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Study of Enzymatic Activity for The Detection of Bacteria in Seawater in Iraq

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Abstract. General Background: Monitoring bacterial contamination in seawater is essential for environmental safety, as fecal coliforms like *Escherichia coli* pose serious health risks. Specific Background: Traditional culture-based methods often underestimate bacterial viability, particularly in stressed or non-cultivable states, limiting their reliability. Knowledge Gap: Limited studies have explored how enzymatic activity can persist in non-cultivable bacteria and how it may be applied as a sensitive detection tool in marine environments. Aims: This study aimed to assess the persistence of β-galactosidase and β-glucuronidase activity in *E. coli* under varying stress conditions, including chlorine exposure, temperature, antibiotic treatment, and freeze-thaw cycles, to evaluate their potential as indicators of bacterial survival in seawater. Results: The findings revealed that under severe treatments (chlorine, high temperature), enzymatic activity declined simultaneously with cultivability, whereas under milder stress (chloramphenicol, freeze-thaw), enzymatic activity persisted despite significant loss of cultivability. Comparative modeling and docking analyses confirmed the structural and functional properties of β-galactosidase relevant to bacterial adaptation. Novelty: This research demonstrates that enzymatic activity may serve as a marker for viable but non-cultivable bacteria, bridging the gap between culture-based detection and molecular approaches. Implications: These results provide a foundation for developing rapid, sensitive monitoring tools for marine water quality, enhancing early detection of fecal contamination.

Highlights:

1. Enzymatic activity is used to assess bacterial viability in different stress conditions.
2. Severe treatments cause simultaneous loss of cultivability and enzymatic activity.
3. Mild treatments reduce cultivability more than enzymatic activity, indicating partial persistence.

Keywords: Enzymatic Activity, Bacteria, Freeze-Thaw Cycles, Chloramphenicol, Seawater

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Introduction

Among the many problems encountered during the first studies of enzyme kinetics, the most important was certainly the lack of understanding of the effect of the hydrogen ion concentration, H+.[1] In aqueous solution, the proton concentration varies between 1 M and 10–14 M, an extremely wide range that is commonly expressed using a logarithmic scale, pH= –log[H+], to reduce the size of the figures manipulated. All enzymes are profoundly influenced by pH, and no substantial progress could be made until MICHAELIS and his collaborators made pH control a routine part of any serious enzyme study. The concept of a buffer to control the concentration of free proton and the pH scale which allows it to be expressed, were introduced by SØRENSEN [2], in a publication demonstrating the importance of the concentration of the hydrogen ion in enzymatic studies. MICHAELIS, however, had already started working in this direction, [3]and it was only shortly after that the first paper in a long series appeared on the effects of pH on enzymes [4]. Although there is still some disagreement regarding the interpretation of the effects of pH on enzyme kinetics, the practical importance of pH remains unchanging: it is impossible to envisage a kinetic study without adequate pH control.[5]

The physiological state of bacteria can be assessed by measuring their enzymatic activity. The activities of β-galactosidase and β-glucuronidase provide a means to evaluate bacterial contamination using 4-methylumbelliferyl-β-D-galactoside (MUGal) and 4-nitrophenyl-β-D-glucuronide (MUGLu) as respective substrates.[6][7]

This measurement is based on the enzymatic hydrolysis of the substrate, resulting in the production of a fluorescent compound: methylumbelliferone. This alternative method for detecting indicators of fecal contamination is notable for its sensitivity and rapidity compared to traditional methods for enumerating fecal coliforms [8], which is crucial for making prompt decisions during coastal pollution events.

All methods for enumerating thermotolerant coliforms rely on lactose acidification and fermentation. However, Munro demonstrated a decrease in this activity after 9 to 10 days of exposure to seawater for E. coli [9]. Other studies have reported the emergence of atypical lactose-negative colonies of E. coli.[10, 11]

Several studies have utilized these techniques to detect fecal coliforms in estuarine waters, food samples, and clinical specimens [12]). These techniques provide a reliable estimate of fecal contamination, with a strong correlation observed between the presence of fecal coliforms and enzymatic reactivity [13, 14]. The detection limit for this technique is estimated at 10^2-10^3 fecal coliforms per 100 mL, aligning with European standards for bathing waters.

The significance of nutrients present in seawater has been demonstrated in relation to enzymatic expression [15]. Both β-galactosidase and β-glucuronidase (see fig 1) are inducible enzymes whose expression depends on specific physiological and genetic factors within bacteria [16]). Lactose serves as an inductive factor for β-galactosidase; when lactose is added to a bacterial culture in a lactose-free medium, β-galactosidase and permease are synthesized almost simultaneously. The presence of lactose can increase the abundance of these proteins by a factor of ten [17, 18]. Further, the comparative modeling, structural annotation, domain identification, and structural comparison of the β-galactosidase enzyme have been done. In addition, molecular docking analysis was also performed on the catalytic by using β-D-galactose separately.

However, it has been found that contrary to other studies[19], the addition of lactose did not enhance sensitivity; rather, they observed an inverse effect. In fact, lactose is rarely

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used in induction experiments because the synthesized β-galactosidase cleaves lactose, leading to a decrease in its concentration[7, 20]. To address this issue, Diez (1991) recommended using an analogue containing a sulfur atom, isopropyl-β-D-thiogalactoside (IPTG)[21], to enhance enzymatic response[22].

In marine environments, factors such as nutrient availability, salinity, and temperature create suboptimal conditions for bacterial growth and influence their physiological state. Enzymes like hydrolases are often involved in the assimilation of certain nutrients, making substrates more accessible to bacterial cells. The enzyme 4-methylumbelliferyl-heptanoate-hydrolase (MUHase), produced by a strain of E. coli in seawater, was studied by different researches[23], revealing that increased enzymatic activity correlates with bacterial adaptation to the nutrient conditions of the marine environment.[24]

Salinity is another factor influencing enzymatic activity. It has been observed observed that diluted effluents in seawater exhibited decreased enzymatic response as salinity increased [25-26].

In this study, the activities of β-galactosidase and β-glucuronidase were measured to detect the presence of fecal coliforms and E. coli in seawater. Additionally, treated effluents collected from wastewater treatment plants and diluted with seawater were tested using these techniques over a period of two weeks.

Materials and Methods

A. Materials

Effluents were collected from the outflows of the biological treatment plants located in Baghdad. Table 1 presents characteristics of the Effluents. Values are expressed in mg/L.

The Artificial seawater was filtered through a 0.2 µm membrane and autoclaved for 20 minutes at 120 °C.

The base medium for induction consisted of: Casein hydrolysate (2.5 g), KH_2PO_4 (2 g), K_2HPO_4 (7 g), $(NH_4)_2SO_4$ (1 g), $MgCl_2 \cdot 6H_2O$ (0.1 g), Distilled water (1 L).

The culture media were used for bacterial enumeration containing: Trypticase agar, incubated for 18 hours at 37 °C, Lactose agar of Drigalski, incubated for 18 hours at 42 °C, Lactose agar of MacConkey, incubated for 18 hours at 44 °C. Isopropyl-β-D-thiogalactoside (IPTG) was used as inducer.

The Substrates was prepared as follows: For MUGal, 4-methylumbelliferyl-β-D-galactoside was prepared extemporaneously by dissolving it in phosphate buffer at a concentration of 0.74 mM (0.05 M, pH 8). The solution was heated to approximately 80 °C to ensure complete dissolution and then filtered through a 0.2 µm membrane.For MUGlu, 4-methylumbelliferyl-β-D-glucuronide was also prepared extemporaneously by adding it to Triton water at a concentration of 1 mg/mL.

Triton water was prepared by adding two drops of Triton X to 100 mL of distilled water, followed by autoclaving for 15 minutes at 120 °C. The substrate MUGlu was dissolved with gentle heating and then filtered through a 0.2 µm membrane.A SEQUOIA-TURNER model 450 spectrofluorometer was utilized to measure the fluorescence of the enzymatic reaction product (MUF). Quartz cuvettes that allow UV transmission were used, with readings taken at an excitation wavelength of 360 nm and an emission wavelength of 450 nm.

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B. Measurement of Enzymatic Activity

β-D-Galactosidase Activity: In a shaking water bath maintained at 44 °C, 13.5 mL of phosphate buffer (0.05 M, pH 8) supplemented with sodium lauryl sulfate (SLS) at a concentration of 0.5 mg/mL was combined with 9 mL of the MUGal substrate. An appropriate volume of the sample to be analyzed, based on its activity, was filtered through a Nuclepore filter (0.2 µm). The filter was then introduced into the phosphate buffer-substrate mixture at 70 °C.Fluorescence kinetics were monitored at 5-minute intervals over a period of 30 minutes. For each measurement, 2.5 mL of the sample was taken, mixed with 100 µL of 10 N NaOH, and placed into the measurement cuvette. A blank was prepared for each series, and each sample was measured in duplicate.

β-D-Glucuronidase Activity: The measurement method for β-D-glucuronidase followed the same protocol as that for β-D-galactosidase, using 17 mL of phosphate buffer (0.05 M, pH 6.4) in an Erlenmeyer flask and adding 3 mL of the MUGlu substrate.Fluorescence kinetics were calculated using linear regression fitted by the least squares method. The enzymatic activity per liter is expressed in picomoles of methylumbelliferone (MUF) produced per liter per minute, given by the equation:

$$Actenzym/litre=(dFm/dEm)\cdot(dEc/dFc)\cdot V$$

where:

dFm = average slope of the two replicates
dEm = slope of the control
dEc = slope of the calibration curve
V = volume of the buffer solution in the Erlenmeyer flask in mL

To determine dEc, fluorescence measurements were conducted on solutions containing 0.1 to 1 µM of methylumbelliferone in phosphate buffer using a spectrofluorometer.The enzymatic activity per cell, expressed as picomoles of MUF produced per cell per minute, is calculated using the following expression:

$$Actenzym/cell=(Act/litre)\cdot(number\ of\ bacteria/litre)$$

Eperimental

In the context of this study, various types of experiments were conducted.

Study of the Induced Strain of E. coli H10407

Induction trials for the strain E. coli H10407 were performed using IPTG.

A. Preparation of the Strain

A loopful of the E. coli H10407 culture was inoculated into 9 mL of base medium supplemented with 1 mL of IPTG at a concentration of 2.5 mM. The bacterial culture was incubated for 18 hours at 37 °C with agitation.After incubation, the bacterial culture was centrifuged and washed with physiological saline, and the pellet was resuspended in 100 mL of water (either freshwater or seawater, depending on the experiment). Experiments were conducted on the induced strain to investigate the role of organic matter on survival in seawater and to study variations in enzymatic activity based on the physiological state of the bacteria.

B. Chlorine Treatment

In another experiment, a fraction (10 mL) of cultured bacteria was exposed for

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one hour to concentrated bleach (1 mL). The solution was filtered through a membrane filter (0.22 µm), and the filter was washed with physiological saline before being placed into 10 mL of Instant Ocean at a salinity of 34 %.

C. Destruction of Bacteria by Temperature

A flask containing 10 mL of diluted induced culture in seawater was autoclaved for 10 minutes at 115 °C to analyze the effects on dead bacteria.After all treatments, bacterial counts as well as measurements of β-galactosidase activity were performed on these different samples.

D. Study of Variations in Enzymatic Activity Intensity Based on Physiological State (Growth Phase) of E.coli

To compare enzymatic activity across different growth phases (exponential and stationary phases), two cultures of induced E.coli were prepared: one incubated for 12 hours and another for 38 hours at 37 °C. After washing with physiological saline, bacterial pellets were resuspended in 10 mL of seawater. Optical densities for both samples were measured; the stationary phase sample was diluted to achieve an optical density similar to that of the exponential phase sample. Bacterial counts and measurements of β-galactosidase activity were monitored for these samples maintained at 15 °C in darkness over a period of approximately two weeks

E. Effect of Organic Matter Presence in Freshwater and Seawater

The survival of E. coli (induced) was monitored in both freshwater and seawater at 30 °C. The effluent, filtered through membranes of 0.22 µm (after passing through 3 µm and 0.45 µm filters), was diluted in water at different concentrations (0%, 25%, and 50%) and then sterilized for 20 minutes at 120 °C. Under these various conditions, bacterial counts of E. coli on Trypticase agar as well as β-galactosidase activity were monitored over a one-week period.

F. Study of β-D-Galactosidase Activity Persistence Based on Bacterial Viability

It was deemed important to correlate enzymatic activity with the number of culturable bacteria. Additional experiments were conducted at 15 °C using stressed strains of E. coli subjected to antibiotic treatment (inhibiting protein synthesis), freeze-thaw cycles, heat shock, or lysis to determine whether enzymatic activity could persist when bacteria are dead or non-culturable.

G. Effect of Chloramphenicol

Chloramphenicol is an antibiotic that inhibits protein synthesis. In this experiment, the strain was induced following the previously described protocol, and after 9 hours of incubation, concentrations of chloramphenicol (0, 10, 100, and 250 µg/mL) were added to different samples. The cultures were incubated at 37 °C with agitation for approximately 12 hours. The recovered bacterial pellets were resuspended in 100 mL of Instant Ocean at a salinity of 34 % (without organic matter addition).

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H. Bacterial Lysis by Freeze-Thaw Cycles

A portion of the induced bacterial culture diluted in seawater underwent six freeze-thaw cycles at -80 °C followed by thawing at 44 °C. After adding lysozyme at a concentration of 0.2 µg/mL, samples were incubated at 37 °C for 30 minutes.During a subsequent experiment (09/08), two samples were treated: one in seawater at a salinity of 34 % and another in freshwater.To assess the effectiveness of bacterial lysis, samples were observed under epifluorescence microscopy.

I. Modeling and Structural Annotation

Comparative modeling is an active area of research in which scientists attempt to predict the three-dimensional structure of a protein based on its sequence. The comparative modeling of the target protein was executed using Swiss- model server. The docking was performed using CB-Dock2 to predict their binding sites and affinity towards ligands.

Results and Discussion

A. Effect of Organic Matter Presence in Freshwater and Seawater

Three experiments were conducted using the same quality of organic matter at temperatures of 22 °C. Overall, a good survival rate of E. coli was observed in freshwater over the 6 to 7 days of experimentation, with a slight protective effect noted at 25% and 50% effluent concentrations compared to the control. In seawater at 30 °C, the impact of salinity on E. coli survival was evident. A decline of at least one log unit was observed by the sixth day of the experiment across all samples, with reductions reaching up to three log units for samples containing 50% effluent (Figures 1). In all cases, the addition of organic matter from the effluent did not appear to protect bacterial survival in seawater. These findings are consistent with observations made during a study conducted on the non-induced strain of E. coli H10407.Fiksdal and Midttun [27, 28] noted that the protective capacity of effluent increases when bacterial counts are low, typically around 102 to 104 CFU/mL. In our study, initial counts were approximately 106 CFU/mL, and we observed a pronounced protective effect in seawater when counts reached this range (102 to 104 CFU/mL) at both 25 °C with 25% and 50% effluent concentrations.

Table 1: Characteristics of the Effluents. Values are expressed in mg/L**.**

Date of Sampling	**25/07/ 23**	**22/08/ 23**	**11/08/ 23**	**16/08/ 23**	**21/08/ 23**
Suspend ed Solids (SS)	9.80	4.80	14.40	17.20	34.20
Volatile Solids at 550 °C	8.60	-	13.40	-	32.0

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Total Organic Carbon (TOC)	12.93	9.20	18.30	13.65	27.90
Dissolved Organic Carbon (DOC)	10.71	8.64	17.80	8.65	18.45
Ammonium NH4	33.10	-	47.0	-	53.70
Phosphates PO4	18.0	-	31.10	-	27.70

The T90 values (the time corresponding to a reduction of cultivability by 90% of the bacterial population) were calculated for these different experiments. In freshwater, the addition of effluent increased the T90 value from 4 days to over 20 days. However, this was not observed during the experiment. In seawater, the T90 did not increase in the presence of effluent; rather, an inverse effect was noted. This indicates that in a hostile environment such as seawater, the protective effect of organic matter is not always effective for bacterial survival. The conditions necessary for bacteria to establish mechanisms of halotolerance may not have been met in our study. During these experiments, a decrease in enzymatic activity per liter was observed during the first two days. At a salinity level of 30 % and at a temperature of 25 °C, this decrease in activity was noted only between the second and fourth days. At this salinity level, enzymatic activities per liter varied from 0.1 to 4 µM, depending on the experiment. At a salinity level of 0 % throughout the experiment, values for activity per cell remained relatively stable; however, a gradual increase in activity per cell was observed over time at a salinity level of 30 %.

B. Study of the Persistence of β-D-Galactosidase Activity Based on Bacterial Viability

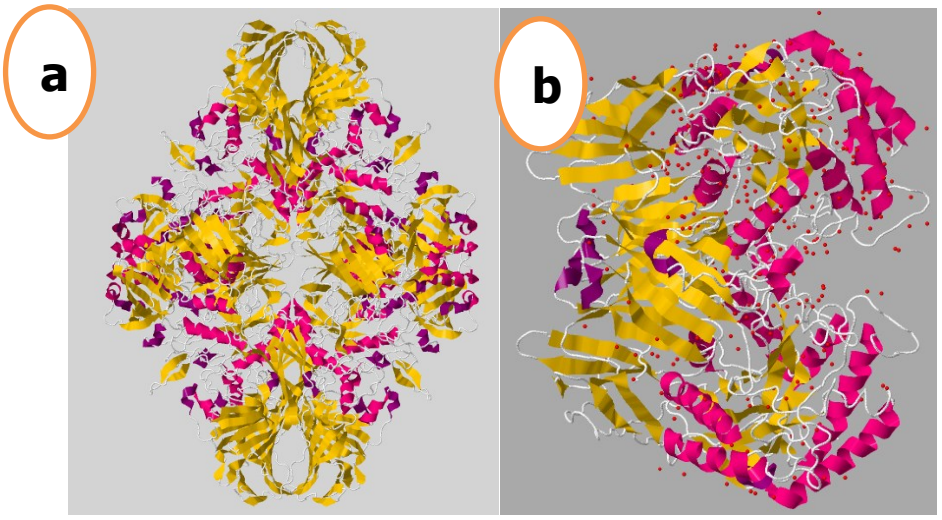

Fig. 1. structure of (a) β-galactosidase and (b) β-glucuronidase.

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The strain of Escherichia coli was subjected to various treatments to assess the persistence of enzymatic activity in relation to the physiological state of the bacteria and the potential for revival in seawater.

C. Assessment of Enzymatic Activity Post-Treatment

The strain was exposed to three different concentrations of chloramphenicol; the results are summarized in Table 2.

Table 2: Effect of Chloramphenicol

Parameters Measured	Control	Sample with 100 µg/ml of chloramphenicol	Sample with 250 µg/ml of chloramphenicol
CFU/ml	2.9 × 107	1.9 × 106	1.9 × 105
AcH (µg·ml⁻¹)	13.59	7.01	2.33
Act/l (µM·min⁻¹)	5.04 × 10⁻11	4.67 × 10-10	1.04 × 10⁻9

In general, it is observed that for chloramphenicol concentrations equal to or less than 100 µg/ml, no significant effects are evident on the culture results; however, there is a slight decrease in enzymatic activity. A concentration of 250 µg/ml of the antibiotic results in a simultaneous loss of cultivability and a reduction in enzymatic activity, although the latter occurs to a lesser extent than the loss of cultivability.

The lysis of bacteria using the freeze-thaw technique resulted in a reduction of bacterial numbers by 2 to 4 log units. A significant decrease in activity per liter is observed following lysis. Depending on the efficiency of the lysis, reductions in activity per liter are noted immediately after lysis, decreasing from 4.22 to 3.10^{-2} µM·min⁻¹ (Table 3).

Table 3: Effect of Lysis by Freeze-Thaw Cycles

Sample	Control	3 freeze-thaw cycles	Control	6 freeze-thaw cycles	Control	3 freeze-thaw cycles with Lysozyme
CFU/ml	3.9 × 106	8.0 × 103	1.9 × 106	1.4 × 10^{-4}	4.3 × 10⁻5	--

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Act/l (μM·min⁻¹)	13.82	6.54	9.62	11.68	4.22	3×10^{-2}
Act/cell (μM·min⁻¹)	3.6×10^{-9}	8.9×10^{-7}	4.95×10^{-9}	7.57×10^{-7}	9.5×10^{-9}	3×10^{-6}

In both types of treatment, there is a drastic reduction in bacterial counts (Table 4).

Table 4: Effect of Bacterial Destruction (Chlorine, Autoclaving)

Sample	Control	Autoclaved Sample	Chlorinated Sample
CFU/ml	1.9 × 10^6	< 10	< 10
Act/l (μM·min⁻¹)	9.41	$< 10^{-3}$	$< 10^{-3}$
Act/cell (μM·min⁻¹)	4.9×10^{-9}	-	-

The count decreases from approximately 106 CFU/ml to fewer than 10 cultivable bacteria per ml. Enzymatic activity also experiences a significant decline; the activity values per liter drop from 9.41 μM·l⁻¹·min⁻¹ for the control sample to values below 10^{-3} for the chlorinated and autoclaved samples. These drastic treatments likely have both a direct effect (cell death) and an indirect effect (protein hydrolysis). At after autoclaving, the decrease in activity may be attributed to the complete destruction of both the enzyme and the cell.

Table 5: The possible pocket in β-galactosidase enzyme.

CurPocket ID	Cavity volume (3 Å)	Center (x, y, z)	Cavity size (x, y, z)
C1	3569	-4, 35, 0	25, 29, 30
C2	3041	0, -46, -2	27, 19, 10
C3	3039	29, -3, 52	29, 14, 27
C4	2573	-26, -2, -57	21, 17, 28
C5	1506	-16, -31, -26	15, 22, 19

D. Simulation results

The 3D structure of the target protein was generated through a swiss model a comparative modeling approach. The predicted structure was visualized using PyMol program (Figure. 2).

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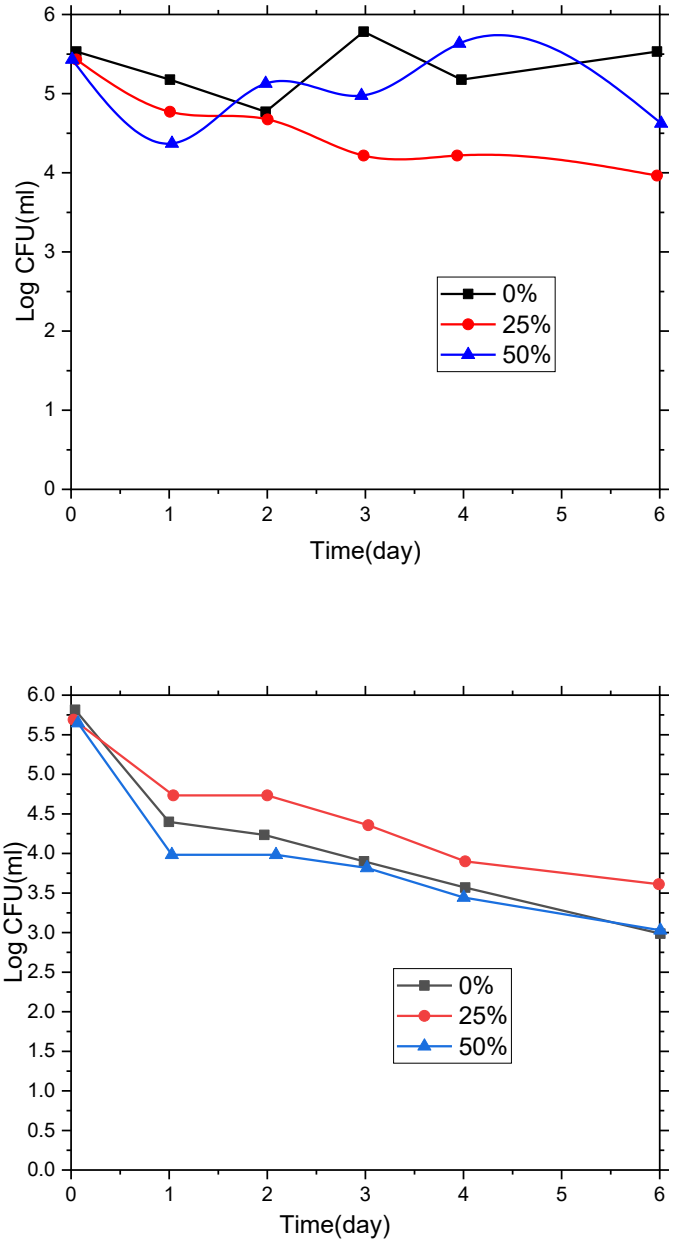

Fig. 2. Survival of E. coli at 25 oC for different concentrations of effluent. (a) salinity 0%, (b) salinity 30%..

The analysis of the modelled structure yielded the corresponding Ramachandran plot for the predicted structure (Figure. 3)

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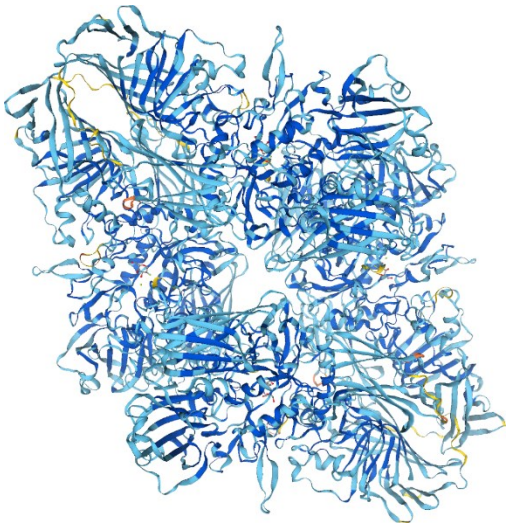

Fig. 3. 3D structure of β-galactosidase enzyme as obtaind using swiss model.

The following anomalies are recorded: the ϕ/ψ angles of 89.1 % residues are in the most favoured regions, 10 % in the additional allowed regions, 0.6 % in the generously allowed regions, 0.2 % in the disallowed regions. The quality model would be expected to have over 90 % in the most favored regions.

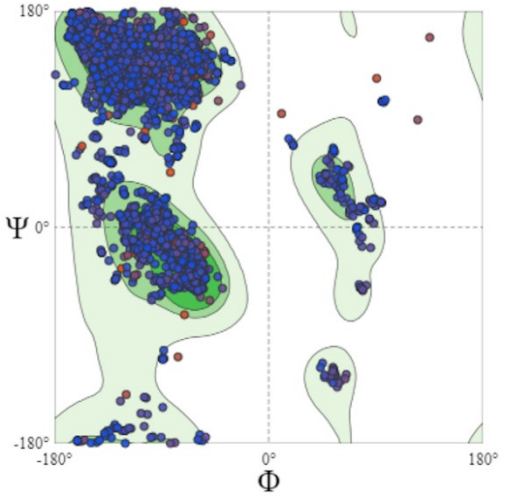

Fig. 4. Ramachandran plot of β-galactosidase enzyme from swiss model.

For further analysis of the enzyme, the possible cavity and been investigated by CB-Dock2 and visualized using pymole and presented in figure 4. Five possible cavities have been identified and listed in table 5.

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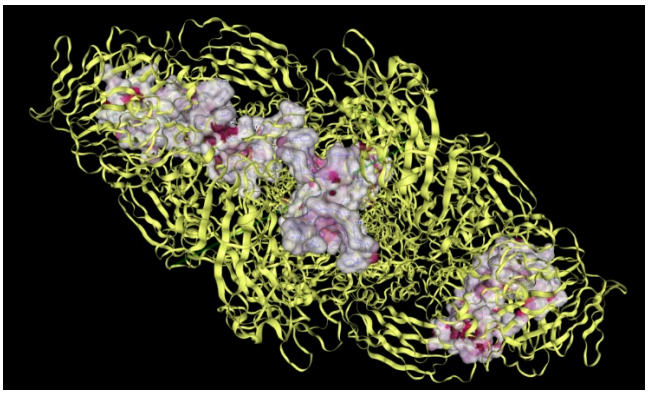

Fig. 5. The possible cavity allowed for β-D-galactose in the enzyme.

Docking calculation revealed that the catalytic residues identified were Glu200 and Glu298 along with interacting ligand β-D-galactose. Molecular docking of both the catalytic residues executed separately with β-D-galactose showed best docking score in both the cases (Figs 5, 6, 7). Table 6 shows the scores of all pockets. It is clear that pockets 2 and 3 have the highest score.

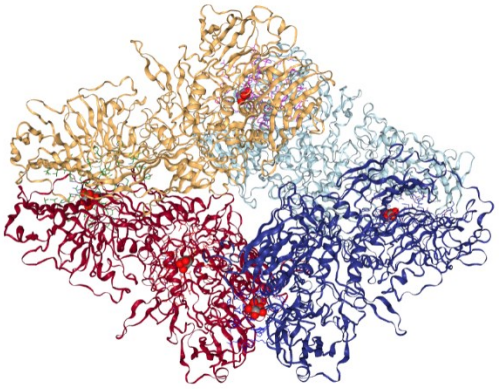

Fig. 6. The binding of β-D-galactose (space filled) at the enzyme active.

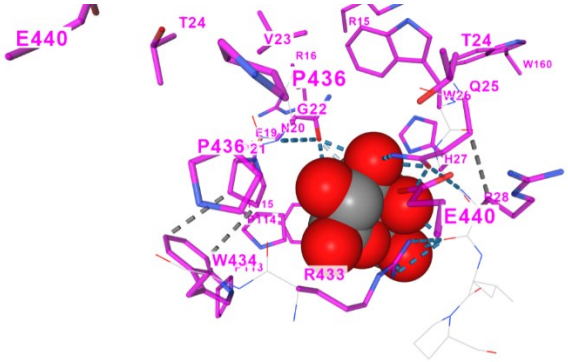

Fig. 7. The figure shows the Ball and stick representation of the molecular docking between β-galactosidase active site E440) and the ligand beta-D-galactos.

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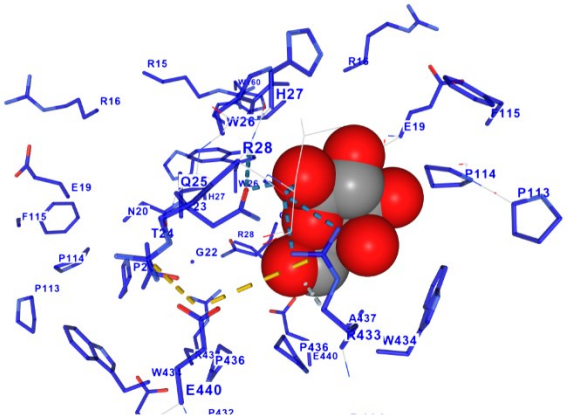

Fig. 8. The figure shows the Ball and stick representation of the molecular docking between β-galactosidase active site R28 and the ligand beta-D-galactos.

Figures 6 7 and 8 show the bond interaction between the catalytic sites with a ligand. While the bond interactions are efficient in both sites, the bond formed between the catalytic site Glu200 was found stronger due to two H-bonds in comparison to the other catalytic site Glu298.

Conclusions

The purpose of these experiments was to study the persistence of enzymatic activity when bacteria are no longer cultivable. During drastic treatments (chlorine exposure and high temperatures), the enzymatic activity of the bacteria disappears simultaneously with their ability to be cultured; the loss of bacterial activity is attributed to cell death and enzyme destruction due to the treatment.

In contrast, during milder treatments (chloramphenicol and freeze-thaw cycles), both the loss of cultivability and the decrease in enzymatic activity are observed simultaneously, but not in the same proportions: the loss of cultivability is often more significant than the reduction in enzymatic activity. It appears that viable but non-cultivable bacteria may retain some enzymatic activity for a period, albeit at a reduced level.

From an analysis standpoint, it can be assumed that if within the region of the enzyme activity, b-D-galactose can be made to accumulate, it will be because it will compete with the substrate and it will also affinity label the active site of the enzyme, making it inactive for any further functioning, thus resulting in a feedback inhibition.

FUNDING

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CONFLICT OF INTEREST

The author declares that no conflicts of interest.

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