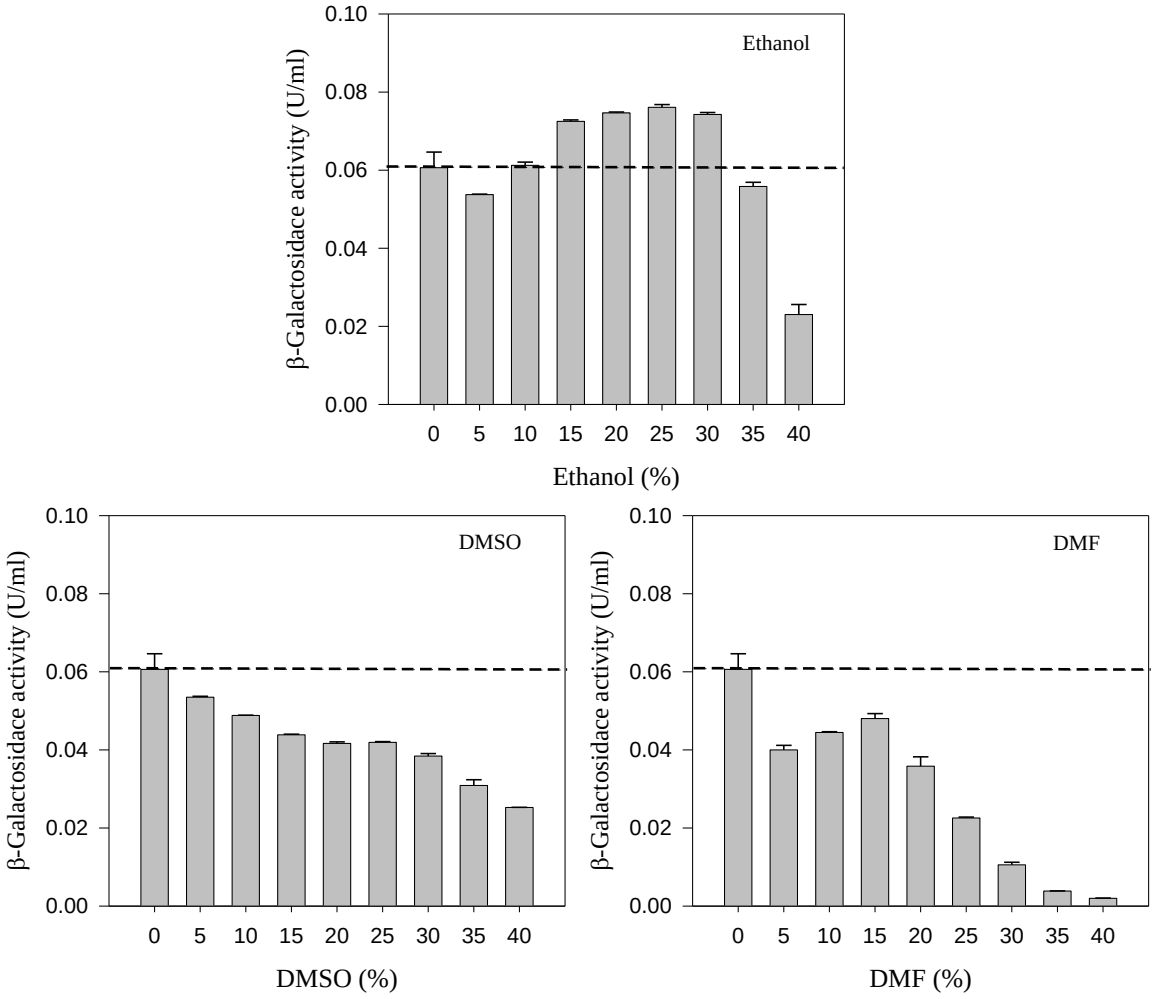

Figure 4.24 Effects of ethanol, DMSO and DMF on cell permeabilization and β-galactosidase activity in *Escherichia coli*

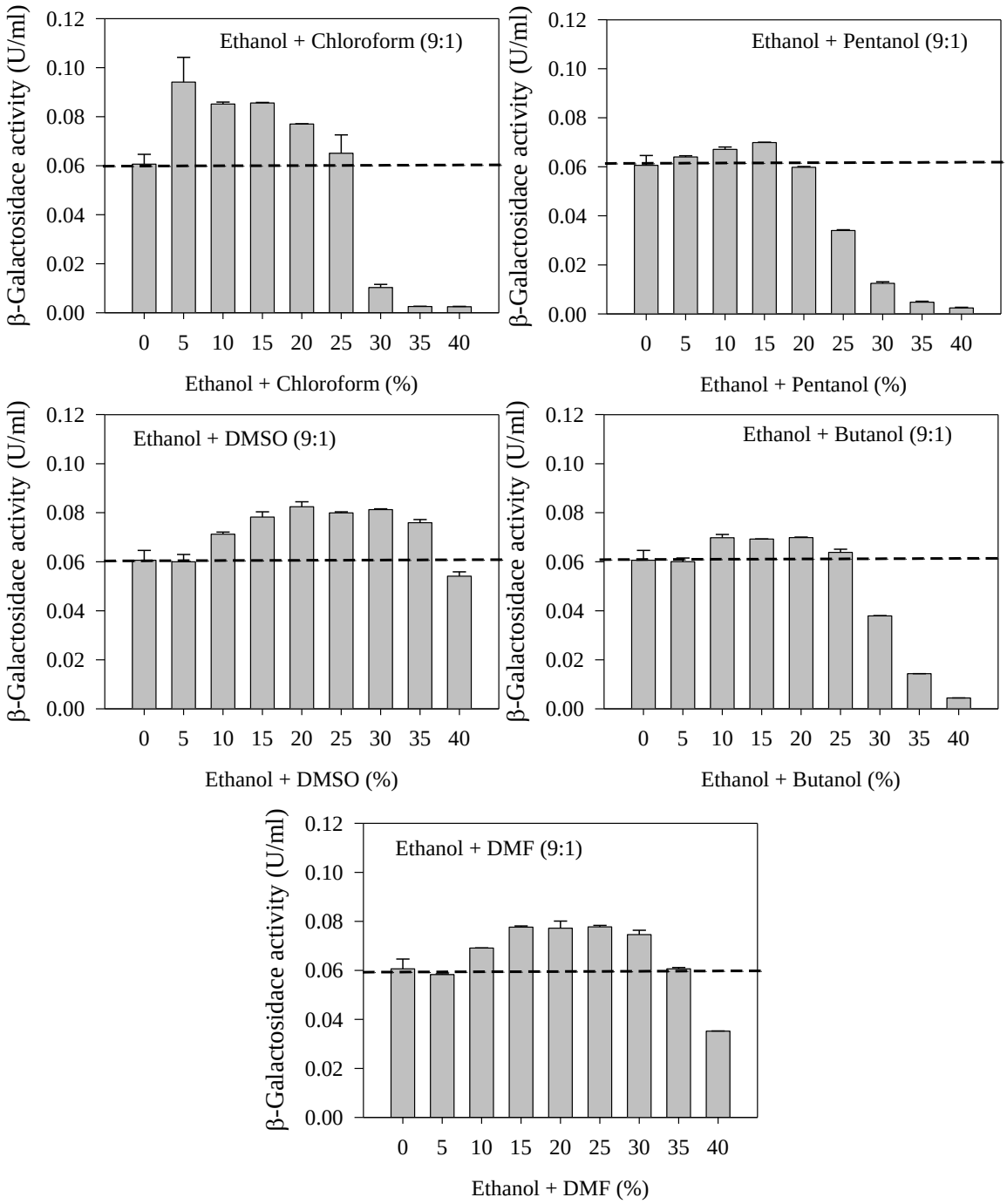

Figure 4.25 Effects of solvent mixtures on cell permeabilization and β-galactosidase activity in *Escherichia coli*

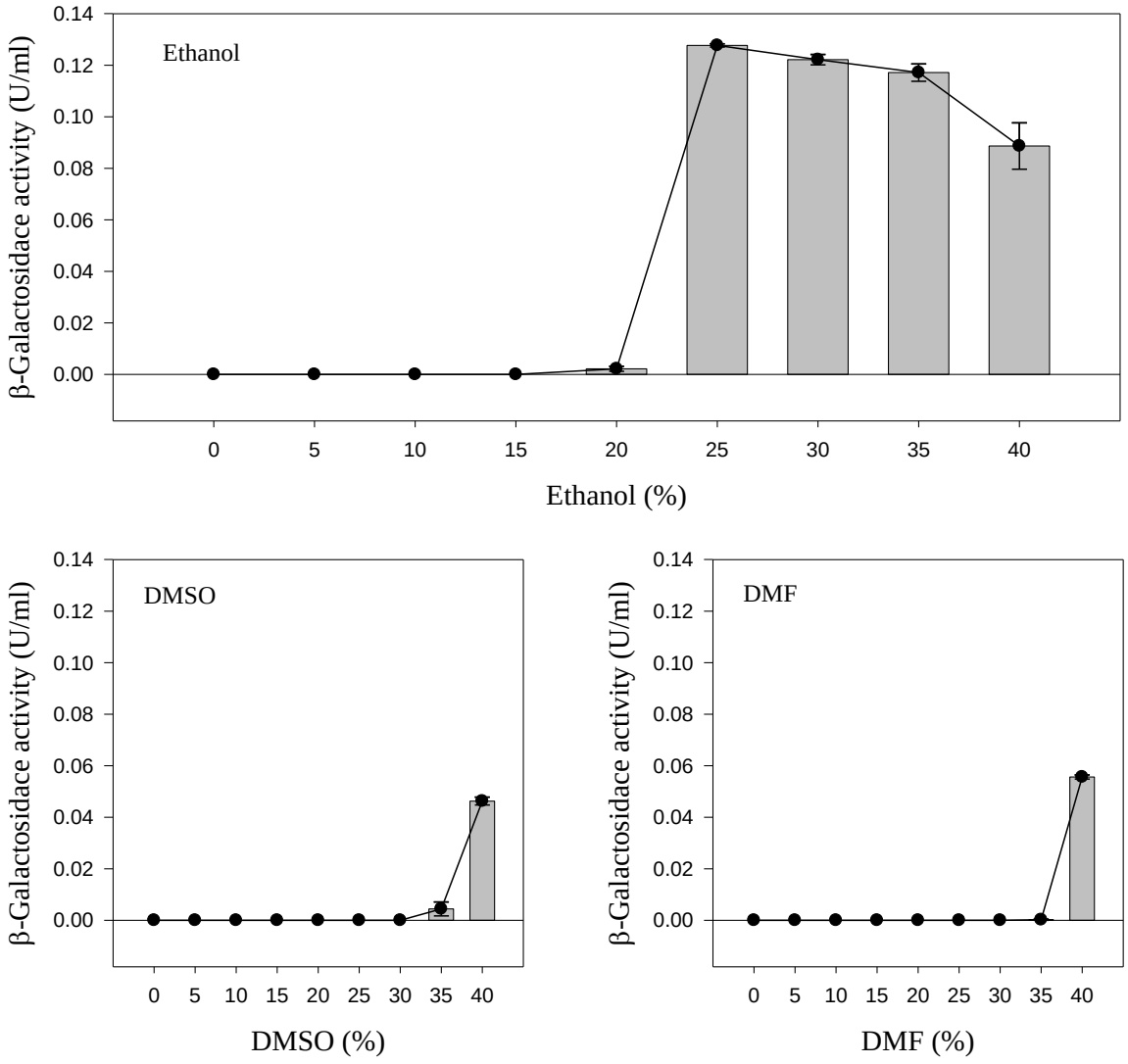

Figure 4.26 Effects of ethanol, DMSO and DMF on cell permeabilization and β-galactosidase activity in *Kluyveromyces marxianus*

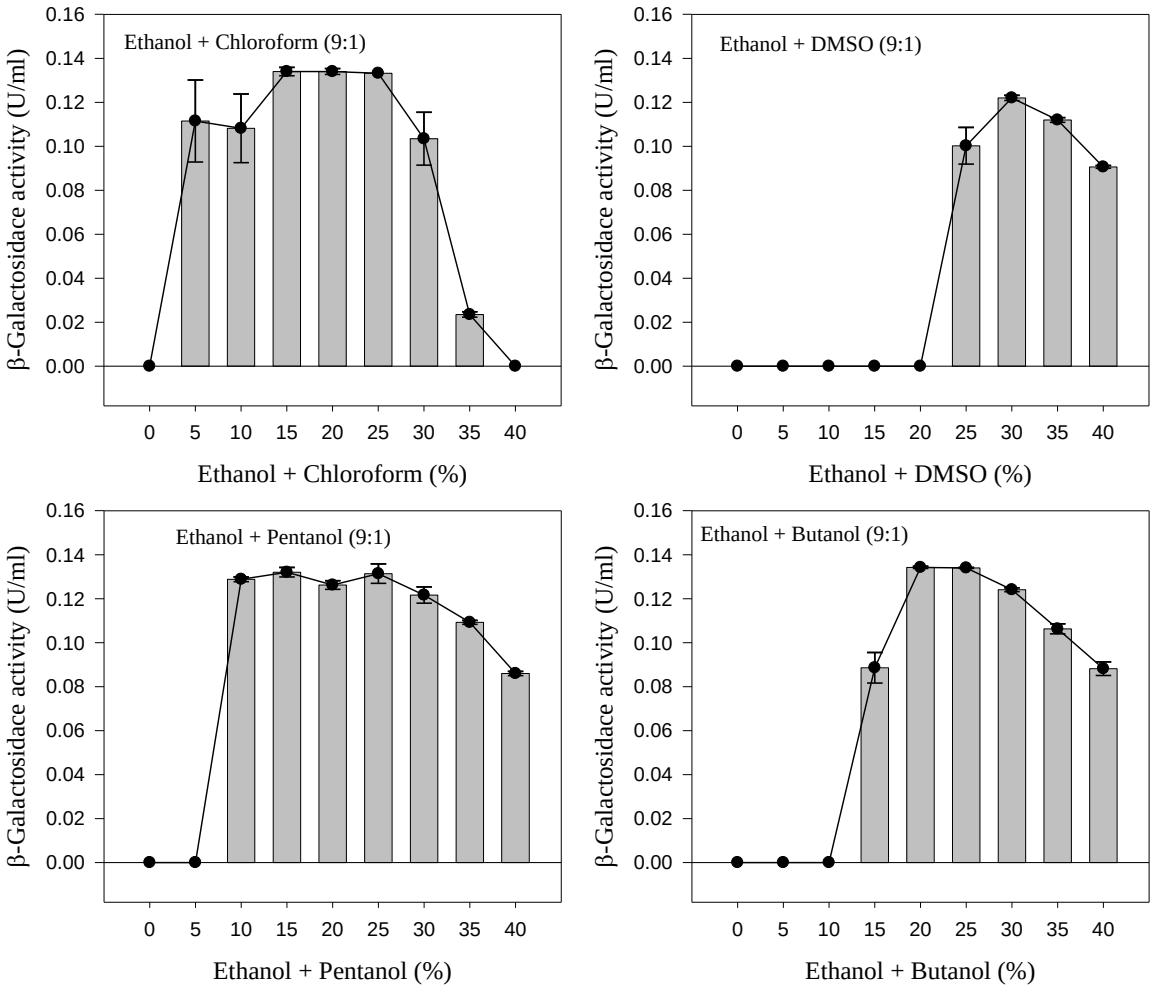

Figure 4.27 Effects of solvent mixtures on cell permeabilization and β-galactosidase activity in *Kluyveromyces marxianus*

4.1.13 Effects of surfactants on cell permeabilization and β-galactosidase activity in *Escherichia coli* and *Kluyveromyces marxianus*

The Effects of surfactants on cell permeabilization and β-galactosidase activity in *E. coli* are shown in Tables 4.28 and 4.29. SDS, CTAB and CPC permeabilized *E. coli* cells, resulting in β-galactosidase activities higher than the background values when no surfactant was applied. However, like the cell-free β-galactosidase, these surfactants exerted inhibitory effects on the whole cell β-galactosidase at high concentrations. At concentrations higher than 0.04% (SDS), 0.006% (CTAB) and 0.002% (CPC), β-galactosidase activity in *E. coli* was inhibited (Fig. 4.28). The best concentrations for the surfactants were 0.02% SDS, 0.002% CTAB and 0.0008% CPC for permeabilization of *E.coli* cells. Triton X-100, SDC, sarcosyl, Tween 20 and Tween 80 were mild surfactants having inhibitory effects at concentrations up to 2%. Concentrations ranging from 0.02% to 2% (Triton X100), 0.0002% to 0.006% (CPC), 0.02% to 2% (SDC), 0.04% to 2% (sarcosyl), 0.02% to 2% (Tween 20) and 0.02% to 1% (Tween 80) could give comparably high β-galactosidase activity in *E. coli* (Fig. 4.29).

The Effects of surfactants on cell permeabilization and β-galactosidase activity in *K. marxianus* are shown in Tables 4.30 and 4.31. Sarcosyl, SDC, CTAB and CPC were good permeabilizers of *K. marxianus* cells for higher β-galactosidase activity than other surfactants (such as Tween 20, Tween 80, SDS, and Triton X-100) that poorly permeabilized the yeast cells (Fig. 4.31). The optimum concentrations are 0.2% (sarcosyl), 0.4% (SDC), 0.4% and 0.6% (CTAB) and 0.0008% (CPC) (Fig. 4.30).

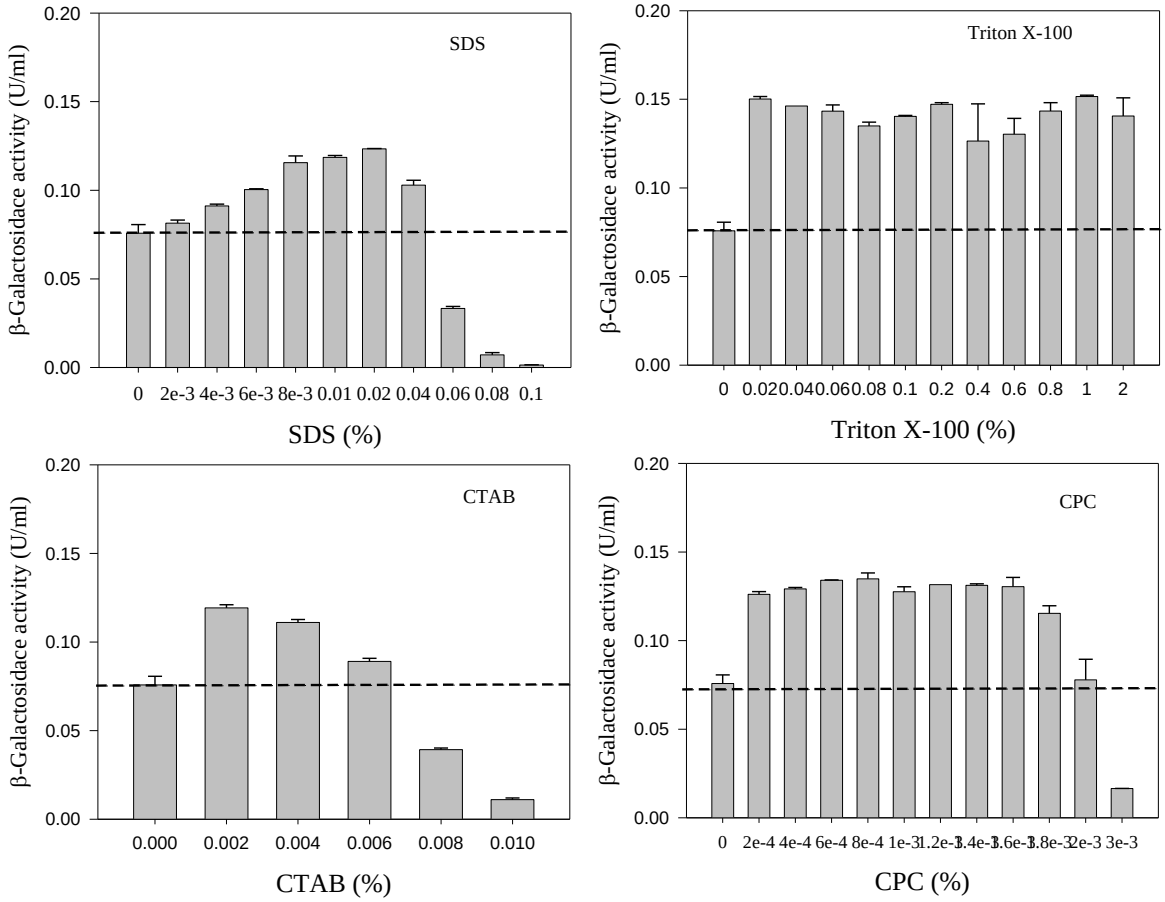

Figure 4.28 Effects of SDS, CTAB, CPC and Triton X-100 on cell permeabilization and β-galactosidase activity in *Escherichia coli*

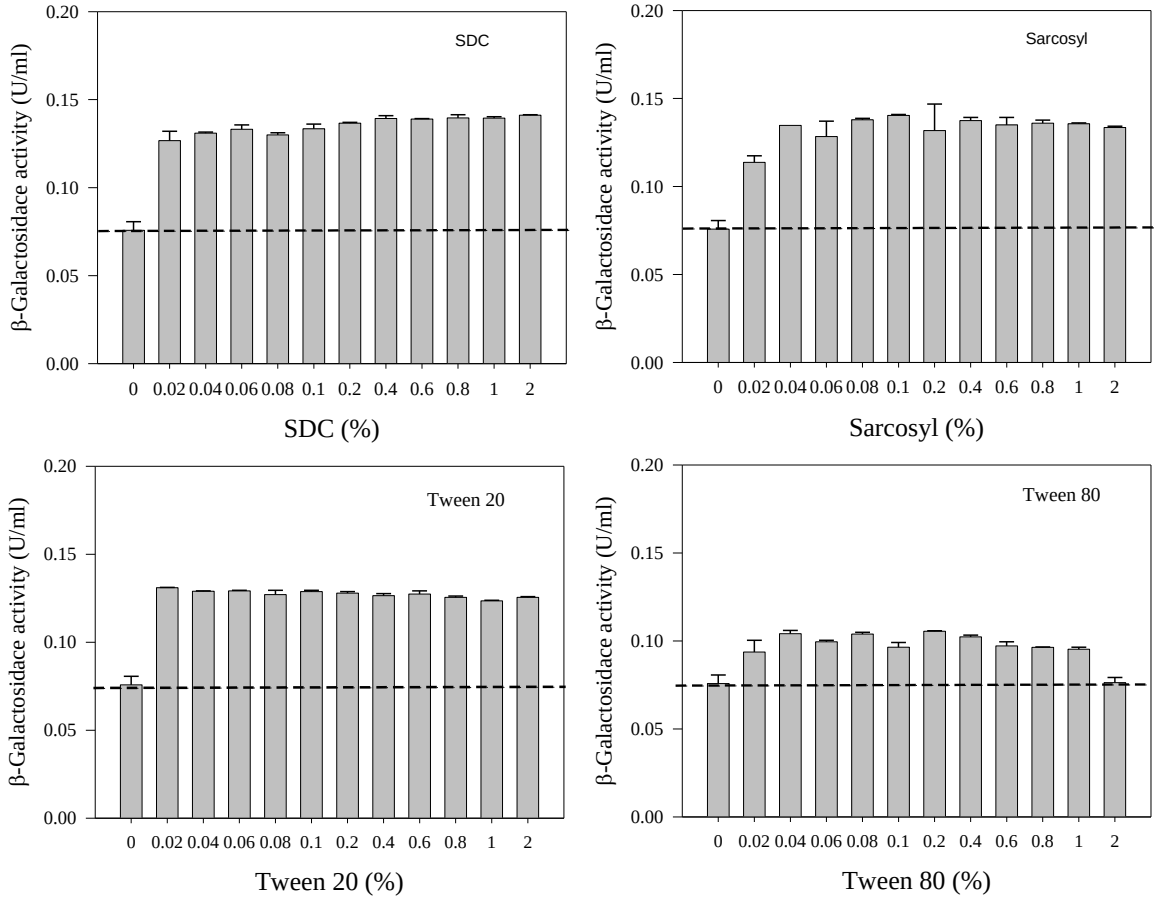

Figure 4.29 Effects of SDC, Sarcosyl, Tween 20 and Tween 80 on cell permeabilization and β-galactosidase activity in *Escherichia coli*

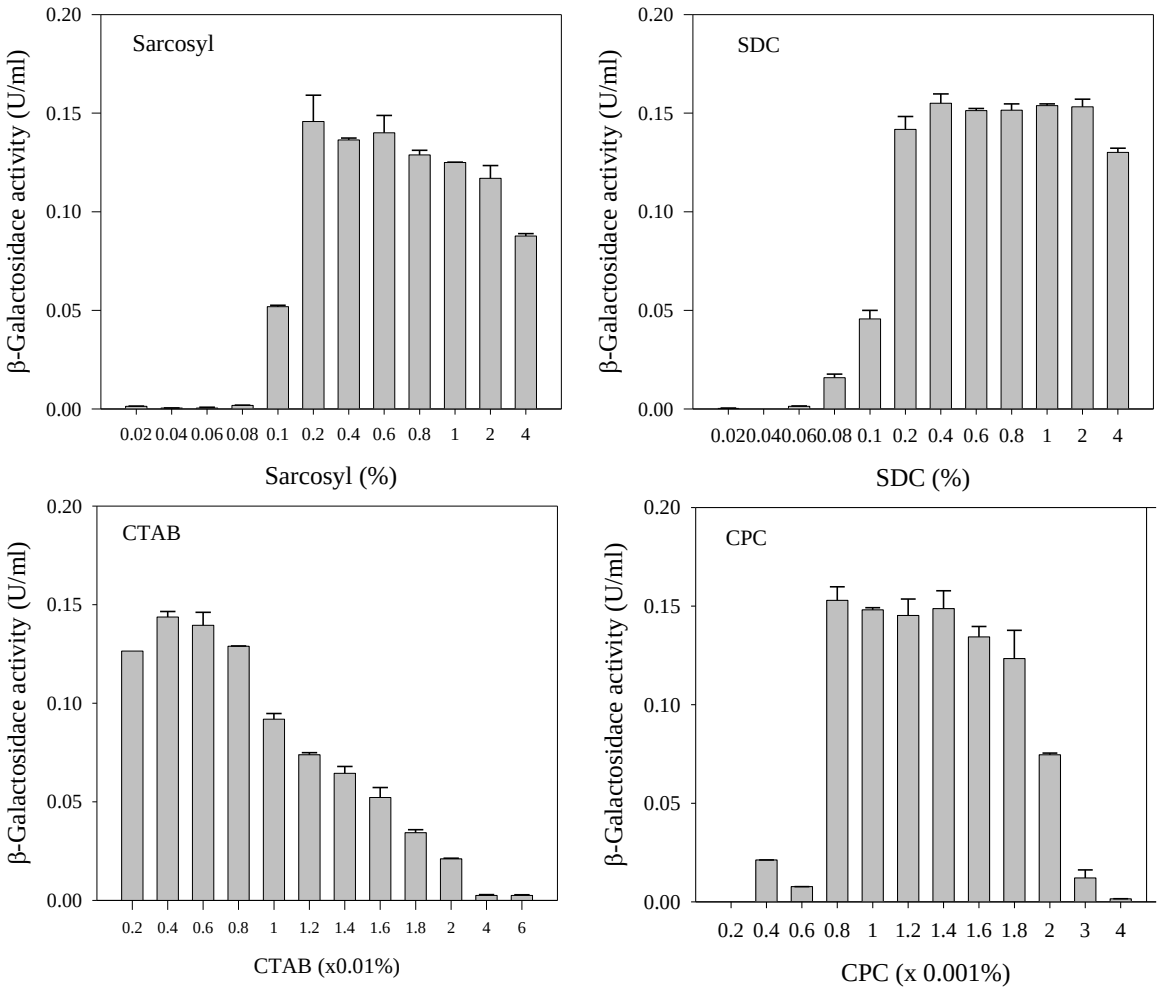

Figure 4.30 Effects of SDC, Sarcosyl, CTAB and CPC on cell permeabilization and β-galactosidase activity in *Kluyveromyces marxianus*

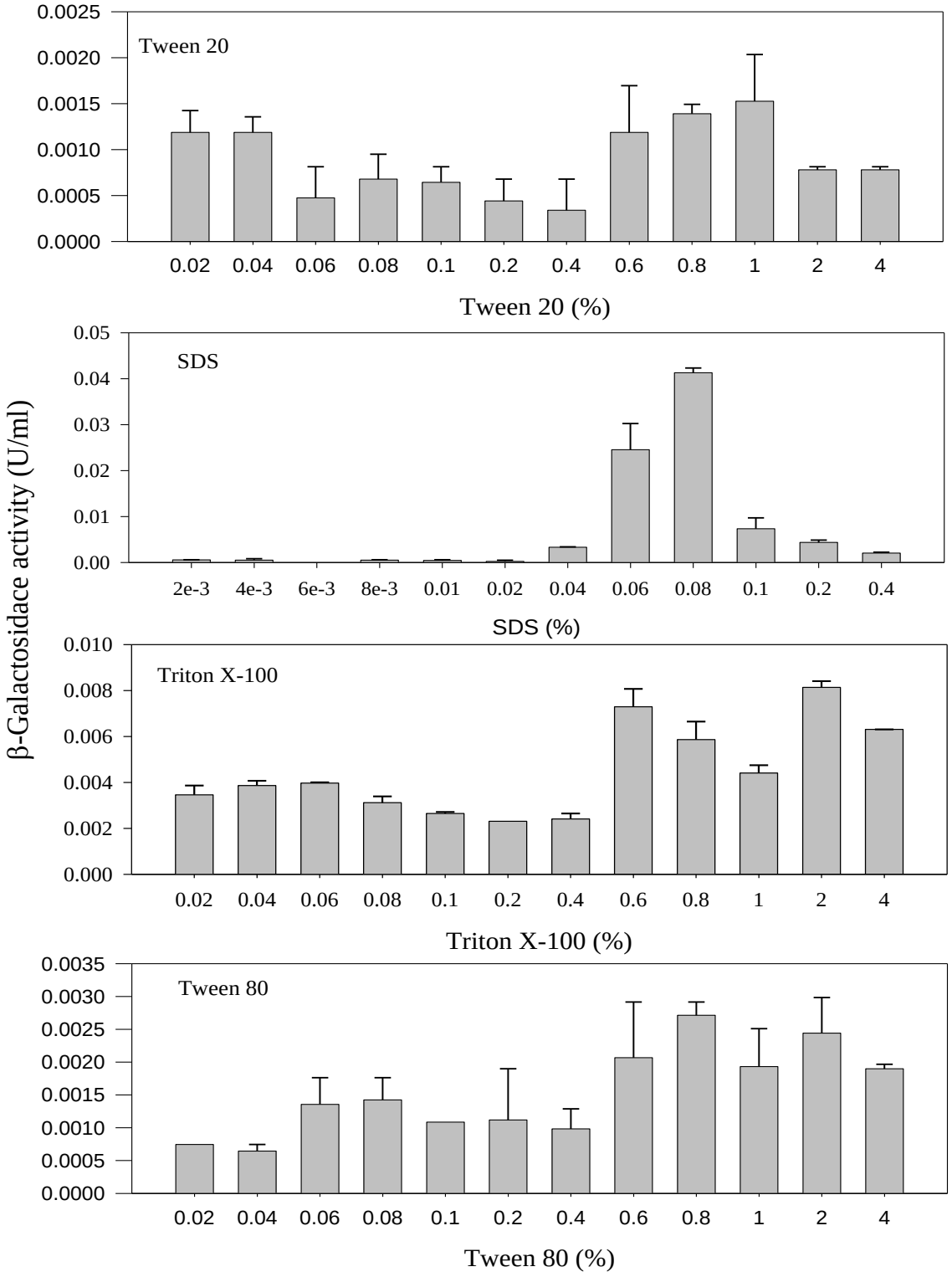

Figure 4.31 Effects of Tween 20, SDS, Tween 80 and Triton X-100 on cell permeabilization and β-galactosidase activity in *Kluyveromyces marxianus*.

4.1.14 β-galactosidase activities in *E. coli* cells permeabilized with selected concentrations of solvents and surfactants

Figures 4.32 and 4.33 show β-galactosidase activities in *E. coli* cells that are permeabilized with selected concentrations of solvents and surfactants. The best concentrations for cell permeabilization and β-galactosidase activity measurement were 20% and 25% (ethanol), 10% and 15% (9:1 ethanol-chloroform and 9:1 ethanol-pentanol mixtures) and 15% and 20% (ethanol-butanol mixture). The best concentrations for the surfactants are 0.1% (sarcosyl), 0.0008% and 0.001% (CPC) and 0.08% and 0.1% (SDC).

4.1.15 β-galactosidase activities in *K. marxianus* cells permeabilized with selected concentrations of solvents and surfactants

Figures 4.34 and 4.35 show β-galactosidase activities in *K. marxianus* cells permeabilized with selected concentrations of solvents and surfactants. The best concentrations for cell permeabilization and β-galactosidase activity measurement are 25% (ethanol), 10% (9:1 ethanol-chloroform mixture), 15% (9:1 ethanol-pentanol mixtures) and 25% (ethanol-butanol mixture). The best concentrations for the surfactants were 0.2% (sarcosyl), 0.0006% and 0.0008% (CPC), 0.2% to 0.8% (SDC) and 0.004% (CTAB).

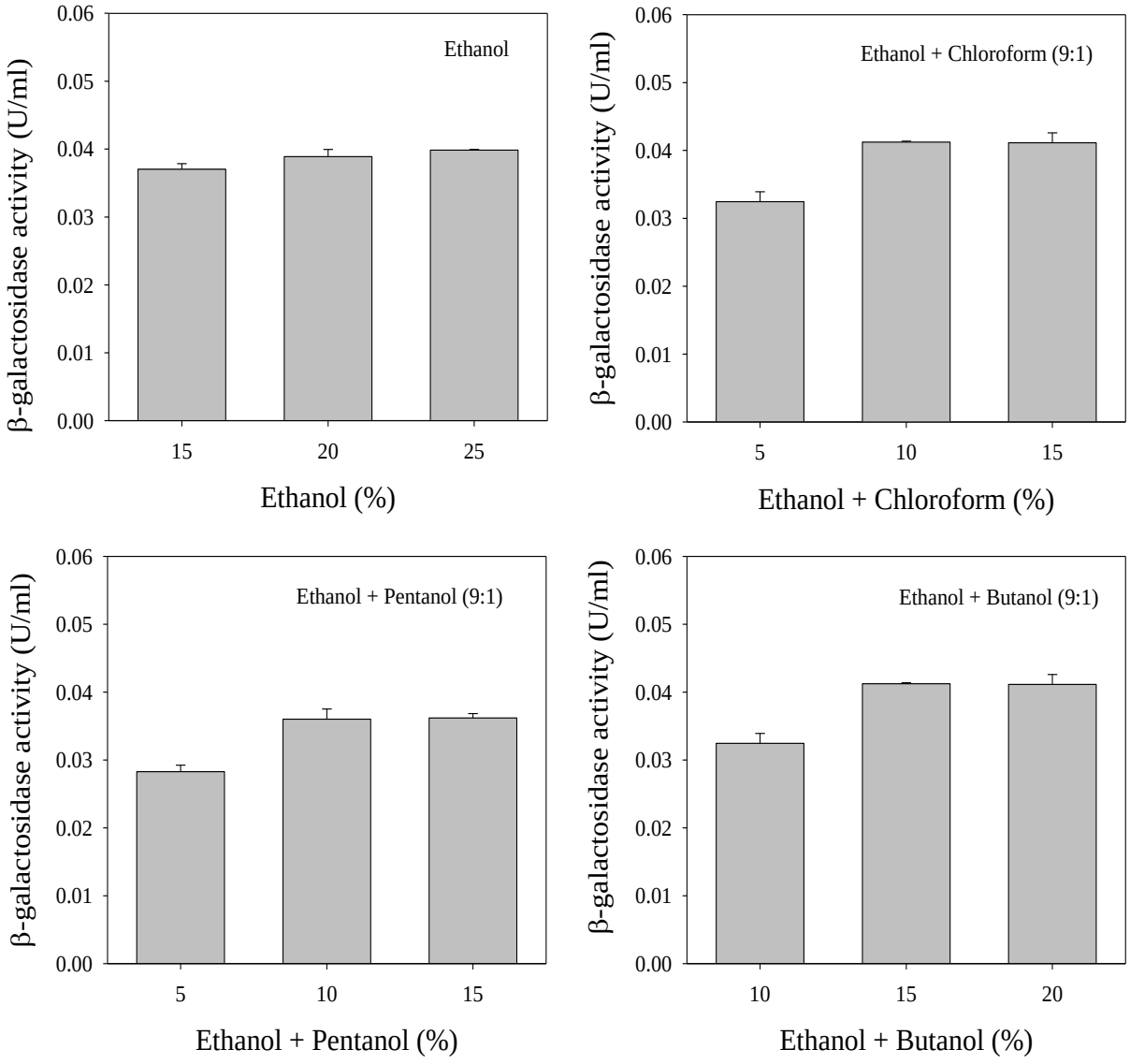

Figure 4.32 β-galactosidase activities in *E. coli* cells permeabilized with selected concentrations of ethanol and mixtures of ethanol with water-immiscible solvents.

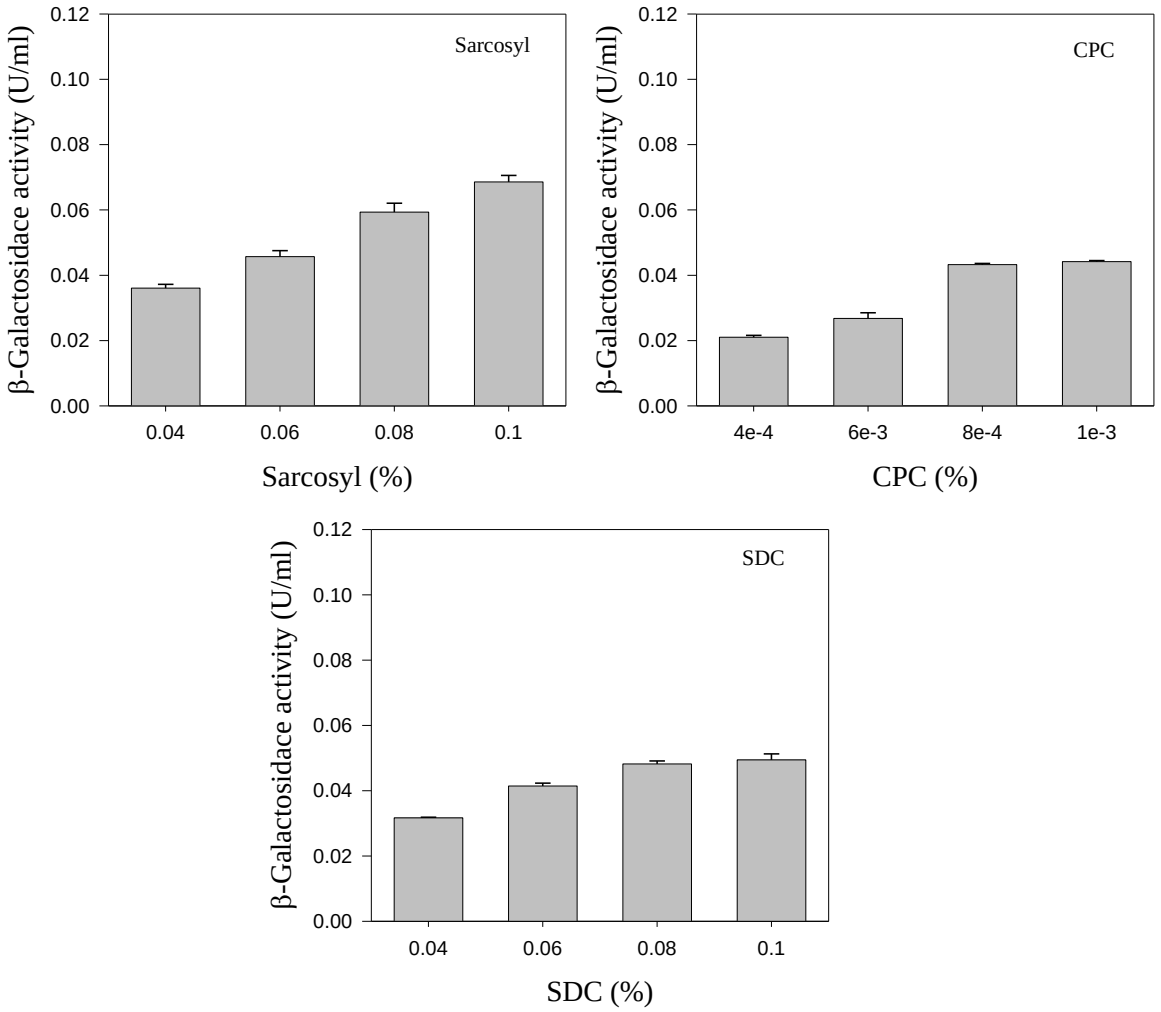

Figure 4.33 β-galactosidase activities in *Escherichia coli* cells permeabilized with sarcosyl, CPC and SDC.

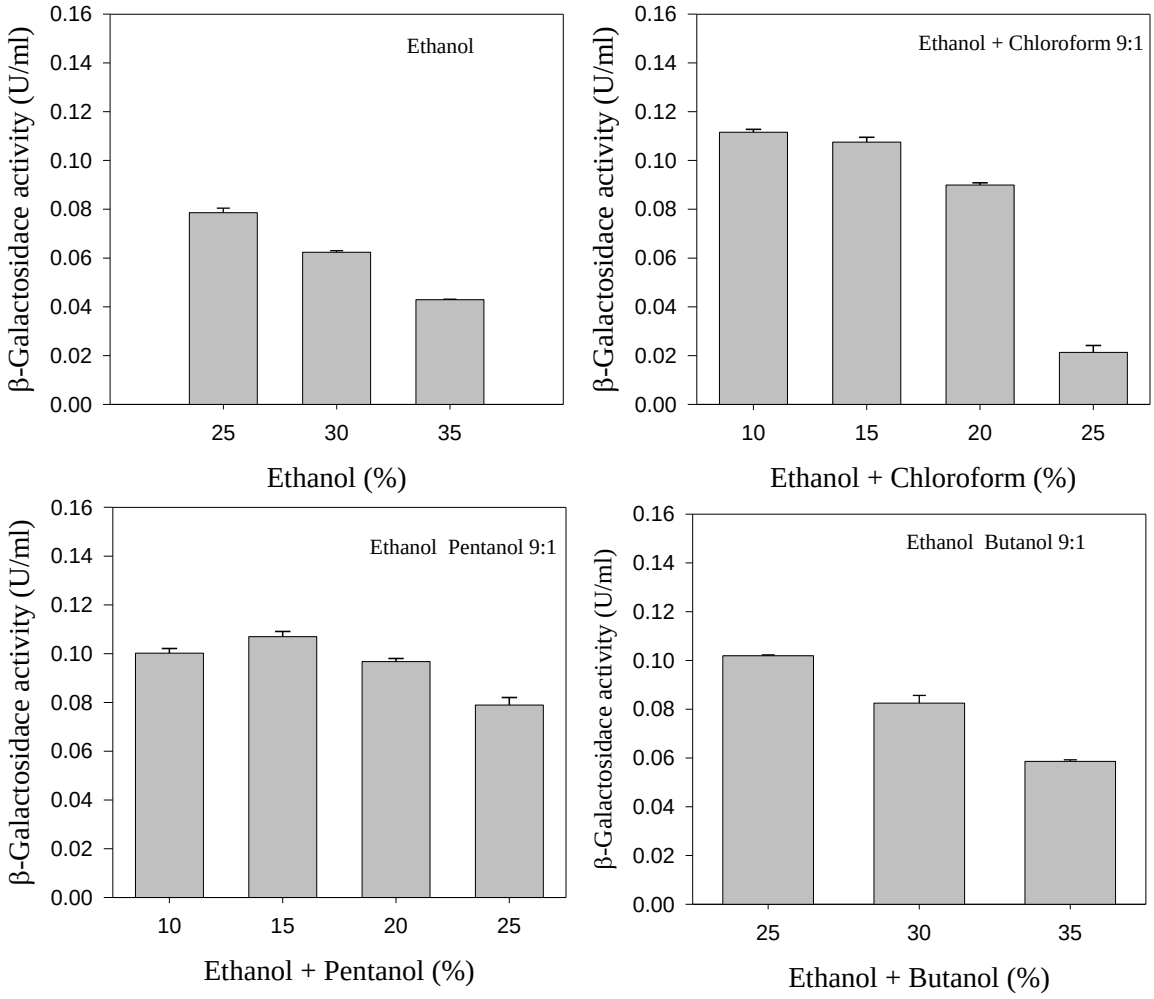

Figure 4.34 β-galactosidase activities in *Kluyveromyces marxianus* cells permeabilized with selected concentrations of ethanol and mixtures of ethanol and water-immiscible solvents.

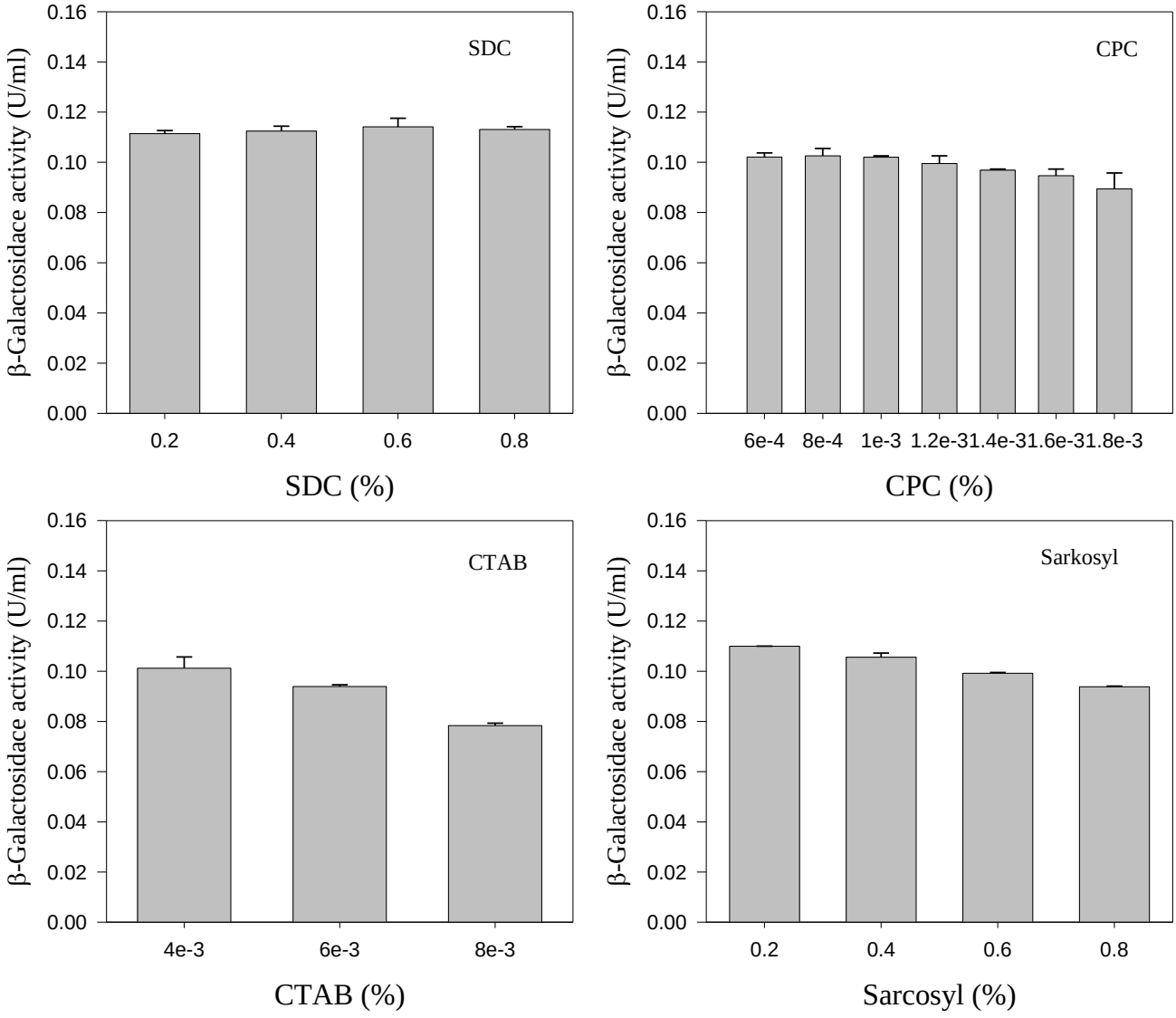

Figure 4.35 β-galactosidase activities in *Kluyveromyces marxianus* cells permeabilized with selected concentrations of SDC, CPC, CTAB and Sarcosyl.

4.1.16 Effect of mercaptoethanol and EDTA on β-galactosidase activity in *E. coli* and *K. marxianus* cells permeabilized with solvents and surfactants

The effects of mercaptoethanol and EDTA on β-galactosidase activity in *E. coli* cells permeabilized with selected concentrations of solvents and surfactants are shown in Figures 4.36 and 4.37. Both EDTA and mercaptoethanol increased the β-galactosidase activity in *E. coli* cells. This increment was higher with EDTA than mercaptoethanol at all concentrations of ethanol and solvent mixtures (Fig. 4.36). The best concentrations for cell permeabilization and β-galactosidase activity measurement in *E coli* are 20 and 25% (ethanol), 10 and 15% (9:1 ethanol-chloroform and 9:1 ethanol-pentanol), and 20% (9:1 ethanol-butanol). Similar trends of the effects of mercaptoethanol and EDTA on β-galactosidase activity of *E. coli* cells were obtained when surfactants were used. All tested concentrations of sarcosyl, SDC and CPC, EDTA, and mercaptoethanol increased β-galactosidase activity in whole *E. coli* cells (Fig. 4.37).

The effects of mercaptoethanol and EDTA on β-galactosidase activity in *K. marxianus* cells permeabilized with selected concentrations of solvents and surfactants are shown in Figures 4.38 and 4.39. Unlike in *E. coli* cells, there was no significant effects of EDTA and mercaptoethanol on solvent and surfactant permeabilization and β-galactosidase activity in *K. marxianus* cells.

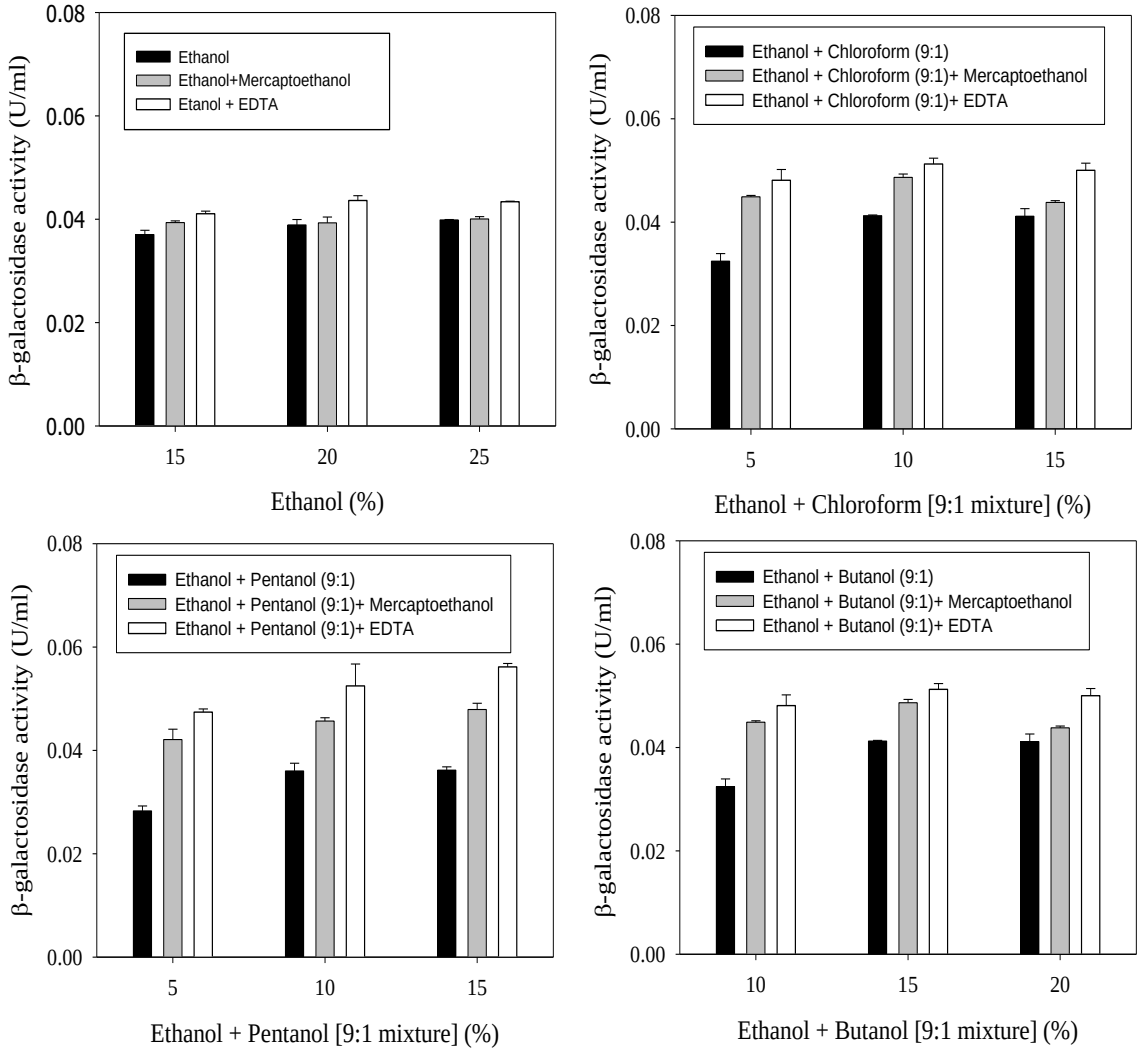

Figure 4.36 Effect of mercaptoethanol and EDTA on β-galactosidase activity in *Escherichia coli* cells permeabilized with solvents

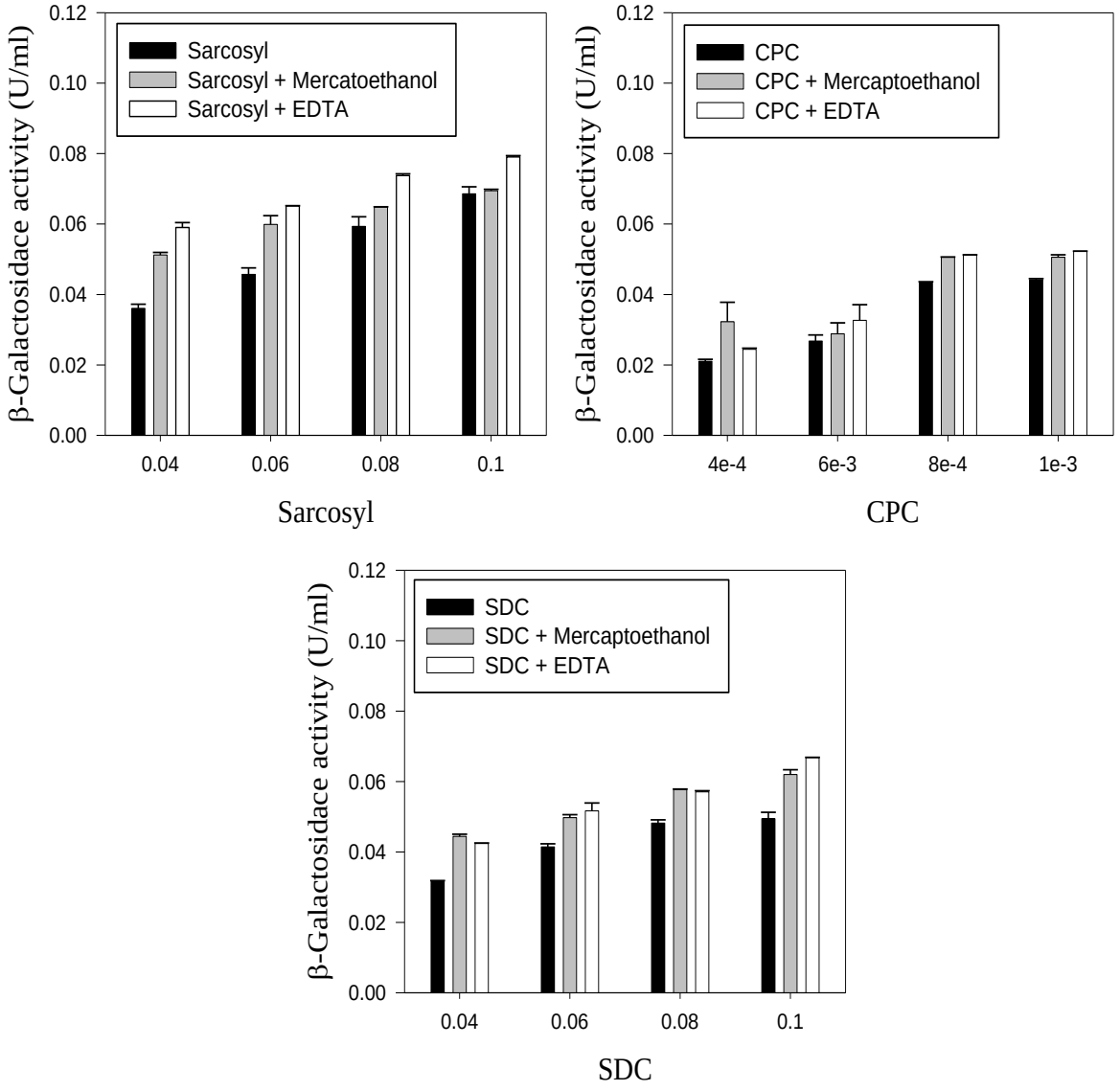

Figure 4.37 Effect of mercaptoethanol and EDTA on β-galactosidase activity in *Escherichia coli* cells permeabilized with surfactants

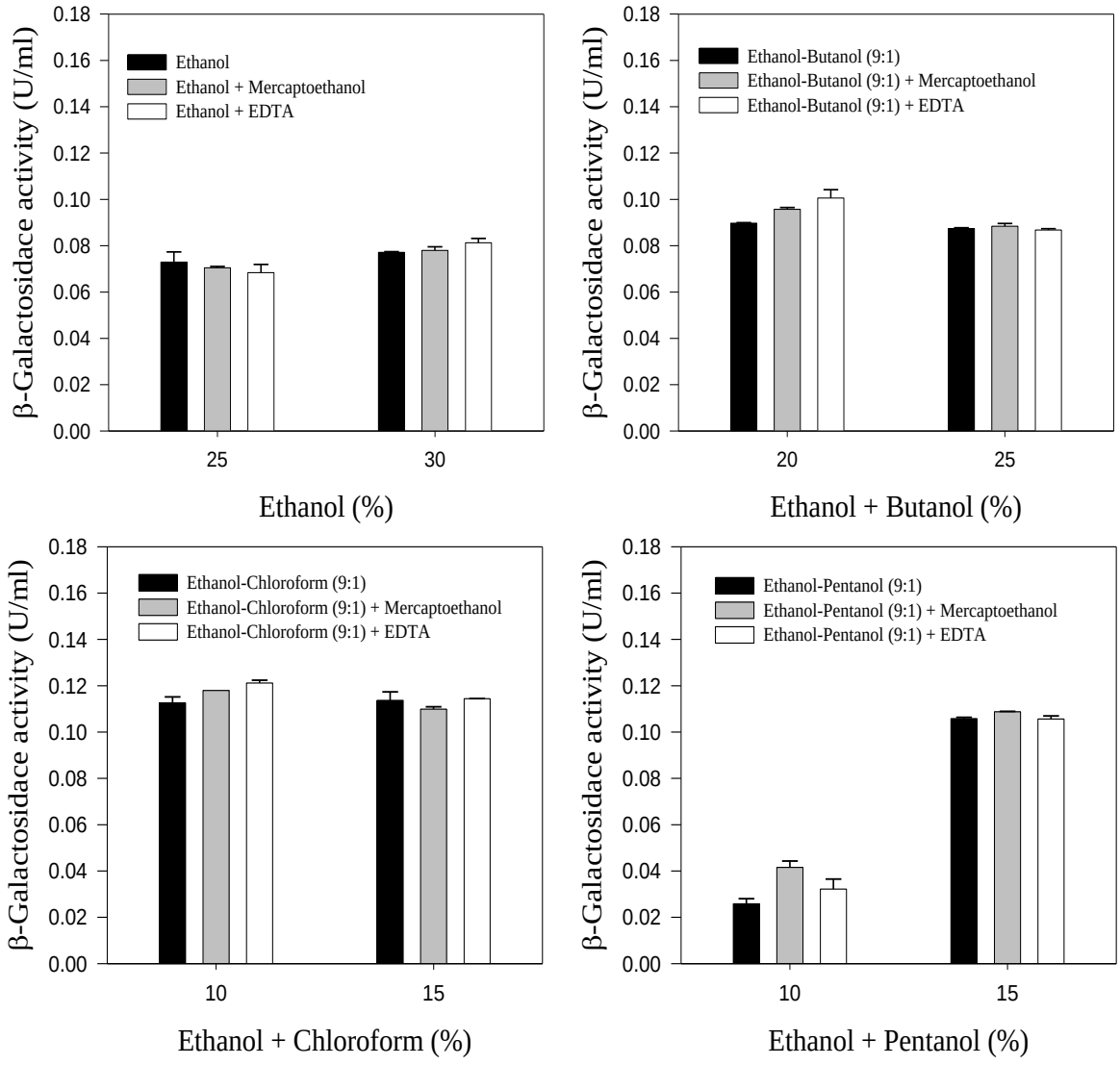

Figure 4.38 Effect of mercaptoethanol and EDTA on β-galactosidase activity in *K. marxianus* cells permeabilized with solvents

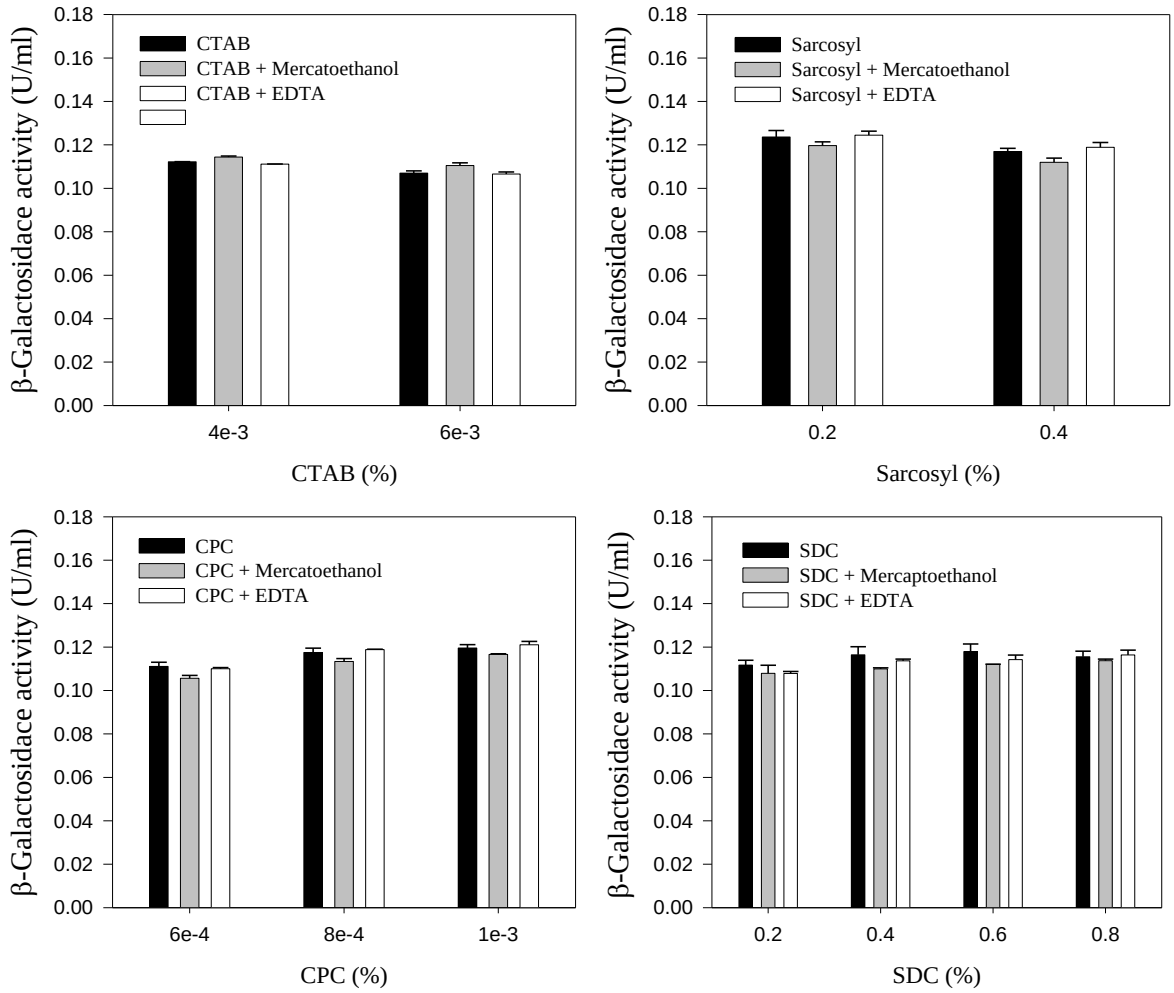

Figure 4.39 Effect of mercaptoethanol and EDTA on β-galactosidase activity in *Kluyveromyces marxianus* cells permeabilized with surfactants.

4.1.17 Comparison of methods for permeabilization *Escherichia coli* for β-galactosidase activity assay

The β-galactosidase activities under various treatments in comparison with the method of Miller (1972) are shown in Figure 4. 40. The treatments are: (0) No permeabilization treatment; (1) SDS + Chloroform method of Miller (1972); (2) 25% ethanol; (3) 5% of 9:1 ethanol-chloroform mixture; (4) 15% of 9:1 ethanol-pentanol mixture; (5) 20% of 9:1 ethanol-butanol mixture; (6) 0.02% SDS; (7) 0.02% Triton X-100; (8) 0.02% Tween 20; (9) 0.02% SDC; (10) 0.1% sarcosyl; (11) 0.2% sarcosyl; (12) 0.002% CTAB; (13) 0.0008% CPC; (14) 0.064% CTAB + 0.032% SDC. The β-galactosidase activity with 5% of 9:1 ethanol-chloroform mixture, 0.0008% CPC, 0.002% CTAB and 0.2% sarcosyl were comparable with Miller's SDS-chloroform treatment. The β-galactosidase activities relative to the Miller's are shown in Table 4.11. The statistical association of the six treatments (designated 1, 3, 10, 11, 12 and 13) with high β-galactosidase activity is shown in Figure 4.40. β-galactosidase activity at 0.1% and 0.2% sarcosyl were statistically different from each other ($P < 0.05$).

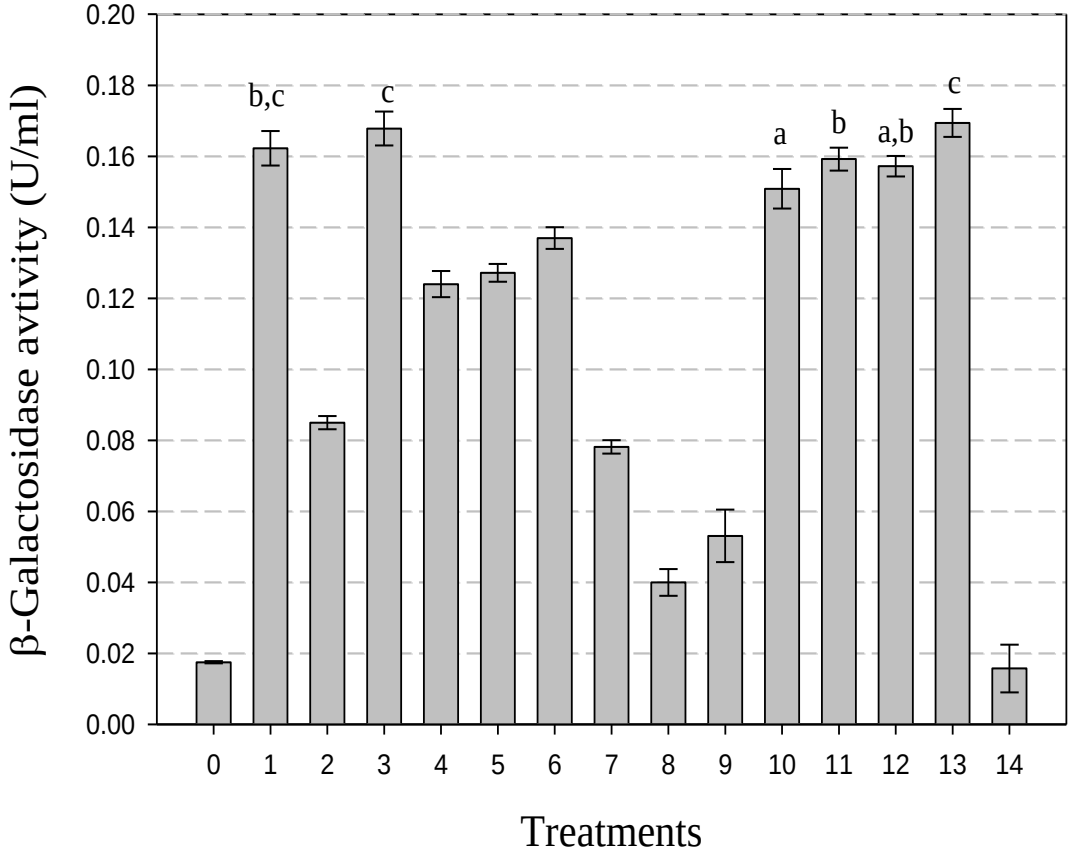

Figure 4.40 β-galactosidase activities in *Escherichia coli* cells under different permeabilization treatments. The values with same letters were not significantly different from each other ($P > 0.05$).

Table 4.11 Permeabilization efficiency of different treatments for β-galactosidase activity assay in *Escherichia coli*

S/N	Solvent, surfactant and solvent mixture	Relative activity (%)
1	SDS + Chloroform (Miller, 1972)	100
2	25% Ethanol	52.4
3	5% Ethanol + Chloroform (9:1)	103.6
4	15% Ethanol + Pentanol (9:1)	76.5
5	20% Ethanol + Butanol (9:1)	78.5
6	0.02% SDS	84.5
7	0.02% Triton X-100	48.2
8	0.02% Tween 20	24.7
9	0.02% Sodium Deoxycholate (SDC)	32.8
10	0.1% Sarcosyl	93.1
11	0.2% Sarcosyl	98.3
12	0.002% CTAB	97.0
13	0.0008% 1-Cetylpyridinium chloride (CPC)	104.6
14	0.064% CTAB+ 0.032% SDC	9.7
14	No treatment	10.8

4.1.18 Comparison of methods for permeabilization *Kluyveromyces marxianus* for β-galactosidase activity Assay

The β-galactosidase activities under various treatments in comparison with the method of Kippert (1995) are shown in Figure 4. 41. The treatments are: (1) 0.133% sarcosyl (Kippert, 1995); (2) 0.15% sarcosyl; (3) 0.2% sarcosyl; (4) 0.0008% of CPC; (5) 0.4% SDC; (6) 0.004% CTAB; (7) 0.004% CTAB + 0.0002% CPC; (8) 0.004% CTAB + 0.0004% CPC; (9) 0.004% CTAB + 0.0006% CPC; (10) 0.004% CTAB + 0.0008% CPC; (11) 15%of 9:1 ethanol:chloroform mixture; (12) 15% of 9:1 ethanol:pentanol mixture; (13) 25% of 9:1 ethanol: butanol mixture. The β-galactosidase activity with 0.15% and 0.2% sarcosyl, 0.4% SDC, 15% of 9:1 ethanol-chloroform mixture and 15% of 9:1 ethanol-pentanol mixture are comparable with Kippert's 0.133% sarcosyl treatment. The β-galactosidase activities relative to Kippert's method are shown in Table 4.12. The statistical association of the six treatments (designated 1, 2, 3, 5, 11 and 12) with high β-galactosidase activity are shown in Figure 4.41. The β-galactosidase activity at 0.15% sarcosyl was higher than 0.2% sarcosyl and 0.133% sarcosyl of Kippert(1995).

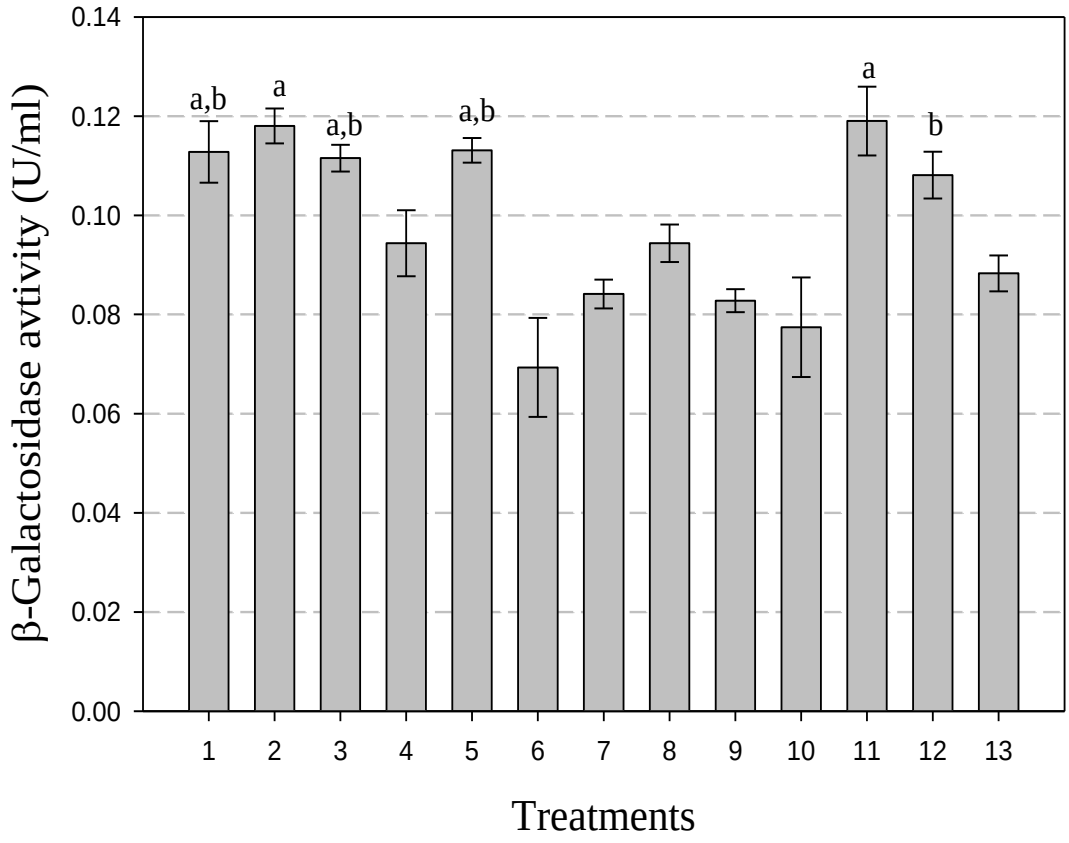

Figure 4.41 β-galactosidase activities in *Kluyveromyces marxianus* cells under different permeabilization treatmentsThe values with same letters were not significantly different from each other (P > 0.05).

Table 4.12 Permeabilization efficiency of different treatments for β-galactosidase activity assay *Kluyveromyces marxianus*

S/N	Solvent, surfactant and solvent mixture	Activity (%)
1	0.133% Sarcosyl	100.0
2	0.15% Sarcosyl	104.4
3	0.2% Sarcosyl	98.7
4	0.0008% CPC	83.5
5	0.4% Sodium Deoxycholate (SDC)	100.1
6	0.004% CTAB	61.3
7	0.004% CTAB + 0.0002% CPC	74.4
8	0.004% CTAB + 0.0004% CPC	83.5
9	0.004% CTAB + 0.0006% CPC	73.2
10	0.004% CTAB + 0.0008% CPC	68.5
11	15% Ethanol + Chloroform (9:1)	105.3
12	15% Ethanol + Pentanol (9:1)	95.7
13	25% Ethanol + Butanol (9:1)	78.1

4.2 Discussion

4.2.1 Inhibition of the activities of cell-free β-galactosidase from *E. coli* and *K. marxianus* by solvents and surfactants

E. coli and *K. marxianus* are well-known for the production of β-galactosidase, a hydrolase enzyme that enables the organisms to breakdown lactose into galactose and glucose (Mattew, 2005; Pansar et al., 2007; Yadav, Bezawada, Yan, Tyagi, & Surampalli, 2014). Assay for β-galactosidase activity requires permeabilization of whole microbial cells with chemical agents to allow penetration of the chromogenic substrate, *o*-nitrophenyl-β-D-galactopyranoside (oNPG), into the intact cells (Kippert, 1995). The enzyme substrate can moderately diffuse into *E. coli* cell to interact with the β-galactosidase. According to Cho, Kim, Yeon, Yoon and Kim (2010), less than 2.3% of *o*NPG was hydrolyzed when the artificial chromogenic substrate was applied to intact *Escherichia coli* cells. This suggests the effective provision of a permeability barrier against *o*NPG penetration into the cell cytoplasm and indicates evidence of background β-galactosidase activity in whole *E. coli* cells without permeabilization. *K. marxianus* has a thicker cell wall with glucans (Gomes et al., 2020) than *E. coli,* making cell permeabilization even more critical for the assay of β-galactosidase activity in *K. marxianus* cells. Organic solvents, including ethanol, chloroform, pentanol, N, N-dimethylformamide, DMSO, and butanol, are commonly employed as permeabilization agents for the assay of β-galactosidase activity in intact bacterial and yeast cells (de Faria et al. 2013; Fenton, 1982; Kumari et al. 2011; Panesar et al. 2007). In addition to organic solvents, surfactants are also applied to permeabilize microbial cell membranes during assay of β-galactosidase activity. Among the wide range of surfactants, sodium dodecyl sulphate, Triton X-100, sodium deoxycholate, Tween 20, Tween 80, cetyltrimethylammonium bromide (CTAB), and sarcosyl are usually selected for cell permeabilization (Guven & Bashan, 1998; Joshi, Gowda, & Bhat, 1987; Kaur, Panesar, Kumar, & Harish, 2009; Miozzari, Niederberger, & Hütter, 1978; Voget, 2018; Yadav et al., 2014). In this study, we evaluated the use of 1-cetylpyridinium chloride, which has not been reported as a permeabilization agent in the assay of β-galactosidase activity.

Some organic solvents and surfactants inhibit the activity of β-galactosidases (Guven & Bashen, 1998; Kippert, 1995). When applied, cell permeabilizing agents could penetrate the cell envelope in bacteria and yeast or cause the leakage of β-galactosidases, inhibiting the

activity of β-galactosidase. During the permeabilization of cells for the production of whole-cell biocatalysts, higher concentrations of these chemical agents could be employed. Cells are usually harvested and washed before determining the β-galactosidases activity of the permeabilized cells (Kumari et al., 2011; Panesar, 2008; Panesar et al., 2007). Nevertheless, the permeabilizing agent remains integral to the reaction mixture during *in situ* assay. This underlined the need to investigate the inhibitory effects of cell permeabilization agents on microbial β-galactosidase activity. Information on the influence of solvents and surfactants on microbial β-galactosidase activity can be useful for understanding and further development of methods for assay of the enzyme in microbial cells.

In this study, we evaluated the inhibitory effects of organic solvents, solvent mixtures, and surfactants on cell-free and cell-bound β-galactosidases with a view to establishing their sub-inhibitory concentrations for the development of methods for rapid *in situ* determination of β-galactosidase activity in *E. coli* and *K. marxianus* cells. The study revealed stimulation of the activity of cell-free β-galactosidase from *Escherichia coli* by 5%, 10%, and 15% ethanol. This corroborates the report of Soto et al.(2017) that 4% ethanol was not inhibitory to the activity of β-galactosidase from *Bacillus circulans* due to the conservation of the enzyme structure. Perhaps the amount of ethanol in the reaction media was not enough to reduce the water activity that affects the enzyme. The effect of ethanol on the secondary structure depends on the ethanol concentration and enzyme type (Bell et al., 2013; Lin et al., 1995; Lin, Wei, Li, & Wang, 2004). A concentration equal to 4% ethanol (equivalent to 0.9M) is in the range (0-2M ethanol) that does not affect the kinetic constants of the β-galactosidase due to the little effect on the secondary structure (Bell et al., 2013). In addition, Bell et al. (2013) reported that at modest concentrations (0 - 2M; 0 - 8.9%), there was little effect of methanol, ethanol, propanol, and butanol on the kinetic constants of β-galactosidase from *Kluyveromyces lactis*. Shifrin and Hunn (1969) opined that 5% ethanol stimulated the activity of β-galactosidase from *E. coli*. It is important to note that low concentrations of ethanol did not stimulate the activity of cell-free β-galactosidase from *K. marxianus*. At 18%, ethanol and N, N-dimethylformamide deactivated the activity of β-galactosidase derived from *E. coli* (Brena, Irazoqui, Giacomini, & Batista-viera, 2003). Information on the inhibitory effects of ethanol on microbial β-galactosidase activity has bearing upon the management of ethanol concentration during the fermentation process. The final concentration of ethanol in

conventional fermentation is limited to 10 or 15% v/v (Leon, Fernandes, Pinheiro, & Cabral, 1998). There is a scarcity of information on the inhibitory effect of DMSO on β-galactosidases from *E. coli* and *K. marxianus*. Nonetheless, Kamran, Bibi, Aman and Qader (2019) reported inhibition of *Aspergillus nidulans* β-galactosidase activity by 29%, 32% and 35% at 1 mM (0.0071% v/v), 5 mM (0.0355% v/v) and 10 mM (0.071% v/v) respectively. This indicated that DMSO is a potent inhibitor of β-galactosidase for *Aspergillus nidulans*. Our study with *E. coli* and K. *marxianus* β-galactosidases, also portrayed DMSO as potent inhibitor of β-galactosidase many folds higher than ethanol. The average estimated No-Observable-Effect-Concentration (NOEC) for DMSO against *E. coli* and *K. marxianus* β-galactosidases were 7. 939 % v/v and 1.414% v/v respectively. At 50% (v/v) of aqueous-organic solvent mixture system, after 5 min preincubation at 37^{o}C, DMSO and DMF completely inhibited the activities of β-galactosidases from *Aspergillus oryzae*, *E. coli* and *Kluyveromyces fragilis* (Yoon & Mckenzie, 2005). This corroborated our report on the toxicity of DMSO and DMF against β-galactosidases from *E. coli* and *K. marxianus*. In comparison, the β-galactosidases of *E. coli* and *K. marxianus* are more tolerant to inhibitory effect of DMSO than the β-galactosidases from *Aspergillus nidulans*. The differences in the response of these β-galactosidases to inhibitory effects of solvents could possibly be attributed to structural differences among the enzymes. In our study, N,N-dimethylformamide had more inhibitory effect than DMSO against β-galactosidase from *E. coli* but have similar inhibitory effect against *K. marxianus* β-galactosidase. The order of toxicity is DMF ≥ DMSO > ethanol. This milder effects of ethanol in comparison to DMSO and DMF have been reported elsewhere (Kamran et al. 2019).

Chloroform, pentanol, and butanol are not miscible with water and thus have solubility issues in assessing their inhibitory effects on β-galactosidase in aqueous systems. Hence, we investigated their effects in binary mixtures with ethanol, DMSO, and DMF. Butanol and pentanol are longer-chain alcohols and, therefore, would be theoretically less inhibitory than ethanol. Information about the inhibitory effects of such mixtures on cell-free β-galactosidase is scarce. It is important to emphasize important observations made with these mixtures. Chloroform, butanol, and pentanol moderated the effects of ethanol, masking the stimulatory effects of ethanol at low concentrations and reducing the median inhibitory concentrations (EC_{50}) and No-Observable-Effect Concentrations. This moderation appeared to increase with the increase in the relative proportions of the water-immiscible solvents in both β-

galactosidases. We further investigated the inhibitory effects of binary mixtures of ethanol with DMSO and DMF and assessed their interactive inhibitory effects against the activities of the β-galactosidases. The TI values and model deviation ratios between the predicted and experimentally derived effect concentrations of the solvent mixtures (ethanol-DMSO and ethanol-DMF mixtures) are around 1.0 and lie between 0.5 and 2.0, suggesting that the deviations are marginal and within the expected inter-laboratory/inter-experiment deviation for most species (Li et al., 2014; Petersen & Tollefsen, 2011). Therefore, the combined effects of the mixture were considered to be additive. The concentrations of the individual solvents and solvent mixtures below NOEC values were sub-inhibitory and recommended for use as permeabilizers during *in situ* β-galactosidase activity measurements in microbial cells. The question is, would these solvents and solvent mixtures be able to permeabilize *E. coli* and *K. marxianus* cells at the sub-inhibitory concentrations? This important consideration will be discussed in a subsequent paragraph.

Sodium dodecyl sulphate (SDS), SDC, Triton X-100, sarcosyl, and CTAB are popular surfactants that have been used to permeabilize bacterial and yeast cells for *in situ* β-galactosidases activity measurements (Joshi et al., 1987; Kippert, 1995; Miozzari, Niederberger, & Hütter, 1978). CPC, Tween 20, and Tween 80 were less prevalent in β-galactosidase assays. Among these surfactants, SDS, CPC, and CTAB were inhibitory to the microbial β-galactosidases. In this study, we observed that the cell-free β-galactosidases from *E. coli* and *K. marxianus* were highly sensitive to the inhibitory effects of SDS. At 0.02% and 0.04% (w/v), SDS was sub-inhibitory to *E. coli* β-galactosidase. These low concentrations of SDS inhibited *K. marxianus* β-galactosidase by 3.9% and 10.1%, respectively. At 0.06%, SDS inhibited the β-galactosidases from *E. coli* and *K. marxianus* by 20% and 12.4%, respectively. Guven and Bashan (1998) have reported that 0.005% of SDS slightly inhibited the activity of purified *E. coli* β-galactosidase. Nonetheless, at 0.02% and 0.1%, the enzyme activity was significantly inhibited (Guven & Bashan, 1998). This report was corroborated by the findings of this study on cell-free β-galactosidase from *E. coli*. In this study, at 0.1%, SDS inhibited *E. coli* and *K. marxianus* β-galactosidases by 30% and 14.7%, respectively. However, a seemingly conflicting report by Muga, Arrondo, Bellon, Sancho and Bernabeu (1993) has been reported. They demonstrated that at 20^{o}C, purified β-galactosidase from *E. coli* was considerably resistant to SDS inactivation up to as high as 2%. Nevertheless, the enzyme

activity decreases with increasing temperature in the presence of SDS. This SDS effect may be attributed to SDS interaction with α-helix and β-structure of the β-galactosidase enzyme (Muga et al. 1993). The differences in these reports could be attributed to variations in temperatures employed in the study. While Muga and co-workers worked at 20^{o}C with purified enzyme, we determined the activity of crude *E. coli* β-galactosidase at 30^{o}C. More recently, Zhou et al. (2021) reported significant inactivation of β-galactosidase from a marine *Bacillus* sp. BY02 by SDS.

In this study, both β-galactosidases were sensitive to the inhibitory effects of CTAB. Concentrations of CTAB up to 0.004 ± 0.001% (*E. coli* β-galactosidase) and 0.0043 ± 0.000% (*K. marxianus* β-galactosidase) were sub-inhibitory. There is a paucity of information on the inhibitory effects of CTAB on cell-free microbial β-galactosidases. However, various concentrations of CTAB have been used to permeabilize cells for β-galactosidase activity assays. Bachhawat, Gowda and Bhat (1996) reported using 0.1% CTAB (0.01 g CTAB/g cell) for the permeabilization of *Kluyveromyces fragilis* cells. *E. coli* cells were permeabilized with 0.01% CTAB solution (Tyler & Magasanik, 1969). An optimum CTAB concentration of 0.06% was used for the permeabilization of *K. marxianus* cells (Kaur et al., 2009). Similarly, the β-galactosidases of *E. coli* and *K. marxianus* were sensitive to the inhibitory effects of CPC. There is a scarcity of information on the inhibitory effects of CPC on cell-free β-galactosidases from microorganisms. CPC with EC_{50} of 0.00138 ± 0.00002% and 0.0010 ± 0.00001% inhibited the enzyme activities more than CTAB.

SDC, Triton X-100, sarcosyl, Tween 20, and Tween 80 were mild to the β-galactosidase enzymes. Effects of Triton X-100 are less deleterious on β-galactosidases due to the absence of charge (Escobar, Bernal, & Mesa, 2013). Triton X-100 has been reported to stabilize β-galactosidases(Escobar et al., 2013; Escobar, Bernal, & Mesa, 2015; Soto et al., 2017). According to Guven and Bashan (1998), 0.1% Triton X-100 and Tween 20 have no significant effect on *E. coli* β-galactosidase activity. In addition, when compared with the absence of the surfactant, 1.5% of Triton X-100 had no inhibitory effect on the enzyme activity and even slightly stimulated β-galactosidase activity. This is in line with the findings of our study. We observed stimulation of cell-free β-galactosidase from *Kluyveromyces marxianus* at Triton X-100 concentrations ranging from 0.2% to 2.0%. A similar stimulatory effect of Triton X-100 was observed with cell-free β-galactosidase from *E. coli* at concentrations above 1.5%. In this

study, Tween 20 had no inhibitory effect on *E. coli* and *K. marxianus* β-galactosidase activity at concentrations ranging from 0.1% to 2%. Tween 80 also stimulated the activity of cell-free *Kluyveromyces marxianus* at concentrations above 0.06% and up to 2%. In the case of *E. coli* β-galactosidase, Tween 80 did not have an inhibitory effect at concentrations ranging from 0.1% to 4%. This mild nature of Triton X-100, Tween 20, and Tween 80 could be attributed to the inability of the surfactants to denature (unfold) the β-galactosidase enzymes. Thus, these surfactants can be used in biochemical assays involving β-galactosidases without affecting enzyme activity. Also in this category is N-lauryl sarcosine (sarcosyl) which has been adjudged as a mild detergent (Yadav et al. 2014).

The metal chelating agent, EDTA has been reported to improve β-galactosidase activity in cells (Prasad et al., 2013). Thus, we investigated the effects of EDTA on the activities of the cell-free β-galactosidases. At EDTA concentration as low as 0.01% (≈ 0.342 mM), the activity of *E. coli* β-galactosidase was inhibited by 4.6%. Concentrations ranging from 0.002% to 0.008% were sub-inhibitory for β-galactosidase from *E. coli*. EDTA was even more potent against *K. marxianus* β-galactosidase. At 0.002% (≈ 0.0684 mM), EDTA inactivated *K. marxianus* β-galactosidase by 10.8%. This probably suggested that the enzymes are metalloenzymes. According to Guven, Kaplan, Guven, Matpan and Dogru (2011), EDTA concentrations up to 20 mM (≈0.58% w/v) did not significantly alter the activity of β-galactosidase purified from *Alicyclobacillus acidocaldarius*. At one (1) mM (≈ 0.029% w/v), EDTA inhibited the activity of β-galactosidase from a marine *Bacillus* sp. BY02 by 53.9% (Zhou et al., 2021). Similarly, one (1) mM EDTA inhibited β-galactosidase from *Bacillus stearothermophilus* by 2.6% (Chen et al. 2008).

4.2.2 Permeabilization of *E. coli* and *K. marxianus* cells by solvents and surfactants for β-galactosidase activity measurements

Chemical cell permeabilization agents alter the cell structure to make it porous so that small molecules, such as substrates and products, can move freely in and out of the intact cell, increasing metabolite production (Alves, Bosso, Rodrigo, & Morioka, 2022; Morioka et al., 2019). Various solvents and surfactants have been used to permeabilize microbial cells during enzyme assays. Solvents interact with membrane-associated sterols and phospholipids, causing their dissolution and subsequent formation of pores in the membrane. Solvents including toluene, chloroform, DMSO, ethanol, methanol, propanol, isopropanol, butanol and

pentanol have been used as cellular permeabilization agents for the production of whole-cell biocatalysts and *in situ* β-galactosidase activity measurements (Kumari et al., 2011; Liu, Fujita, Kondo, & Fukuda, 2000; Marcel, Pinho, Maria, & Passos, 2011). Surfactants are amphiphilic molecules, possessing both hydrophobic and hydrophilic parts in their structures. They are categorized as nonionic (e.g., Triton X-100), anionic (SDS, sodium deoxycholate, sarcosyl), cationic (CTAB, CPC), and Zwitterionic. These permeabilization agents are used to produce whole-cell biocatalysts on the premise that the cell remains intact without cell lysis and the enzymes leak out. This is not a critical consideration when the specific objective is to assay for total enzyme activity in bacterial or yeast cells. However, during permeabilization, intracellular enzymes could leak (Kumari et al., 2011). This necessitated the study of the inhibitory effects of solvents and surfactants against β-galactosidases in permeabilized bacterial and yeast cells.

In this study, we investigated the ability of solvents (ethanol, chloroform, butanol, pentanol, butanol, DMSO, and DMF) and surfactants (SDS, CTAB, Triton X-100, sarcosyl, SDC, CPC, Tween 20 and Tween 80) to permeabilize *E. coli* and *K. marxianus* cells for β-galactosidase activity measurements. DMSO and DMF did not permeabilize *E. coli* cells at concentrations up to 40%, given that there was no increase in β-galactosidase activity beyond the background level. Rather, DMSO and DMF at this concentration range reduced the background β-galactosidase activity in *E. coli* cells. Similarly, DMSO and DMF did not permeabilize *K. marxianus* cells at concentrations up to 30% (DMSO) and 35% (DMF). DMSO could only permeabilize *K. marxianus* cells very poorly at 35% and 40%. On the other hand, DMF only permeabilized *K. marxianus* cells poorly at 40%. The poor cell permeabilization of DMSO has been reported elsewhere. According to Pascual and Herrera (1981), DMSO at 40% failed to permeabilize *Kluyveromyces* sp. cells. In the report of Kippert (1995), DMSO showed poor permeabilization of *Saccharomyces pombe* and *Saccharomyces cerevisiae* cells for β-galactosidase activity assay. Ethanol was used as permeabilizing agent to permeabilize bacterial and yeast cells for β-galactosidase activity assays (Alves et al., 2022; Decleire, Cat, & Huynh, 1987; Panesar et al., 2007; Panesar, 2008; Morioka, de Oliveira, lognesi, & Suguimoto, 2016; Morioka et al., 2019 Rodriguez-Colinas et al., 2011; Yadav et al., 2014; Xia et al., 2021). These studies used 27 % and 75% ethanol to permeabilize yeast cells for β-galactosidase assays in whole-cell biocatalysts. This could be possible because

usually, after cell permeabilization, the solvent is washed off from the cell before assaying for β-galactosidase activity. In our study, we used the solvents and surfactant to permeabilize *E. coli* and *K. marxianus* cells for *in situ* β-galactosidase activity assay where the cells were not washed and permeabilization agent remained the integral part of the reaction mixture. In our procedure, ethanol at concentration ranging between 15% and 30% v/v permeabilized *E. coli* cells to increase the β-galactosidase activity background control value. In the case of *K. marxianus*, cell permeabilization occurred at ethanol concentrations ranging between 25% and 40% v/v. However, there was a reduction in β-galactosidase activity at concentrations above 25%. The optimum ethanol concentration for both organisms is 25%. At this point, it is important to emphasize two important observations in our study. First, comparing *E. coli* and *K. marxianus* cells, there was no β-galactosidase activity detectable in *K. marxianus* cells without application of permeabilization agent after 20 min incubation at 30^{o}C, unlike in *E coli*. This indicates that the *K. marxianus* cell envelope is stronger than that of *E. coli* and that the β-galactosidase in the yeast is located inside the cell cytoplasm, while β-galactosidase in *E. coli* could be cell surface-bound. Second, comparing cell-free and whole-cell galactosidases, the sub-inhibitory concentrations of ethanol did not permeabilize the cells, especially the yeast cells. Also, concentrations of ethanol that inhibited cell-free β-galactosidase did not inhibit cell-bound. This is evidence that intracellular β-galactosidase in both organisms did not leak out of the cells at these ethanol concentrations and that the cell walls protected the intracellular β-galactosidases. These observations were also true for the ethanol mixtures with chloroform, butanol or pentanol. The deactivation of *E. coli* and *K. marxianus* β-galactosidase activity at higher concentrations of ethanol and solvent mixtures (significantly at 25% - 40% in *E. coli* and 35%-40% in *K. marxianus*) suggests that the intracellular β-galactosidase of *E. coli* may have leaked out of the cell to be exposed to the inhibitory effects of ethanol and solvent mixtures. After repeated experiments, the selected optimal concentrations of the solvents for both organisms were ethanol (25%), 9:1 ethanol-chloroform (10% and 15%), 9:1 ethanol-pentanol (15%) and 9:1 ethanol-butanol (20%) under the assay conditions (20 min permeabilization time, 30 min post-permeabilization incubation with *O*NPG at 30^{o}C, in Z-buffer of pH 7.0). Solvent mixtures have been used to permeabilize yeast cells for subsequent determination of β-galactosidase activity (Stred, Tomti, Sturdik, & Kremnick, 1993; Flores et al., 1994; Numano & Sungur, 2004; Kumari et al. 2011). A 9:1

ethanol-chloroform mixture has been used to permeabilize *Kluyveromyces* cells for β-galactosidase activity assay (Champluvier, Kamp, & Rouxhet, 1988; Stred'ansky et al. 1993). Stred'ansky et al. (1993) reported an optimum condition of 37^{o}C, pH 9.5 -10.5, 0.1 -0.5 M phosphate buffer and 1% chloroform after 1.5 – 2 h treatment.to extract over 90% of the intracellular β-galactosidase from *Kluyveromyces marxianus* cells. This amount of chloroform is equivalent to using 10% of 9:1 ethanol-chloroform mixture in a 2 ml final volume, as in our study. This concentration of 9:1 ethanol-chloroform mixture equally gave optimal permeabilization of *K. marxianus* cells in our study. According to Flores et al. (1994), no β-galactosidase activity was detected in *Kluyveromyces lactis* cells when treated with 10% ethanol. Treatment with 2% chloroform and 5:1 ethanol-chloroform mixture resulted in almost equal β-galactosidase activity.

We also investigated the ability of surfactants to permeabilize *E. coli* and *Kluyveromyces* cells. All the tested surfactants could permeabilize *E. coli*, with Tween having the least permeabilization ability. In the case of *K. marxianus*, Tween 20, SDS, Triton X-100 and Tween 80 were poor permeabilizers of *K. marxianus* cells. For further studies on *K. marxianus* cells, SDC, CPC, CTAB and sarcosyl were selected. On the other hand, SDS, Triton X-100, Tween 20, SDC, CTAB and Sarcosyl were selected for further studies on *E. coli* cells. The optimum concentrations of these surfactants for *K. marxianus* were 0.004% (CTAB), 0.2% (sarcosyl), 0.0006-0.001% (CPC) and 0.2-0.8% (SDC). Optimal surfactant concentrations for *E. coli* were 0.02% (SDS, Triton X-100, Tween 20 and SDC), 0.1 and 0.2% (sarcosyl), 0.002% CTAB and 0.0008% (CPC). With the exception of CPC, which has not been widely studied, these surfactants have been reportedly used at varying concentrations to permeabilize microbial cells for β-galactosidase activity assay in whole bacterial and yeast cells.

Investigation indicated that mercaptoethanol and EDTA potentiated β-galactosidase activity, especially in *E. coli* cells. The absence of mercaptoethanol resulted in about a 30% decrease in β-galactosidase activity in recombinant *Saccharomyces* species (Kippert, 1995). EDTA has been reported to aid in releasing β-galactosidase from Gram-negative bacterial cell walls (Geciova et al., 2000). The SDS-chloroform method reported by Miller (1972) has been widely used for permeabilization of bacterial cells for β-galactosidase activity assay. According to the Miller method, SDS and chloroform were applied at final concentrations of 0.0043% (w/v) SDS and 8.7% (v/v) chloroform, respectively. Since the publication of Miller's

method, other researchers have reported variable proportions of SDS and chloroform for the permeabilization of microbial cells. Griffith and Wolf (2002) reported the application of 0.0017% or 0.0018% final concentration of SDS and 3.4% or 3.6% final concentration of chloroform to permeabilize *E. coli* K-12 strain HB 301 cells for β-galactosidase activity assay. Dutton et al. (1990) applied 0.0016% SDS and 1.6% chloroform to permeabilize *E. coli* cells to assay β-galactosidase activity. However, Dutton et al. (1990) further reported that 0.32% SDS does not inhibit the *E. coli* β-galactosidase activity and could replace a combination of 0.0016% SDS and 1.6% chloroform. In this study, 0.002% SDS showed poor permeabilization of *E. coli* cells, and 0.1% SDS almost totally inhibited β-galactosidase activity in *E. coli* cells. This implies that 0.32% SDS reported by Dutton et al. (1990) would totally inhibit β-galactosidase activity in our own *E. coli*. Rezaee (2003) used 0.0016% SDS and 3.2% chloroform to permeabilize yeast cells for a rapid and sensitive assay of β-galactosidase activity with 5-bromo-4-chloro-3-indolyl-ß-D galactopyranoside (X-Gal). In the case of our own *K. marxianus*, while working with *o*NPG, SDS showed poor cell permeabilization and low β-galactosidase activity at SDS concentrations up to 0.04%. Optimum cell permeabilization and β-galactosidase activity in *K. marxianus* cells was obtained at 0.08% SDS. However, this optimum activity was much lower than that of sarcosyl, CTAB, SDC and CPC.

A combination of CTAB and SDC has been used for permeabilization of microbial cells Zhang and Bremer (1996) permeabilized *E. coli* cells with 0.04% CTAB and 0.02% SDC in the first instance for 30 min to 2h at 30^{o}C. This concentration was later diluted by a substrate mixture containing 0.002% CTAB and 0.001% SDC, resulting in the final concentration of 0.0043% CTAB and 0.0016% CTAB in the assay reaction mixture, which was further incubated for 1-3h. The protocol of Zhang and Bremer (1996) is time-consuming, and the sequential addition of CTAB and SDC as components of the substrate mixture is unnecessary. Therefore, a method has been suggested using a final concentration of 0.064% CTAB and 0,032% SDC to permeabilize cells for β-galactosidase activity assay with *o*NPG while adopting Miller unit measurement (https://research.pomona.edu/jane-liu/files/2012/08/Beta-Galactosidase-Assay-A-better-Miller-LIU-LAB.pdf). In our study, the optimal CTAB concentration was 0.002% and 0.004% for *E. coli* and *K. marxianus* respectively.

4.2.3 Comparison of methods

Miller's SDS-chloroform method has been the reference method for assay of β-galactosidase activity in bacterial cells. However, the method has drawbacks due to the water insolubility of chloroform. Chloroform interferes with optical density or absorbance readings and is incompatible with standard microtitre plates (Schaefer et al. 2016). In addition, chloroform may have variable permeabilization and unevenly permeabilized cells due to water insolubility. An alternative method involving the use of lysozyme and Popculture reagent was suggested by Schaefer et al. (2016). However, lysozyme and Popculture are expensive reagents, making the method very costly. Other methods involving CTAB and SDC have also been suggested (Toulouse et al., 2017). This study was embarked upon to develop alternative methods for β-galactosidase activity assay in whole microbial cells. We came up with optimal concentrations of several solvents and surfactants (working with Z-buffer pH 7.0, 20 min permeabilization and 30 min post-permeabilization incubation time) for permeabilization and β-galactosidase activity assay in *E. coli* and *K. marxianus* cells. Our methods were compared with the Miller method. Comparative analysis showed that 5% of 9:1 ethanol-chloroform mixture and 0.0008% CPC could produce even better results than the Miller method. However, there was no statistical difference between the Miller and the methods above. The ethanol helped to disperse the chloroform, making it more evenly distributed in the aqueous reaction mixture. CPC is highly soluble in water and would not affect microtitre plates. The fact that CPC permeabilized *E. coli* cells at a very low concentration makes it even more cost-effective. These suggested methods could thus replace Miller's SDS-chloroform method. Other treatments that gave reasonably good results are 0.1% sarcosyl, 0.2% sarcosyl and 0.004% CTAB applications under the assay condition. They could thus be further optimized for a possibly better result.

In the case of *K. marxianus*, the reference method for comparison is the method suggested by Kippert (1995) for rapid determination permeabilization and accurate quantitative determination of β-galactosidase activity in yeast cells. Kippert (1995) used a final concentration of 0.133% sodium lauryl sarcosinate (sarcosyl) in Z-buffer. We adopted the method of Kipert (1995) but with different concentrations of solvents and surfactants for the permeabilization and quantitative determination of β-galactosidase activity in *K. marxianus*. A comparative analysis of the method indicated that 0.15% sarcosyl treatment of *K. marxianus*

produced even higher β-galactosidase activity than Kippert's 0.133% sarcosyl would suggest for *Saccharomyces* species. β-galactosidase activity equal to 0.15% sarcosyl was produced by 15% of the 9:1 ethanol-chloroform mixture. Other treatments that produced β-galactosidase activity that was not statistically different from Kippert's 0.133% sarcosyl were 15% of a 9:1 ethanol-pentanol mixture and 0.4% SDC. Other treatments that produced reasonably high but lower β-galactosidase activity than the sarcosyl treatment were 0.0008% CPC and 25% of a 9:1 ethanol-butanol mixture. These promising methods could further be optimized for an improved result. This indicates that Kippert's method could be replaced. However, since the assay result could depend on the specific organism and other factors, further investigation with a wide variety of yeasts and other experimental conditions would be necessary.

CHAPTER FIVE

CONCLUSION AND RECOMMENDATIONS

5.1 Conclusion

The study investigated the inhibitory effects of surfactants, solvents and solvent mixtures on the activities of β-galactosidases from *E. coli* and *K. marxianus* with a view to establishing their sub-inhibitory concentrations for *in situ* assay of β-galactosidase activity in intact cells. The inhibitory effects of solvents on the cell-free β-galactosidase activity were in the order: DMF > DMSO > ethanol (*E. coli*) and DMF >DMSO ≥ ethanol (*K. marxianus*). CPC, SDS and CTAB have dose-dependent inhibitory effects on the β-galactosidases. On the other hand, Triton X-100, SDC, sarcosyl, Tween 20 and Tween 80 have no dose-dependent inhibition of the enzyme activities at concentrations up to 4%. Unlike *K. marxianus*, ethanol had a hormetic effect on *E. coli* cell-free β-galactosidase activity. Ethanol-DMSO and ethanol-DMF mixtures had additive effects on the activities of cell-free β-galactosidases from *E.coli* and *K. marxianus.*

With the exception of ethanol and 9:1 ethanol-chloroform mixture for *E.coli*, the sub-inhibitory concentrations of the solvents and solvent mixtures were not optimal for permeabilization of *E. coli* and *K. marxianus* cells. Also, with the exception of CTAB, sub-inhibitory concentrations of the inhibitory surfactants (CTAB, SDS and CPC) were not optimal for permeabilization of *E. coli* and K. *marxianus* cells for *in-situ* β-galactosidase activity. Concentrations of 9:1 mixtures of ethanol and water-immiscible solvents ranging from 10% to 25% were good for permeabilization of the yeast and bacterial cells. The best surfactant concentrations for permeabilizing *E. coli* cells for *in-situ* β-galactosidase activity were 0.08% and 0.1% SDC, 0.1% (sarcosyl), 0.0008% and 0.001% (CPC). Similarly, the best concentrations of surfactants for *K. marxianus* permeabilization were 0.0008% (CPC), 0.15% (sarcosyl), 0.2 to 0.8% (SDC) and 0.004% (CTAB). Surfactants like Tween 20, Tween 80, SDS and Triton X-100 were not good for permeabilization of *K. marxianus cells*. The study suggested that CPC could potentially be used for permeabilization of *E. coli* and *K marxianus* cells for *in-situ* β-galactosidase activity assays. In the case of *E. coli*, CPC produced a result slightly greater than the classical Miller's method. Investigations indicated that mercaptoethanol and EDTA potentiated the activity of β-galactosidase more in E. coli than in

K. marxianus, highlighting the importance of the inclusion of EDTA and mercaptoethanol in the reaction mixture.

Compared with existing classical methods, we concluded that 5% of 9:1 ethanol-chloroform mixture and 0.0008% CPC produced better results than Miller's SDS-chloroform method for assay of β-galactosidase activity in *E. coli*. These methods do not have solubility issues and could replace Miller's SDS-chloroform method. Sarcosyl at 0.1% and 0.2% produced high β-galactosidase activity (93.1% and 98.3% of Miller's method, respectively), which could be optimized for better results. Similarly, compared with Kippert's 0.133% sarcosyl, 0.15% sarcosyl, and 15% 9:1 ethanol-chloroform mixture produced higher β-galactosidase activity in *K. marxianus*. CPC at 0.0008% produced 83.5% of β-galactosidase activity *in K. marxianus*, suggesting that CPC could also be applied for β-galactosidase activity assay in intact cells.

In addition to the recommended methods that could replace the existing methods, these promising concentrations of solvents and surfactants could further be optimized to obtain better results in the *in-situ* assay of β-galactosidase activity in microbial cells.

5.2 Recommendations

Based on the findings of this study, we postulated the following recommendations for practical applications and future research.

a) A new protocol using 0.0008% CPC and 5% of 9:1 ethanol-chloroform mixture is recommended for β-galactosidase activity assay in *E. coli*.
b) Assay of β-galactosidase activity in *K. marxianus* with 0.15% sarcosyl and 15% of 9:1 ethanol-chloroform mixture to obtain higher β-galactosidase activity than Kippert's 0.133% sarcosyl.
c) The inclusion of EDTA and mercaptoethanol in the reaction mixture during permeabilization of *E. coli* and *K. marxianus* cells since they help to enhance the β-galactosidase activity in microbial cells, especially in *E. coli*.
d) Sarcosyl (0.1%, 0.2%) and 0.002% of CTAB concentrations for permeabilization and β-galactosidase activity in *E. coli* should be optimized in further studies since they gave good results.
e) CPC at 0.0008% and 25% of 9:1 ethanol-butanol mixture for β-galactosidase activity assay in *K. marxianus* should be optimized in future research since they gave good results.

f) Further investigation with a wide variety of bacteria and yeasts would be necessary since the β-galactosidase activity assay result could depend on the specific organism.

Contributions to Knowledge

a. Sub-inhibitory concentrations of surfactants, solvents and solvents mixtures for β-galactosidases from *E. coli* and *K. marxianus* were established by this study.
b. The ethanol-DMSO and ethanol-DMF binary mixture was reported to have an additive effect on the activities of cell-free β-galactosidases from *E. coli* and *K. marxianus*.
c. 1-Cetylpyridinium chloride (CPC) could potentially be used to permeabilize *E. coli* and *K. marxianus* cells for β-galactosidase activity assay. In *E. coli*, the application of 0.0008% CPC resulted in 4.6% increase in β-galactosidase activity compared to Miller's SDS-chloroform method.
d. An assay procedure with 5% of 9:1 ethanol-chloroform mixture resulted in a 3.6% increase of β-galactosidase activity compared to Miller's SDS-chloroform method for cell permeabilization and *in situ* β-galactosidase activity assay in *E. coli*.
e. In *K. marxianus*, cell permeabilization and β-galactosidase activity assay with 15% of 9:1 ethanol-chloroform mixture resulted in a 5.3% increase of β-galactosidase activity compared to the classical 0.133% sarcosyl of Kippert.
f. An assay procedure for cell permeabilization, and β-galactosidase activity assay in *K. marxianus* with 0.15% sarcosyl resulted in a 4.4% increase of β-galactosidase activity compared to Kippert's 0.133% sarcosyl.
g. These research findings aligned with Sustainable Development Goal number 3 (SDG 3), which stated that the research must improve healthcare and wellbeing. This simply implied that it ensures healthy lives of all ages and help to reduce diseases and sickness. One significant application of β-galactosidase is in healthcare, such as producing Galactooligosaccharide (GOS), solving the problem of lactose intolerance and diagnosing and treating diseases like galactosemia.
h. GOS encourages the growth of *E.coli* and *Bifidobacteria* in the gastrointestinal tract and intestine, respectively. This microbes is necessary for the proper functioning of the part of the body found, and confers health benefits such as supporting gut health and immunity. Also, galactosemia is a rare genetic disorder that affects the body's ability to break down galactose.

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APPENDICES

APPENDIX 1.0 MacFarland turbidity standards for bacteria

Table 1.0 : McFarland turbidity standards for bacteria

Standard	Cells/ml (x10^9)	ABS1	ABS2	ABS3	Mean	SD
0.5	0.15	0.1	0.097	0.099	0.099	0.002
1	0.3	0.163	0.158	0.161	0.161	0.003
2	0.6	0.304	0.358	0.331	0.331	0.027
3	0.9	0.463	0.453	0.458	0.458	0.005
4	1.2	0.559	0.61	0.585	0.585	0.026
5	1.5	0.77	0.754	0.762	0.762	0.008
6	1.8	0.955	0.931	0.943	0.943	0.012
7	2.1	1.071	1.033	1.052	1.052	0.019
8	2.4	1.239	1.168	1.204	1.204	0.036

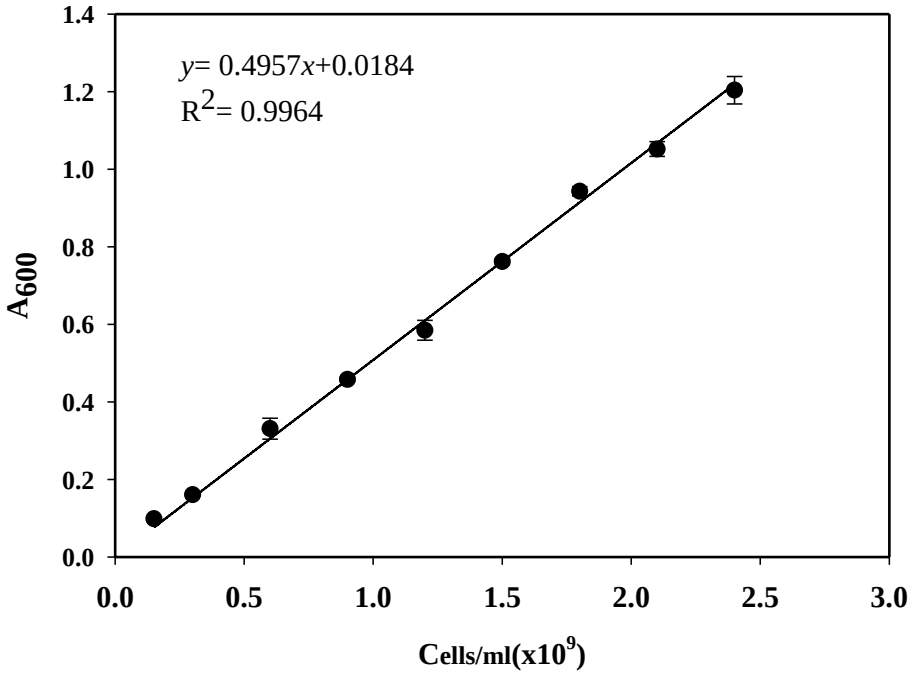

Figure 1: Calibration curve with McFarland turbidity standards for standardization of inoculum

APPENDIX 2.0 Statistical Analysis of water-miscible solvents against β-galactosidase from *Escherichia coli* and *Kluyveromyces marxianus*

A) For *E.coli*

i) EC50 (%)

ANOVA

Replicates

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	496.071	2	248.036	1109.154	.000
Within Groups	1.342	6	.224		
Total	497.413	8			

Post Hoc Tests

Homogeneous Subsets

Replicates

Duncan

Water miscible solvents EC50(%) for *E.coli*	N	Subset for alpha = 0.05		
		1	2	3
DMF	3	16.14300		
DMSO	3		28.34400	
Ethanol	3			33.92200
Sig.		1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

NB: There is a significant difference between them since P <0.05.

ii) NOEC (%)

ANOVA

Replicates

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	296.266	2	148.133	226.111	.000
Within Groups	3.931	6	.655		
Total	300.197	8			

Post Hoc Tests

Homogeneous Subsets

Replicates

Duncan

Water miscible solvents NOEC(%) for *E.coli*	N	Subset for alpha = 0.05		
		1	2	3
DMF	3	.39800		
DMSO	3		7.93900	
Ethanol	3			14.43900
Sig.		1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

NB: There is a significant difference between them since P <0.05.

B) For *K. marxianus*

i) EC50 (%)

ANOVA

Replicates

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	344.148	2	172.074	155.119	.000
Within Groups	6.656	6	1.109		
Total	350.804	8			

Post Hoc Tests
Homogeneous Subsets

Replicates

Duncan

Water miscible solvents EC50(%) for *K. marxianus*	N	Subset for alpha = 0.05	
		1	2
DMSO	3	19.85600	
DMF	3	20.76600	
Ethanol	3		33.40500
Sig.		.331	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

NB: There is a significant difference between them since P <0.05. But, values in the same subset, are not significantly different from each other.

ii) NOEC (%)

ANOVA

Replicates

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	5.757	2	2.879	2.456	.166
Within Groups	7.032	6	1.172		
Total	12.790	8			

Post Hoc Tests
Homogeneous Subsets

Replicates

Duncan

Water miscible solvents NOEC (%) for *K. marxianus*	N	Subset for alpha = 0.05
		1
DMSO	3	1.41400
DMF	3	2.51400
Ethanol	3	3.36800
Sig.		.077

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

NB: There is no significant difference between them since p>0.05.

APPENDIX 3.0 Statistical analysis of Binary mixtures of water-miscible and water-immiscible solvents against β-galactosidase from *Escherichia coli*

A) For Ethanol and water-immiscible solvents

i) EC50 (%)

ANOVA

Replicates

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	382.808	5	76.562	218.183	.000
Within Groups	4.211	12	.351		
Total	387.019	17			

Post Hoc Tests

Homogeneous Subsets

Replicates

Duncan

Ethanol: Water-immiscible solvents EC50(%) for *E.coli*	N	Subset for alpha = 0.05				
		1	2	3	4	5
Ethanol+Pentanol 7:3	3	15.83900				
Ethanol+Butanol 7:3	3	16.14500				
Ethanol+Chloroform 9:1	3		20.94600			
Ethanol+Pentanol 9:1	3			22.34300		
Ethanol+Butanol 9:1	3				26.38900	
Ethanol+Chloroform 7:3	3					27.98300
Sig.		.539	1.000	1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

NB: There is a significant difference between them since P <0.05. But, values in the same subset, are not significantly different from each other.

ii) NOEC (%)

ANOVA

Replicates

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	535.269	5	107.054	390.219	.000
Within Groups	3.292	12	.274		
Total	538.561	17			

Post Hoc Tests

Homogeneous Subsets

Replicates

Duncan

Ethanol: Water-immiscible solvents NOEC (%) for *E.coli*	N	Subset for alpha = 0.05			
		1	2	3	4
Ethanol+Pentanol 7:3	3	1.07500			
Ethanol+Butanol 7:3	3	1.83500			
Ethanol+Pentanol 9:1	3		4.59000		
Ethanol+Butanol 9:1	3		4.92000		
Ethanol+Chloroform 9:1	3			10.33400	
Ethanol+Chloroform 7:3	3				16.81700
Sig.		.101	.455	1.000	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

NB: There is a significant difference between them since P <0.05. But, values in the same subset, are not significantly different from each other.

B) For DMSO and water-immiscible solvents

i) EC50 (%)

ANOVA

Replicates

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	215.272	5	43.054	28.669	.000
Within Groups	18.022	12	1.502		
Total	233.293	17			

Post Hoc Tests
Homogeneous Subsets

Replicates

Duncan

DMSO: Water-immiscible solvents EC50(%) for *E.coli*	N	Subset for alpha = 0.05		
		1	2	3
DMSO+Butanol 7:3	3	19.20700		
DMSO+Pentanol 9:1	3		21.71300	
DMSO+Chloroform 9:1	3		22.24000	
DMSO+Pentanol 7:3	3			26.87400
DMSO+Butanol 9:1	3			27.71000
DMSO+Chloroform 7:3	3			28.38000
Sig.		1.000	.608	.177

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

There is a significant difference between them since P <0.05. But, values in the same subset, are not significantly different from each other.

ii) NOEC (%)

ANOVA

Replicates

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	113.782	5	22.756	41.711	.000
Within Groups	6.547	12	.546		
Total	120.329	17			

Post Hoc Tests
Homogeneous Subsets

Replicates

Duncan

DMSO: Water-immiscible solvents NOEC (%) for *E.coli*	N	Subset for alpha = 0.05		
		1	2	3
DMSO+Pentanol 7:3	3	.00000		
DMSO+Butanol 9:1	3	.66700		
DMSO+Pentanol 9:1	3	1.14100		
DMSO+Butanol 7:3	3		3.69300	
DMSO+Chloroform 9:1	3		4.93400	
DMSO+Chloroform 7:3	3			6.98300
Sig.		.096	.062	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

There is a significant difference between them since P <0.05. But, values in the same subset, are not significantly different from each other.

C) For DMF and water-immiscible solvents

i) EC50 (%)

ANOVA

Replicates

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	78.002	5	15.600	110.303	.000
Within Groups	1.697	12	.141		
Total	79.699	17			

Post Hoc Tests
Homogeneous Subsets

Replicates

Duncan

DMF: Water-immiscible solvents EC50(%) for *E.coli*	N	Subset for alpha = 0.05				
		1	2	3	4	5
DMF+Pentanol 7:3	3	8.51700				
DMF+Chloroform 7:3	3	9.16600	9.16600			
DMF+Chloroform 9:1	3		9.54500			
DMF+Butanol 7:3	3			11.02800		
DMF+Pentanol 9:1	3				12.86000	
DMF+Butanol 9:1	3					14.30900
Sig.		.056	.241	1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

There is a significant difference between them since P <0.05. But, values in the same subset, are not significantly different from each other.

ii) NOEC

ANOVA

Replicates

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	9.117	5	1.823	39.675	.000
Within Groups	.551	12	.046		
Total	9.668	17			

Post Hoc Tests

Homogeneous Subsets

Replicates

Duncan

DMF: Water-immiscible solvents NOEC(%) for E.coli	N	Subset for alpha = 0.05		
		1	2	3
DMF+Pentanol 7:3	3	.31200		
DMF+Chloroform 9:1	3		.69400	
DMF+Butanol 7:3	3		.82500	
DMF+Chloroform 7:3	3		.95000	
DMF+Butanol 9:1	3			1.97200
DMF+Pentanol 9:1	3			2.29500
Sig.		1.000	.189	.090

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

There is a significant difference between them since P <0.05. But, values in the same subset, are not significantly different from each other.

APPENDIX 4.0 Statistical analysis of Binary mixtures of water-miscible and water-immiscible solvents against β-galactosidase from *Kluyveromyces marxianus*

A) For Ethanol and water- immiscible solvents

i) EC50 (%)

ANOVA

Replicates

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	1198.034	5	239.607	176.678	.000
Within Groups	16.274	12	1.356		
Total	1214.308	17			

Post Hoc Tests
Homogeneous Subsets

Replicates

Duncan

Ethanol: Water-immiscible solvents EC50 (%) for *K.marxianus*	N	Subset for alpha = 0.05				
		1	2	3	4	5
Ethanol+Chloroform 9:1	3	9.64800				
Ethanol+Chloroform 7:3	3		12.59900			
Ethanol+Butanol 7:3	3			17.82300		
Ethanol+Pentanol 7:3	3				24.85900	
Ethanol+Pentanol 9:1	3				25.61500	
Ethanol+Butanol 9:1	3					33.42200
Sig.		1.000	1.000	1.000	.442	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

There is a significant difference between them since P <0.05. But, values in the same subset, are not significantly different from each other.

ii) NOEC (%)

ANOVA

Replicates

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	136.029	5	27.206	27.651	.000
Within Groups	11.807	12	.984		
Total	147.835	17			

Post Hoc Tests
Homogeneous Subsets

Replicates

Duncan

Ethanol: Water-immiscible solvents NOEC (%) for *K.marxianus*	N	Subset for alpha = 0.05			
		1	2	3	4
Ethanol+Chloroform 9:1	3	.54000			
Ethanol+Butanol 7:3	3	1.21100			
Ethanol+Chloroform 7:3	3		3.63200		
Ethanol+Pentanol 9:1	3			6.04900	
Ethanol+Pentanol 7:3	3			6.30700	6.30700
Ethanol+Butanol 9:1	3				8.05900
Sig.		.424	1.000	.756	.051

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

There is a significant difference between them since P <0.05. But, values in the same subset, are not significantly different from each other.

B) For DMSO and water-immiscible solvents

i) EC50 (%)

ANOVA

Replicates

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	1962.769	5	392.554	25.132	.000
Within Groups	187.438	12	15.620		
Total	2150.207	17			

Post Hoc Tests
Homogeneous Subsets

Replicates

Duncan

DMSO: Water-immiscible solvents EC50 (%) for *K.marxianus*	N	Subset for alpha = 0.05		
		1	2	3
DMSO+Pentanol 9:1	3	.00000		
DMSO+Butanol 7:3	3	.00000		
DMSO+Chloroform 9:1	3		16.15600	
DMSO+Chloroform 7:3	3		20.13000	20.13000
DMSO+Pentanol 7:3	3		22.26000	22.26000
DMSO+Butanol 9:1	3			26.32600
Sig.		1.000	.096	.092

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

There is a significant difference between them since P <0.05. But, values in the same subset, are not significantly different from each other.

ii) NOEC (%)

ANOVA

Replicates

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	41.726	5	8.345	11.436	.000
Within Groups	8.757	12	.730		
Total	50.483	17			

Post Hoc Tests
Homogeneous Subsets

Replicates

Duncan

DMSO: Water-immiscible solvents NOEC (%) for *K.marxianus*	N	Subset for alpha = 0.05		
		1	2	3
DMSO+Pentanol 9:1	3	.00000		
DMSO+Butanol 7:3	3	.00000		
DMSO+Chloroform 9:1	3	.28800		
DMSO+Butanol 9:1	3	1.56600	1.56600	
DMSO+Chloroform 7:3	3		2.30100	
DMSO+Pentanol 7:3	3			4.21300
Sig.		.059	.313	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

There is a significant difference between them since P <0.05. But, values in the same subset, are not significantly different from each other.

C) For DMF and water-immiscible solvents

i) EC50 (%)

ANOVA

Replicates

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	113.233	5	22.647	8.907	.001
Within Groups	30.512	12	2.543		
Total	143.745	17			

Post Hoc Tests
Homogeneous Subsets

Replicates

Duncan

DMF: Water-immiscible solvents EC50(%) for K.marxianus	N	Subset for alpha = 0.05			
		1	2	3	4
DMF+Pentanol 7:3	3	11.17900			
DMF+Chloroform 7:3	3	12.18300	12.18300		
DMF+Chloroform 9:1	3		14.27400	14.27400	
DMF+Butanol 7:3	3			15.43600	
DMF+Pentanol 9:1	3			16.81700	16.81700
DMF+Butanol 9:1	3				18.41600
Sig.		.456	.134	.087	.243

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

b.

There is a significant difference between them since P <0.05. But, values in the same subset, are not significantly different from each other.

ii) NOEC (%)

ANOVA

Replicates

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	8.660	5	1.732	6.970	.003
Within Groups	2.982	12	.249		
Total	11.642	17			

Post Hoc Tests

Homogeneous Subsets

Replicates

Duncan

DMF: Water-immiscible solvents NOEC (%) for K.marxianus	N	Subset for alpha = 0.05		
		1	2	3
DMF+Pentanol 9:1	3	.08100		
DMF+Chloroform 7:3	3		.97600	
DMF+Butanol 9:1	3		1.32000	1.32000
DMF+Pentanol 7:3	3		1.88700	1.88700
DMF+Chloroform 9:1	3			1.98600
DMF+Butanol 7:3	3			2.01900
Sig.		1.000	.054	.137

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

There is a significant difference between them since P <0.05. But, values in the same subset, are not significantly different from each other.

APPENDIX 5.0 Statistical Analysis for EC_{50}, NOEC, Toxic Index and Combined Effect of ethanol-DMSO mixtures on cell-free β-galactosidase from *E.coli*

Ethanol: DMSO IN *E.coli*

A)

ANOVA

OBSERVED EC50 for ***E.coli***

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	45.337	5	9.067	3.813	.027
Within Groups	28.535	12	2.378		
Total	73.873	17			

Post Hoc Tests

OBSERVED EC50 for *E.coli*

Duncan

Ethanol: DMSO	N	Subset for alpha = 0.05		
		1	2	3
DMSO	3	28.1120		
ETHANOL	3	29.2460	29.2460	
Ethanol: DMSO (7:3)	3		30.9870	30.9870
Ethanol: DMSO (9:1)	3		31.2560	31.2560
Ethanol: DMSO (6:4)	3		31.3460	31.3460
Ethanol: DMSO (8:2)	3			33.0450
Sig.		.385	.148	.155

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

NB: There is a significant difference between them since P <0.05. But, values in the same subset, are not significantly different from each other.

B)

ANOVA

CA-PREDICTED EC50 for ***E.coli***

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.286	3	.095	.163	.918
Within Groups	4.682	8	.585		
Total	4.968	11			

Post Hoc Tests

Homogeneous Subsets

CA-PREDICTED EC50 for ***E.coli***

Duncan

Ethanol: DMSO	N	Subset for alpha = 0.05
		1
Ethanol: DMSO (6:4)	3	28.89700
Ethanol: DMSO (7:3)	3	29.03400
Ethanol: DMSO (8:2)	3	29.17200
Ethanol: DMSO (9:1)	3	29.31100
Sig.		.549

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

NB: There is no significant difference between them since p> 0.05, and all values are in the same subset

C)

ANOVA

NOEC for ***E.coli***

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	76.735	5	15.347	14.510	.000
Within Groups	12.692	12	1.058		
Total	89.427	17			

Post Hoc Tests
Homogeneous Subsets

NOEC for ***E.coli***

Duncan

Ethanol: DMSO	N	Subset for alpha = 0.05	
		1	2
DMSO	3	6.40700	
Ethanol: DMSO (7:3)	3		10.74100
ETHANOL	3		11.26500
Ethanol: DMSO (6:4)	3		11.60100
Ethanol: DMSO (9:1)	3		12.16600
Ethanol: DMSO (8:2)	3		12.68500
Sig.		1.000	.056

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

NB: There is a significant difference between them since P <0.05. But, values in the same subset, are not significantly different from each other.

D) FOR OBSERVED EC50 AND CA-PREDICTED EC50 FOR ETHANOL:DMSO 9:1

ANOVA

REPLICATES

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	5.675	1	5.675	11.257	.028
Within Groups	2.016	4	.504		
Total	7.691	5			

NB: There is a significant difference between them since P <0.05.

E) FOR OBSERVED EC50 AND CA-PREDICTED EC50 FOR ETHANOL:DMSO 8:2

ANOVA

REPLICATES

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	22.500	1	22.500	10.885	.030
Within Groups	8.268	4	2.067		
Total	30.768	5			

There is a significant difference between them since P <0.05.

F) FOR OBSERVED EC50 AND CA-PREDICTED EC50 FOR ETHANOL:DMSO 7:3

ANOVA

REPLICATES

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	5.721	1	5.721	6.999	.057
Within Groups	3.270	4	.817		
Total	8.991	5			

There is no significant difference between them since p>0.0

G) FOR OBSERVED EC50 AND CA-PREDICTED EC50 FOR ETHANOL: DMSO 6:4

ANOVA

REPLICATES

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	8.996	1	8.996	25.942	.007
Within Groups	1.387	4	.347		
Total	10.384	5			

There is a significant difference between them since P <0.05.

APPENDIX 6.0 Statistical Analysis for EC_{50}, NOEC, Toxic Index and Combined Effect of ethanol-DMF mixtures on cell-free β-galactosidase from *E.coli*

A)

ANOVA

OBSERVED EC50 for ***E.coli***

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	298.815	5	59.763	264.081	.000
Within Groups	2.716	12	.226		
Total	301.531	17			

Post Hoc Tests

Homogeneous Subsets

OBSERVED EC50 for ***E.coli***

Duncan

Ethanol: DMF	N	Subset for alpha = 0.05				
		1	2	3	4	5
DMF	3	18.24400				
Ethanol: DMF (6:4)	3		27.36800			
Ethanol: DMF (7:3)	3		28.01700	28.01700		
Ethanol: DMF (8:2)	3			28.39500		
Ethanol: DMF (9:1)	3				29.49700	
Ethanol	3					30.68700
Sig.		1.000	.121	.350	1.000	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

NB: There is a significant difference between them since P <0.05. But, values in the same subset, are not significantly different from each other.

B)

ANOVA

CA-Predicted EC50 for ***E.coli***

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	35.500	3	11.833	10.585	.004
Within Groups	8.944	8	1.118		
Total	44.444	11			

Post Hoc Tests

Homogeneous Subsets

CA-Predicted EC50 for ***E.coli***

Duncan

Ethanol: DMF	N	Subset for alpha = 0.05		
		1	2	3
Ethanol: DMF (6:4)	3	24.09100		
Ethanol: DMF (7:3)	3	25.45400	25.45400	
Ethanol: DMF (8:2)	3		26.98100	26.98100
Ethanol: DMF (9:1)	3			28.70300
Sig.		.153	.115	.081

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

NB: There is a significant difference between them since P <0.05. But, values in the same subset, are not significantly different from each other.

C)

ANOVA

NOEC for ***E.coli***

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	161.977	5	32.395	46.930	.000
Within Groups	8.283	12	.690		
Total	170.260	17			

Post Hoc Tests

Homogeneous Subsets

NOEC for ***E.coli***

Duncan

Ethanol: DMF	N	Subset for alpha = 0.05			
		1	2	3	4
DMF	3	1.89700			
Ethanol: DMF (6:4)	3		7.28300		
Ethanol: DMF (7:3)	3		8.02600		
Ethanol: DMF (8:2)	3		8.23700		
Ethanol: DMF (9:1)	3			9.92900	
Ethanol	3				11.55600
Sig.		1.000	.205	1.000	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

NB: There is a significant difference between them since P <0.05. But, values in the same subset, are not significantly different from each other.

D) FOR OBSERVED EC50 AND CA-PREDICTED EC50 FOR ETHANOL:DMF 9:1

ANOVA

REPLICATES

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.946	1	.946	1.499	.288
Within Groups	2.523	4	.631		
Total	3.469	5			

There is no significant difference between them since p>0.05

E) FOR OBSERVED EC50 AND CA-PREDICTED EC50 FOR ETHANOL:DMF 8:2

ANOVA

REPLICATES

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	2.999	1	2.999	4.949	.090
Within Groups	2.424	4	.606		
Total	5.423	5			

There is no significant difference between them since p>0.05

F) FOR OBSERVED EC50 AND CA-PREDICTED EC50 FOR ETHANOL:DMF 7:3

ANOVA

REPLICATES

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	9.853	1	9.853	16.041	.016
Within Groups	2.457	4	.614		
Total	12.311	5			

There is a significant difference between them since P <0.05.

G) FOR OBSERVED EC50 AND CA-PREDICTED EC50 FOR ETHANOL: DMF 6:4

ANOVA

REPLICATES

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	16.108	1	16.108	28.107	.006
Within Groups	2.292	4	.573		
Total	18.400	5			

There is a significant difference between them since P <0.05

H) FOR ETHANOL (ETHANOL: DMF) AND (ETHANOL: DMSO) OBSERVED EC50 FOR *E. coli*

ANOVA

REPLICATES

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	3.115	1	3.115	.660	.462
Within Groups	18.887	4	4.722		
Total	22.001	5			

There is no significant difference between them since p >0.05I) FOR ETHANOL (ETHANOL: DMF) AND (ETHANOL: DMSO) NOEC FOR *E. coli*

ANOVA

REPLICATES

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.127	1	.127	.064	.813
Within Groups	7.945	4	1.986		
Total	8.072	5			

There is no significant difference between them since p >0.05

APPENDIX 7.0 Statistical Analysis for EC_{50}, NOEC, Toxic index and combined effect of ethanol-DMSO mixtures on cell-free β-galactosidase from *Kluyveromyces marxianus*

A)

ANOVA

OBSERVED EC50 for *Kluyveromyces marxianus*

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	3.160	5	.632	.913	.505
Within Groups	8.307	12	.692		
Total	11.466	17			

Post Hoc Tests

Homogeneous Subsets

OBSERVED EC50 for *Kluyveromyces marxianus*

Duncan

TREATMENTS	N	Subset for alpha = 0.05
		1
ETHANOL	3	18.93000
DMSO	3	19.11400
Ethanol: DMSO (6:4)	3	19.35000
Ethanol: DMSO (9:1)	3	19.66200
Ethanol: DMSO (8:2)	3	19.82300
Ethanol: DMSO (7:3)	3	20.15300
Sig.		.129

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

There is no significant difference between them since p>0.05, and all the values are in the same subset.

B)

ANOVA

CA-Predicted EC50 for *Kluyveromyces marxianus*

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.000	3	.000	.000	1.000
Within Groups	18.609	8	2.326		
Total	18.610	11			

Post Hoc Tests
Homogeneous Subsets

CA-Predicted EC50 for *Kluyveromyces marxianus*

Duncan

Ethanol: DMSO	N	Subset for alpha = 0.05
		1
Ethanol: DMSO (6:4)	3	18.8800
Ethanol: DMSO (7:3)	3	18.8860
Ethanol: DMSO (8:2)	3	18.8910
Ethanol: DMSO (9:1)	3	18.8970
Sig.		.990

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

There is no significant difference between them since p>0.05, and all the values are in the same subset.

C)

ANOVA

NOEC for *Kluyveromyces marxianus*

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	1.444	5	.289	.999	.459
Within Groups	3.471	12	.289		
Total	4.915	17			

Post Hoc Tests

Homogeneous Subsets

NOEC for *Kluyveromyces marxianus*

Duncan

TREATMENTS	N	Subset for alpha = 0.05
		1
DMSO	3	2.21500
Ethanol: DMSO (8:2)	3	2.22200
Ethanol: DMSO (6:4)	3	2.23500
ETHANOL	3	2.65100
Ethanol: DMSO (9:1)	3	2.65900
Ethanol: DMSO (7:3)	3	2.95300
Sig.		.154

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

There is no significant difference between them since p>0.05, and all the values are in the same subset.

D) FOR OBSERVED EC50 AND CA-PREDICTED EC50 FOR ETHANOL:DMSO 9:1

ANOVA

REPLICATES

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.878	1	.878	.493	.521
Within Groups	7.118	4	1.779		
Total	7.996	5			

There is no significant difference between them since p > 0.05

E) FOR OBSERVED EC50 AND CA-PREDICTED EC50 FOR ETHANOL:DMSO 8:2

ANOVA

REPLICATES

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	1.303	1	1.303	.851	.409
Within Groups	6.127	4	1.532		
Total	7.430	5			

There is no significant difference between them since p > 0.05

E) FOR OBSERVED EC50 AND CA-PREDICTED EC50 FOR ETHANOL:DMSO 7:3

ANOVA

REPLICATES

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	2.408	1	2.408	1.818	.249
Within Groups	5.299	4	1.325		
Total	7.707	5			

There is no significant difference between them since p > 0.05

F) FOR OBSERVED EC50 AND CA-PREDICTED EC50 FOR ETHANOL : DMSO 6:4

ANOVA

REPLICATES

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.331	1	.331	.262	.636
Within Groups	5.060	4	1.265		
Total	5.391	5			

There is no significant difference between them since p > 0.05

APPENDIX 8.0. Statistical Analysis for EC_{50}, NOEC, Toxic Index and combined effect of ethanol-DMF mixtures on cell-free β-galactosidase from *Kluyveromyces marxianus*

A)

ANOVA

OBSERVED EC50 FOR *Kluyveromyces marxianus*

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	192.006	5	38.401	53.739	.000
Within Groups	8.575	12	.715		
Total	200.581	17			

Post Hoc Tests

Homogeneous Subsets

OBSERVED EC50 for *Kluyveromyces marxianus*

Duncan

Ethanol: DMF	N	Subset for alpha = 0.05				
		1	2	3	4	5
DMF	3	13.58700				
Ethanol: DMF (6:4)	3		17.17000			
Ethanol: DMF (8:2)	3		17.81900	17.81900		
Ethanol: DMF (7:3)	3			19.30000	19.30000	
Ethanol: DMF (9:1)	3				19.71700	
Ethanol	3					24.47500
Sig.		1.000	.366	.053	.557	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

NB: There is a significant difference between them since P <0.05. However, values in the same subset are not significantly different from each other.

B)

ANOVA

CA-Predicted EC50

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	28.325	3	9.442	9.625	.005
Within Groups	7.848	8	.981		
Total	36.172	11			

Post Hoc Tests
Homogeneous Subsets

CA-Predicted EC50

Duncan

Ethanol: DMF	N	Subset for alpha = 0.05		
		1	2	3
Ethanol: DMF (6:4)	3	18.52500		
Ethanol: DMF (7:3)	3	19.72100	19.72100	
Ethanol: DMF (8:2)	3		21.08100	21.08100
Ethanol: DMF (9:1)	3			22.64400
Sig.		.177	.131	.089

Means for groups in homogeneous subsets are displayed.
a. Uses Harmonic Mean Sample Size = 3.000.

NB: There is a significant difference between them since P <0.05. However, values in the same subset are not significantly different from each other.

C)

ANOVA

NOEC for *K.marxianus*

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	120.075	5	24.015	72.084	.000
Within Groups	3.998	12	.333		
Total	124.073	17			

Post Hoc Tests
Homogeneous Subsets

NOEC for *K.marxianus*

Duncan

TREATMENTS	N	Subset for alpha = 0.05			
		1	2	3	4
Ethanol: DMF (6:4)	3	.43700			
DMF	3	.92000	.92000		
Ethanol: DMF (7:3)	3	1.38100	1.38100		
Ethanol: DMF (9:1)	3		1.77900		
Ethanol: DMF (8:2)	3			4.11500	
ETHANOL	3				7.91200
Sig.		.080	.108	1.000	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

NB: There is a significant difference between them since P <0.05. However, values in the same subset are not significantly different from each other.

D)

ANOVA

TI

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.032	3	.011	39.718	.000
Within Groups	.002	8	.000		
Total	.034	11			

Post Hoc Tests
Homogeneous Subsets

TI

Duncan

Ethanol: DMF	N	Subset for alpha = 0.05		
		1	2	3
Ethanol: DMF (8:2)	3	.84500		
Ethanol: DMF (9:1)	3	.87000		
Ethanol: DMF (6:4)	3		.92600	
Ethanol: DMF (7:3)	3			.97800
Sig.		.098	1.000	1.000

Means for groups in homogeneous subsets are displayed.
a. Uses Harmonic Mean Sample Size = 3.000.

NB: There is a significant difference between them since P <0.05. However, values in the same subset are not significantly different from each other.

E) FOR OBSERVED EC50 AND CA-PREDICTED EC50 FOR ETHANOL: DMF 9:1

ANOVA

REPLICATES

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	12.851	1	12.851	13.198	.022
Within Groups	3.895	4	.974		
Total	16.746	5			

There is a significant difference between them since P <0.0

F) FOR OBSERVED EC50 AND CA-PREDICTED EC50 FOR ETHANOL: DMF 8:2

ANOVA

REPLICATES

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	15.961	1	15.961	19.285	.012
Within Groups	3.310	4	.828		
Total	19.271	5			

There is a significant difference between them since P <0.05.

G) FOR OBSERVED EC50 AND CA-PREDICTED EC50 FOR ETHANOL: DMF 7:3

ANOVA

REPLICATES

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.266	1	.266	.320	.602
Within Groups	3.328	4	.832		
Total	3.594	5			

There is no significant difference between them since p>0.05

H) FOR OBSERVED EC50 AND CA-PREDICTED EC50 FOR ETHANOL: DMF 6:4

ANOVA

REPLICATES

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	2.754	1	2.754	2.692	.176
Within Groups	4.092	4	1.023		
Total	6.846	5			

There is no significant difference between them since p>0.05

I) FOR ETHANOL (ETHANOL: DMF) AND ETHANOL (ETHANOL: DMF) FOR *K. marxianus*

ANOVA

REPLICATES

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	46.121	1	46.121	45.426	.003
Within Groups	4.061	4	1.015		
Total	50.182	5			

There is a significant difference between them since P <0.05.

APPENDIX 9.0 Statistical analysis of inhibitory concentrations and NOEC of some surfactants and EDTA against the activity of cell-free β-galactosidase from *Escherichia coli* and *Kluyveromyces marxianus*

A) For *Escherichia coli*

i) EC50 (%)

ANOVA

REPLICATES

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.004	3	.001	171.097	.000
Within Groups	.000	8	.000		
Total	.004	11			

Post Hoc Tests
Homogeneous Subsets

REPLICATES

Duncan

EC50(%) of Surfactants and EDTA for *E.coli*	N	Subset for alpha = 0.05		
		1	2	3
CPC	3	.00138000		
SDS	3	.00320000		
CTAB	3		.01410000	
EDTA	3			.04830000
Sig.		.462	1.000	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

NB: There is a significant difference between them since P <0.05. However, values in the same subset are not significantly different from each other.

ii) NOEC (%)

ANOVA

REPLICATES

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.000	3	.000	37.660	.000
Within Groups	.000	8	.000		
Total	.000	11			

Post Hoc Tests

Homogeneous Subsets

REPLICATES

Duncan

NOEC(%) of Surfactants and EDTA for *E.coli*	N	Subset for alpha = 0.05	
		1	2
SDS	3	0E-8	
CPC	3	.00001900	
EDTA	3	.00070000	
CTAB	3		.00400000
Sig.		.165	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

NB: There is a significant difference between them since P <0.05. However, values in the same subset are not significantly different from each other.

B) **For *Kluyveromyces marxianus***

i) EC50 (%)

ANOVA

REPLICATES

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.430	3	.143	376.879	.000
Within Groups	.003	8	.000		
Total	.433	11			

REPLICATES

Duncan

EC50 (%) of Surfactants and EDTA for *Kluyveromyces marxianus*	N	Subset for alpha = 0.05	
		1	2
CPC	3	.00100000	
EDTA	3	.01100000	
CTAB	3	.01130000	
SDS	3		.44500000
Sig.		.552	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

Post Hoc Tests
Homogeneous Subsets

NB: There is a significant difference between them since P <0.05. However, values in the same subset are not significantly different from each other.

i) NOEC (%)

ANOVA

REPLICATES

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.000	3	.000	26.224	.000
Within Groups	.000	8	.000		
Total	.000	11			

Post Hoc Tests
Homogeneous Subsets

REPLICATES

Duncan

NOEC(%) of Surfactants and EDTA for *Kluyveromyces marxianus*	N	Subset for alpha = 0.05		
		1	2	3
EDTA	3	0E-8		
CPC	3	.00023000		
SDS	3		.00200000	
CTAB	3			.00430000
Sig.		.687	1.000	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

NB: There is a significant difference between them since P <0.05. But, values in the same subset are not significantly different from each other

APPENDIX 10 Statistical analysis for Comparison of permeabilization treatments for β-galactosidase activity assay in *Kluyveromyces marxianus*

ANOVA

REPLICATES

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.000	5	.000	2.145	.129
Within Groups	.000	12	.000		
Total	.001	17			

Post Hoc Tests
Homogeneous Subsets

REPLICATES

Duncan

Comparison test for *K.marxianus using the* Kippert series	N	Subset for alpha = 0.05	
		1	2
Ethanol + Pentanol (9:1); 15%	3	.108000	
Sarcosyl; 0.2%	3	.112000	.112000
Sarcosyl; 0.133% (Kippert, 1995)	3	.113000	.113000
Sodium Deoxycholate (SDC); 0.4%	3	.113000	.113000
Sarcosy; 0.15%	3		.118000
Ethanol + Chloroform (9:1); 15%	3		.119000
Sig.		.261	.130

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

NB: There is no significant difference between them since P >0.05 and values in the same subset are not significantly different from each other

APPENDIX 11 Statistical analysis for Comparison of permeabilization treatments for β-galactosidase activity assay in *Escherichia coli*

ANOVA

REPLICATES

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.001	5	.000	5.545	.007
Within Groups	.000	12	.000		
Total	.001	17			

Post Hoc Tests
Homogeneous Subsets

REPLICATES

Duncan

Comparison test for *E.coli* using Miller series	N	Subset for alpha = 0.05		
		1	2	3
Sarcosyl; 0.1%	3	.151000		
CTAB; 0.002%	3	.157000	.157000	
Sarcosyl; 0.2%,	3	.159000	.159000	
0.005 % SDS + 8.7% Chloroform;(Miller, 1972)	3		.162000	.162000
Ethanol + Chloroform (9:1); 5%	3		.163000	.163000
1-Cetylpyridinium chloride (CPC); 0.0008%	3			.169000
Sig.		.058	.153	.092

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

NB: There is a significant difference between them since P <0.05. However, values in the same subset are not significantly different from each other.