Asian Journal of Biotechnology and Bioresource Technology

Volume 10, Issue 4, Page 112-123, 2024; Article no.AJB2T.126742
ISSN: 2457-0125

Inhibition of *Escherichia coli* Cell-Free β-Galactosidase Activity by Binary Mixtures of Water-miscible Solvents

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

Article Information

DOI: https://doi.org/10.9734/ajb2t/2024/v10i4224

Open Peer Review History:
This journal follows the Advanced Open Peer Review policy. Identity of the Reviewers, Editor(s) and additional Reviewers, peer review comments, different versions of the manuscript, comments of the editors, etc are available here: https://www.sdiarticle5.com/review-history/126742

Original Research Article

Received: 07/09/2024
Accepted: 11/11/2024
Published: 18/11/2024

ABSTRACT

The inhibitory effects of ethanol-dimethylsulfoxide (DMSO) and ethanol-N,N-dimethylformamide (DMF) binary mixtures on the activity of cell-free β-galactosidase from *Escherichia coli* were assessed. The concentration-response relationships of the individual solvent and the mixtures were fitted to a Gormpertz model to estimate the median inhibitory concentrations (EC_{50}) and No-Observable-Effect-Concentration (NOEC) thresholds. The EC_{50} values estimated are 29.246 ± 2.986% (ethanol), 28.112 ± 0.471% (DMSO), and 18.244 ± 0.674% (DMF). The NOEC values are 11.265 ± 1.121% (ethanol), 6.4047 ± 0.564% (DMSO), and 1.897 ± 0.427% (DMF). The order of inhibitory effects is DMF > DMSO > ethanol. The mixture NOEC values ranged from 10.081% (6:4 ethanol-DMSO mixture) to 13.619% (9:1 ethanol-DMSO mixture). In the case of ethanol-DMF

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Cite as: *Opurum, Christian C., Obioma K. Mejeha, Christian O. Nweke, and Oluchukwu R. Nwangwu. 2024. "Inhibition of Escherichia Coli Cell-Free β-Galactosidase Activity by Binary Mixtures of Water-Miscible Solvents". Asian Journal of Biotechnology and Bioresource Technology 10 (4):112-23. https://doi.org/10.9734/ajb2t/2024/v10i4224.*

mixtures, NOEC values ranged from 6.843% in 6:4 ethanol-DMF mixture to 10.599% in 9:1 ethanol-DMF mixture. All solvent mixtures with higher EC_{50} and NOEC values are less inhibitory than the individual DMSO and DMF. The inhibitory effects of the ethanol-DMSO and ethanol-DMF binary mixtures were predicted using the concentration addition (CA) model, toxic index (TI) and model deviation ratio (MDR). The TI for the ethanol-DMSO mixtures ranged from 1.076 ± 0.046 to 1.142 ± 0.032, while that of ethanol-DMF ranged from 1.027 ± 0.015 to 1.136 ± 0.024. These values are marginally higher than 1; thus, the combined effect is considered additive. The study provides fundamental information on the sub-inhibitory concentrations of ethanol, DMSO, and DMF, or their mixtures, for use in the permeabilization of *E. coli* cells for a β-galactosidase activity assay.

Keywords: Ethanol; N; N-dimethylformamide; dimethylsulfoxide; concentration addition; toxic index.

1. INTRODUCTION

β-Galactosidase is an enzyme with historical and scientific relevance [1]. It is present in various species such as bacteria, fungi (molds and yeasts), microalga, and plants, particularly vegetables [2]. Xavier et al. [3] stated that the enzyme has garnered much attention because of the prevalence of lactose intolerance in the human population and the significance of milk in the diet. It has been estimated that over 70% of people worldwide, across all age groups, are intolerant to lactose. Perini et al. [4] listed some clinical symptoms of lactose intolerance, including abdominal pain, diarrhea, etc. β-Galactosidases break down lactose, or β-galactopyranosides, into a variety of trans-galactosylation products called galactooligosaccharides (GOS), which act as prebiotics and have several health benefits [3]. In addition, the activity and biosynthesis of β-galactosidases have been used as microbial responses in assessing chemical toxicity [5-8]. These applications necessitated β-galactosidase assays in microbial cells.

The spectrophotometric methods involving chromogenic substrate, o-nitrophenyl-ß-D-galactopyranoside (*O*NPG), have been commonly used to assay β-galactosidase activity in microbial cells [9]. Due to the intracellular localization of β-galactosidase, permeabilization of microbial cells during *in-vitro* β-galactosidase assay is essential for *O*NPG to enter the cell and interact with the enzyme [10]. Various organic solvents, including ethanol, dimethylsulfoxide (DMSO) and N, N-dimethylformamide (DMF), have been used as cell-permeabilizing agents to assess β-galactosidase activity in microbial cells [11-16]. These organic solvents could hinder the activity of β-galactosidases from microorganisms. The effects of alcohols on the steady-state kinetic parameters of the model enzyme β-galactosidase were studied by Bell et al. [17]. The accumulation of ethanol and other alcohols following non-oxidative metabolism is highly stressful, inhibits metabolic activity, and can ultimately kill the cell.

During the production of whole-cell biocatalysts, high concentrations of solvents are used to permeabilize microbial cells, which are subsequently washed before assaying for β-galactosidase activity in permeabilized cells. Kumari et al. [15] noted that loss of enzyme activity may result from the permeabilization of microbial cells with high concentrations of organic solvents, which will also lead to an underestimation of enzyme activity as a result of solvent toxicity or washing of the cell after permeabilization. Accurate determination of β-galactosidase activity in whole microbial cells would, therefore, require optimal concentration of the organic solvents. Permeabilization of microbial cells with solvents may result in leakage of β-galactosidase from intact cells and exposure of the enzymes to inhibitory effects of the solvents. Not much was known about the inhibitory effects of ethanol, DMSO, and DMF and their binary mixtures on the cell-free β-galactosidase activity of *E. coli*.

In this study, a β-galactosidase activity inhibition test was performed to establish sub-inhibitory concentrations of ethanol, DMSO and DMF as well as their mixtures. The sub-inhibitory concentrations would potentially be applicable in the permeabilization of *Escherichia coli* cells for *in-situ* β-galactosidase activity assay and in the solvent-β-galactosidase systems.

2. MATERIALS AND METHODS

2.1 Reagents

The reagents used are analytical grade chemicals. The enzyme substrate, *o*-nitrophenyl-β-D-galactopyranosid (ONPG), 2-nitrophenol, Na_2HPO_4, $NaH_2PO_4.H_2O$, KCl, $MgSO_4.7H_2O$ and

Na_2CO_3 were purchased from Merck, Germany. Ethanol, dimethylsulfoxide (DMSO) and N-N-dimethylformamide (DMF) were purchased from Guangdong Guanghua Sci-Tech. Co. Ltd (GHTECH), China.

2.2 Test Organism

The test bacterium, *E. coli,* was isolated from a stool specimen on Eosin Methylene Blue (EMB) agar (Himedia). The biochemical characteristics of the bacterium were confirmed by morphology, Gram reaction, motility, aerobic growth, catalase, oxidase, urease, methyl red, Voges Proskauer (VP), citrate utilization, urease, hydrogen sulfide (H_2S), lactose fermentation tests according to the method of Barrow and Feltham [18]. The identity of the bacterium was further confirmed by its pink colour on chromogenic Urinary Tract Infection (UTI) agar (Sisco Research Laboratory(SRL) PVT. Ltd, Mumbai, India.). The test organism was stocked on Nutrient agar slant at 4◦C.

2.3 Screen Test for β-Galactosidase Production

E. *coli* was cultivated for 48 h at 30°C in a culture medium modified from Panasar et al. [14]. The medium contained (g/l): casamino acid, 5.0; peptone, 5.0; yeast extract, 3.0; lactose, 5; Ammonium sulphate, 2.0; KH_2PO_4, 1.0. 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 and incubating for 10 min at 30◦C. After incubation, 0.2 ml of 0.2% ONPG was added to the cell suspension and incubated at 30◦C. A yellow color production within a few min indicates a positive test.

2.4 Production of Crude β-Galactosidase

E. *coli* was grown for 48 h at 30°C in the culture medium stated above. The cells were harvested by centrifugation and washed twice in Z buffer (pH of 7.0). The Z-buffer contained 8.54 g Na_2HPO_4, 5.5 g $NaH_2PO_4.H_2O$, 0.75 g KCl and 0.25 g $MgSO_4.7H_2O$ in 1 litre of distilled water. Working Z-buffer was prepared by adding 140 µl of β-mercaptoethanol to 50 ml of Z-buffer. The bacterial cells were suspended in the Z-buffer to a cell density of $1.17x10^9$ cells/ml (A_{600} = 0.6). The cell suspension was cooled under ice. 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.

2.5 β-Galactosidase Activity Inhibition by Individual Solvents

Inhibitions of the activity of cell-free β-galactosidase from *E. coli* by individual solvents 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 over solvent concentrations of 5% to 70%. The reaction mixture in each culture tube contained requisite volumes of respective solvents and 0.2 ml of Z-buffered 0.2% *O*NPG. Requisite volumes of distilled water were added to obtain a 1.6 ml reaction mixture. Then, the reaction was initiated by adding 0.4 ml of Z-buffered crude enzyme to obtain a 2 ml final volume of the reaction mixture. 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 solution. The absorbance of the 2-nitrophenol (2-NP) solution produced in each tube was measured at 420nm in a spectrophotometer (Searchtech Instrument, 752N).

2.6 β-Galactosidase Activity Inhibition by Solvent Mixtures

Binary mixtures of water-miscible solvents (ethanol-DMSO and ethanol-DMF) were combined in four binary mixture ratios (9:1, 8:2, 7;3 and 6:4). The inhibition assay with the mixtures and the individual solvents for each binary mixture were done simultaneously to avoid false conclusion on the combined effect of the mixtures [19]. Inhibitions of the activity of cell-free β-galactosidase from *E*. *coli* by the binary mixtures of solvents were determined according to the procedure described for individual solvents above. Each binary mixture was prepared and added to the reaction mixture like 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 experimentally [20].

2.7 Relative Inhibitions of β-Galactosidase Activity

In each assay, β-galactosidase activity relative to the control was computed as shown in Eq. 1.

$$\beta\text{-Galactosidase activity}\,(\%) = \frac{A_{\text{Test}}}{A_{\text{Control}}} \times 100 \quad (1)$$

Where $A_{Control}$ is the enzyme activity in control, and A_{Test} is the enzyme activity in the tests containing varying concentrations of solvents or their mixtures.

2.8 Determination of Effective Concentrations (ED_K) and NOEC

The concentration-response relationships of the individual solvent and mixtures were fitted with the 4-parameter Gormpertz model (Eq. 2).

$$y = c + (d - c)\exp\left(-\left(\frac{x}{a}\right)^b\right) \quad (2)$$

Where *y* is the response, *x* is the concentration of the effector, *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 Gormpertz function for *K*% inhibition of β-galactosidase activity, Eq (2) was reparameterized using the defining relationship described by Nweke et al. [21]. Eq. (3) was solved for a^b to obtain Eq. (4).

$$c + \frac{100 - \mathrm{K}}{100}(d - c) = c + (d - c)\exp\left(-\left(\frac{x}{a}\right)^b\right) \quad (3)$$

$$a^b = -\frac{{ED_{\mathrm{K}}}^b}{\ln\left(\frac{100 - \mathrm{K}}{100}\right)} \quad (4)$$

Substituting a^b into Eq. (2) resulted in a sigmoid concentration-response model (Eq. 5) for incorporation of any effective concentrations (ED_K).

$$y = c + (d - c)\exp\left[\left(\frac{x}{ED_{\mathrm{K}}}\right)^b \ln\left(\frac{100 - \mathrm{K}}{100}\right)\right] \quad (5)$$

The No-Observed-Effect-Concentrations (NOEC) is the highest concentration of individual solvent or their mixtures 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 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, as shown in Eq. (6).

$$\mathrm{CV}(\%) = \frac{\mathrm{SD}}{\mathrm{Mean}} \times 100 \quad (6)$$

Thus, if the CV is taken as *K*, the inhibitor concentration that inhibited β-galactosidase activity by CV% (EDCV) was taken to be NOEC and computed by fitting the concentration-response data into Eq. 5. During curve fitting, the upper asymptote (*d*) was fixed at 100%, and the effect at infinite concentration (*c*) was fixed at zero. The ED_{50} values were estimated by curve-fitting concentration-response data while setting K at 50.

2.9 Prediction of Inhibitory Effects of Solvent Mixtures

The inhibitory effects of the ethanol-DMSO and ethanol-DMF binary mixtures on the activities of crude β-galactosidase from *E. coli* were predicted from the inhibitory effects of the individual solvents by using the concentration addition (CA) model. The CA model can be written in Eq. (7) [22].

$$EC_{x(mix)} = \left(\sum_{i=1}^{n} \frac{\pi_i}{EC_{xi}}\right)^{-1} \quad (7)$$

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 Eq. (7), the inhibitory effects of the mixtures were predicted as described elsewhere [20, 23, 24]. The total concentration of each mixture that elicited 1 – 99% relative β-galactosidase activities was calculated in steps of 1%. In the first step, the EC_x values for 1 – 99% enzyme activity were calculated for each component from the Gormpertz dose-response model that fitted the individual dose-response data. In the second step, the EC_x values were substituted into Eq. (7) to obtain each mixture's 1 – 99% ECx(mix) values. The resulting 99 concentration/response pairs were plotted as a line chart, which visualized the CA-predicted dose-response curve.

2.10 Computation of the Toxic Index (TI)

To evaluate the interactive effect of the mixtures on the β-galactosidase activity, 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. (8) [20, 23].

$$\mathrm{TI} = \sum_{i=1}^{n} \frac{C_i}{EC_{50i}} = \sum_{i=1}^{n} \frac{\pi_i EC_{50mix}}{EC_{50i}} \quad (8)$$

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 [25].

2.11 Computation of the Model Deviation Ratio (MDR)

The model deviation ratios (MDR) were calculated as the ratio of the predicted EC_{50} to the experimentally observed EC_{50} (Eq. 9). The MDR greater than 1 indicated synergistic interaction. In contrast, a value of less than 1 indicated antagonistic interaction. MDR value of 1 indicated additivity (no interaction) [24, 26].

$$\mathrm{MDR} = \frac{\text{Predicted } \mathrm{EC}_{50}}{\text{Observed } \mathrm{EC}_{50}} \quad (9)\,.$$

2.12 Statistical Analysis

Quantitative data are presented as means ± standard deviation. The ANOVA and Duncan post hoc test 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.

3. RESULTS AND DISCUSSION

3.1 Inhibition of Cell-Free β-Galactosidase Activity by Individual Solvents

The inhibition of the activities of cell-free β-galactosidase from *E. coli* by water-miscible solvents, ethanol, dimethyl sulfoxide (DMSO), and N, N-dimethylformamide (DMF) is shown in Fig. 1. The observed concentration-response data were well-described by the Gormpertz model. There was stimulation of the activity of cell-free β-galactosidase 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. 1). DMSO and DMF progressively inhibited *E. coli* cell-free β-galactosidase from 5% until complete inhibition occurred at 50% DMSO and 40% DMF. The EC_{50} and NOEC values for the individual solvents are shown in Tables 1 and 2. The EC_{50} and NOEC values for ethanol from experiments 1 and 2 are not significantly different from each other ($P > 0.05$).

3.2 Inhibition of Cell-Free β-Galactosidase by Solvent Mixtures

Inhibitions of the activities of cell-free β-galactosidase by ethanol-DMSO binary mixtures are shown in Fig. 2. 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 1 shows the EC_{50}, NOEC, TI, MDR, and the combined effects of ethanol-DMSO mixtures on the activity of cell-free β-galactosidase. The CA model predicted statistically equal EC_{50} values ($P > 0.05$) among all the ethanol-DMSO mixtures. However, the CA model predicted significantly lower EC_{50} values than the observed in all the ethanol-DMSO mixtures except for the 7:3 mixture. The NOEC values of the mixture are not significantly different from each other ($P > 0.05$). The TI and MDR values for all the mixtures are slightly above and below 1.000, respectively, and are not significantly different from each other ($P > 0.05$).

Inhibitions of the activities of cell-free β-galactosidase by binary mixtures of ethanol and DMF were shown in Fig. 3. 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%. All concentration-response curves were described using the Gormpertz model.

Table 2 shows the *EC*$_{50}$, NOEC, TI, MDR, and the combined effects of ethanol-DMF mixtures on the activity of cell-free β-galactosidase. The CA model predicted lower EC_{50} values than the observed in all the ethanol-DMF mixtures. However, observed and CA-predicted EC_{50} values for 9:1 and 8:2 ethanol-DMF mixtures are not significantly different from each other. With the exception of the 9:1 ethanol-DMF mixture, the NOEC values for all the mixtures are not statistically different from each other. The TI values for 9:1 and 8:2 mixtures are not significantly different from each other ($P > 0.05$). Similarly, TI values for 7:3 and 6:4 ethanol-DMF mixtures are not statistically different ($P > 0.05$). However, the TI values are marginally higher than 1.000 in all the mixtures. The MDR values, which are not significantly different from each other, are slightly lower than 1.000 in all the mixture ratios.

Table 1. Median inhibitory concentrations (EC_{50}) of ethanol-DMSO mixtures, NOEC, toxic index, and combined effect of ethanol-DMSO mixtures on cell-free β-galactosidase

Solvent/Solvent mixture	EC_{50} (%)		NOEC (%)	TI	Combined Effect
	Observed	CA-predicted			
Ethanol	29.246 ± 2.986^{ab}	-	11.265 ± 1.121^{b}	-	-
DMSO	28.112 ± 0.471^{a}	-	6.407 ± 0.564^{a}	-	-
Ethanol: DMSO (9:1)	31.256 ± 0.642^{bc}	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 ± 1.026^{bc*}	29.034 ± 0.763^{a*}	10.741 ± 0.660^{b}	1.076 ± 0.046^{a}	Additive
Ethanol: DMSO (6:4)	31.346 ± 0.345^{bc}	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} and NOEC values with the same superscript letter are not significantly different from each other ($p > 0.05$). Within rows, EC_{50} values with asterisks are not significantly different from each other ($p > 0.05$). The MDR values for 9:1, 8:2, 7:3 and 6:4 mixture ratios are 0.938 ± 0.005, 0.884 ± 0.027, 0.937 ± 0.006 and 0.922 ± 0.014 respectively.

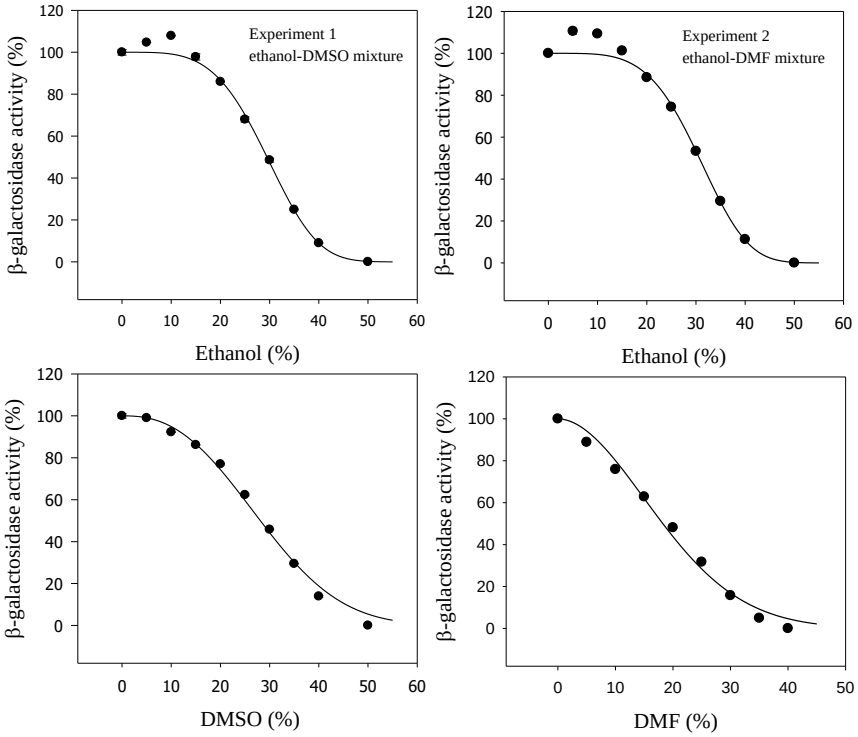

Fig. 1. Inhibition of the activities of cell-free β-galactosidase by individual ethanol, DMSO, and DMF in a different experimental batches with ethanol-DMSO and ethanol-DMF binary mixtures. The solid line represents the Gormpertz model fit to the observed data.

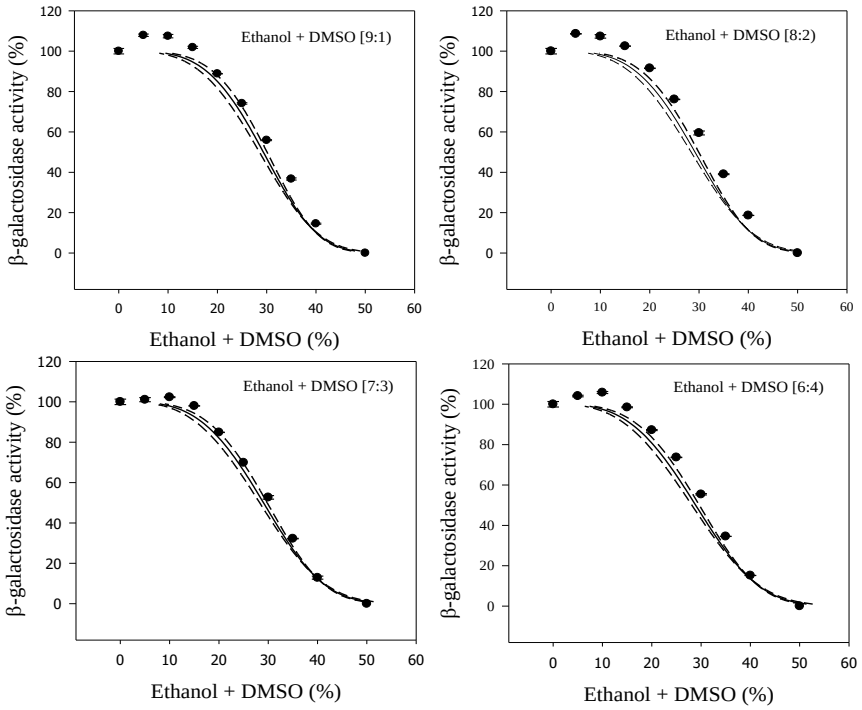

Fig. 2. Inhibition of the activities of cell-free β-galactosidase from *Escherichia coli* by binary mixtures of ethanol and DMSO. The solid and dashed lines represent the mean and 95% confidence limit of CA model-predicted concentration-response relationships, respectively

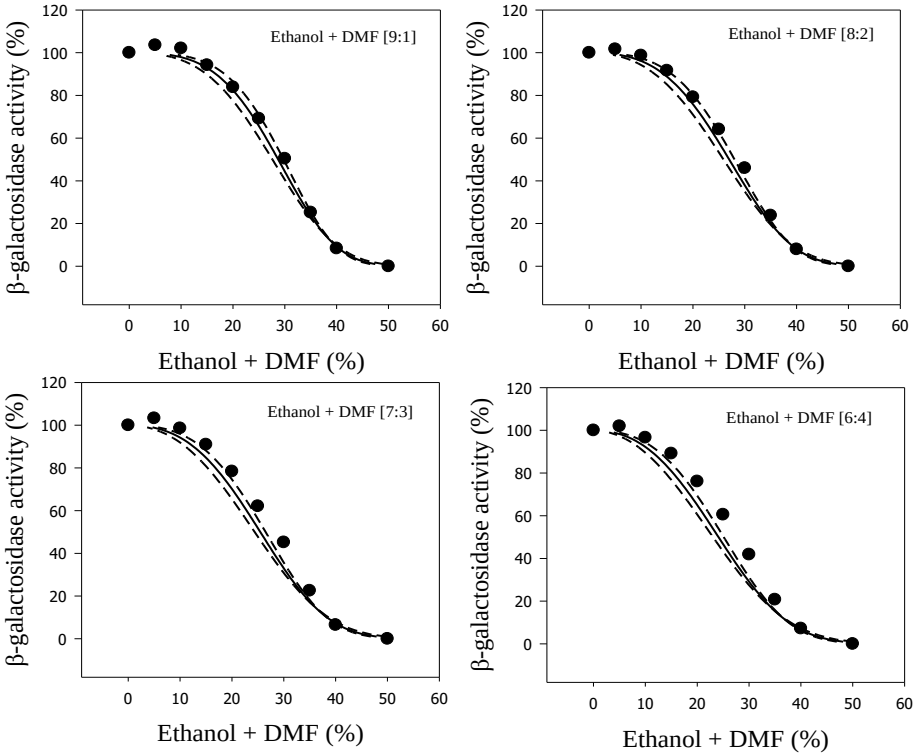

Fig. 3. Inhibition of the activities of cell-free β-galactosidase by binary mixtures of ethanol and DMF. The solid and dashed lines represent the mean and 95% confidence limit of CA model-predicted concentration-response relationships, respectively

E. *coli* is well-known for the production of β-galactosidase, a hydrolase enzyme that enables the bacterium to break down lactose into galactose and glucose. Assay for β-galactosidase activity requires permeabilization of microbial cells with chemical agents to allow penetration of the chromogenic substrate, *o*-nitrophenyl-β-D-galactopyranoside (*O*NPG), into the intact cells [27]. However, *E*. *coli* has thin cell wall porins in the outer membrane, enabling the transport of molecules. The enzyme substrate can moderately diffuse into the cell to interact with the β-galactosidase. According to Cho et al [28], less than 2.3% of *O*NPG was hydrolyzed when the artificial chromogenic substrate was applied to intact *E*. *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. Similarly, in cell lysis or excessive permeabilization, permeabilizing agents could access the cytoplasmic contents, resulting in inhibition of β-galactosidase activity. This underlined the need to investigate the inhibitory effects of cell permeabilization agents on *E.coli* β-galactosidase.

Ethanol, DMF, and DMSO are organic solvents commonly employed to permeabilize microbial cells, allowing intracellular enzymes like β-galactosidase to be released from the cell. At high concentrations, these solvents can readily hinder the activity of β-galactosidase. There was stimulation of the activity of cell-free β-galactosidase from *E*. *coli* by 5%, 10%, and 15% ethanol. This corroborates the report of Soto et al. [29] that 4% ethanol was not inhibitory to the activity of β-galactosidase from *Bacillus circulans* due to the conservation of the enzyme structure. Probably, 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 [17, 30, 31]. A concentration equal to 4% ethanol (equivalent to 0.9M) is within 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 [17]. In a similar experiment, Hamed et al. [32] investigated the effects of ethanol (0-20%) on the enzymatic activity of β-galactosidase from *E*. *coli* DH5α. They reported that 10% ethanol stimulated β-galactosidase activity by 15%. At 15%, ethanol slightly inhibited β-galactosidase activity, while 20% inhibited β-galactosidase activity by approximately 30% [32]. Also, Bell et al. [17] reported that at modest concentrations (0 - 2M), there was little effect of methanol, ethanol, propanol, and butanol on the kinetic constants of β-galactosidase from *Kluyveromyces lactis*. At 18% and 36%, ethanol and DMF deactivated the activity of cell-free β-galactosidase derived from *E*. *coli* with a half-life of 55 h and 1.9 h, respectively [33]. Karan et al. [34] noted that assessment of the effects of organic solvents on β-galactosidase plays an essential role in its industrial applications. They investigated the effects of organic solvents on the activity and stability of β-galactosidase from the antarctic haloarchaeon *Halorubrum lacusprofuundi*. They reported that 5% or 10% ethanol inhibited β-galactosidase activity by 30-35%.

Table 2. Median inhibitory concentrations (EC_{50}) of ethanol-DMF mixtures, NOEC, toxic index, and combined effect of ethanol-DMF mixtures on cell-free β-galactosidase

Solvent/Solvent mixture	EC_{50} (%)		NOEC (%)	TI	Combined Effect
	Observed	CA-predicted			
Ethanol	30.687 ± 0.726^{e}	-	11.556 ± 1.648^{d}	-	-
DMF	18.244 ± 0.674^{a}	-	1.897 ± 0.427^{a}	-	-
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^{bc*}	8.237 ± 0.486^{b}	1.052 ± 0.019^{a}	Additive
Ethanol: DMF (7:3)	28.017 ± 0.346^{bc}	25.454 ± 1.053^{ab}	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, values with the same superscript letter are not significantly different from each other ($p > 0.05$).
Within rows, EC_{50} values with asterisks are not significantly different from each other ($p > 0.05$).
The MDR values for 9:1, 8:2, 7:3 and 6:4 mixture ratios are 0.973 ± 0.026, 0.950 ± 0.028, 0.908 ± 0.026 and 0.880 ± 0.029 respectively

There is a scarcity of information on the inhibitory effect of DMSO on β-galactosidases from *E*. *coli*. However, Kamran et al. [35] 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 *A*. *nidulans*. Our study with *E*. *coli* β-galactosidase also portrayed DMSO as a potent inhibitor of β-galactosidase, more than ethanol. The average estimated NOEC for DMSO against *E*. *coli* β-galactosidases was 6.407% v/v. At 50% (v/v) of the 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* [12]. This corroborated our report on the toxicity of DMSO and DMF against β-galactosidases from *E*. *coli.* In comparison, the β-galactosidase from *E*. *coli* tolerated the inhibitory effect of DMSO more than the β-galactosidase from *A*. *nidulans*. The differences in the response of these β-galactosidases to the inhibitory effects of solvents could 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*. Both DMF and DMSO are more inhibitory than ethanol against *E*. *coli* β-galactosidase activity. The milder effects of ethanol compared to DMSO and DMF have been reported elsewhere [35].

We further investigated the interactive inhibitory effects of ethanol-DMSO and ethanol-DMF binary mixtures against the activities of the β-galactosidase. The model deviation ratios between the predicted and experimentally observed 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 [36, 37]. Therefore, the combined effects of the mixtures were considered to be additive. The concentrations of the individual solvents and solvent mixtures below NOEC values are sub-inhibitory and recommended for cell permeabilization during *in-situ* β-galactosidase activity measurements in *E*. *coli*. Whether these concentrations would be suitable for permeabilizing *E*. *coli* cells for accurate *in-situ* β-galactosidase activity assay would be a subject of further research.

4. CONCLUSION

This study investigated the inhibition of *E*. *coli* cell-free β-galactosidase activity by ethanol, DMSO, and DMF and their binary mixtures. The subinhibitory levels of the water-miscible solvents include concentrations up to 13.204%, 6.971%, and 2.314% of ethanol, DMSO, and DMF, respectively. In the ethanol-DMSO mixtures, the NOEC of all the mixture ratios is not statistically different from the NOEC of ethanol, the least toxic component. On the other hand, adding DMF to ethanol resulted in a significant decrease in the NOEC values of the ethanol-DMF mixtures. Analyzing the data revealed that the binary mixture of solvents had additive effects on the activity of cell-free β-galactosidase from *E*. *coli*.

DISCLAIMER (ARTIFICIAL INTELLIGENCE)

Author(s) hereby declare that NO generative AI technologies such as Large Language Models (ChatGPT, COPILOT, etc.) and text-to-image generators have been used during the writing or editing of this manuscript.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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Peer-review history:
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https://www.sdiarticle5.com/review-history/126742