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Oryginal Research

Investigation of the Inhibitory Effects of Chemical Compounds on Eco-Efficiency: an Analysis Based on the Activity of *Bacillus licheniformis*

Kambiz Najafi, Nahid Haghnazari*, Kambiz Davari, Fatemeh Keshavarzi**

Department of Biology, Sanandaj Branch, Islamic Azad University, Sanandaj, Iran

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Abstract

The penicillinase enzyme plays an important role in local ecological efficiency and resistance to antibiotic agents. *Bacillus licheniformis*, the causative agent of septicemia, has a high penicillinase activity. The main goal of this study was to identify selective inhibitors for clinical applications. To achieve this, the inhibitory effects of four chemical compounds on the activity of the Bacillus licheniformis (*B. licheniformis*) penicillinase enzyme were evaluated. *B. licheniformis* PTCC 1320 (NCIB6346) was cultured in tryptic soy broth (TSB) (30 g/L) liquid culture medium and was adjusted to 0.5 McFarland turbidity standard (4-hr culture). Mueller-Hinton broth (MHB) was used to adjust the turbidity. Then, the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values of benzylpenicillin antibiotic (penicillin G) were determined. Then, a concentration of 8 mg/ml of citrate was chosen and consistently added to all the tubes (serial tubes). Enzyme inhibition by Citra and other inhibitors was evaluated by measuring MIC and MBC in the presence of each compound separately. Finally, the results were confirmed by molecular modeling data. The MIC and MBC levels of benzylpenicillin were determined to be 2 and 4 µg/mL, respectively. However, in the presence of citrate, the MIC and MBC values of benzylpenicillin were found to be 0.125 and 0.25 µg/mL, respectively. Moreover, molecular docking results showed that the active site of penicillinase had an anionic cavity where amino acid anions (Thr 235, Arg 24, Ser 70, Ser 130, Tyr 135, Asn 170, and Asn 104) were present and stabilized the intermediates formed in this site by inducing electrostatic interactions. Citrate could competitively attach to this site. The other three compounds (diethyl malate, malic anhydride, and malic acid) showed similar binding patterns to citrate, but were smaller in size and had fewer active groups: their interactions were less strong and weaker than those of citrate can enhance ecological efficiency. The results show that citrate is the best inhibitory compound for *B. licheniformis* penicillinase as well as for promoting ecological construction.

Keywords: ecological efficiency, *Bacillus licheniformis,* penicillinase, inhibitor, molecular modeling

*e-mail: N.haghnazari@iausdj.ac.ir
**e-mail: k.davari@iausdj.ac.ir

Introduction

Penicillinase enzyme catalyzes the conversion of penicillin to penicilloic acid without coenzymes. Based on the IUPAC Committee (International Union of Pure and Applied Chemistry) classification, the official name of this enzyme is penicillin amide hydrolase, and its number is EC 3.5.2.6. This enzyme belongs to the third group of enzymes, namely hydrolases, and is also known by various names such as penicillin beta-lactamase and beta-lactam amide hydrolase [1].

The initial classification of beta-lactamases was reported based on function, sensitivity to inhibitors, and genomic location. This division divided beta-lactamases into two general categories: chromosomal and plasmid-mediated [2]. Further classified beta-lactamases into two broad categories based on enzyme-specific substrates and sensitivity to inhibitors: penicillinases with benzylpenicillin as a specific substrate and cephalosporinases with cephalosporin as a specific substrate [3]. Class A beta-lactamases are referred to as penicillinases, with penicillinase as the main substrate [4-6].

Studies conducted in the field of identifying microorganisms that produce the penicillinase enzyme have shown that this enzyme is produced by different microorganisms such as bacteria, mycobacteria, fungi, and yeasts; thus, the enzyme can be obtained from complete bacterial cells, extracellular culture media, fungal spores, and crude extracts of cells. *Bacillus licheniformis* can be mentioned among the microorganisms that produce penicillinase enzyme and belong to the group of Gram-negative bacteria. *B. licheniformis* is known to cause septicemia, eye infections, gastrointestinal inflammation, blood poisoning, and food poisoning.

The *B. licheniformis* penicillinase enzyme is a class A serine hydrolase. In recent years, two penicillinase-inducing strains (NCTC 749 and 6346), referred to as *B. subtilis*, have been subjected to enzymological studies [6-8] in relation to penicillinase, its secretion and induction [6-9], and cognitive [10] and physiological immunity. In fact, all ten strains of *B. licheniformis* produce wild-type penicillinase and, based on their immunological and enzymatic properties, clearly fall into one or more distinct subtypes of strains 749 and 6346, which have previously been studied [11].

Examining the amino acid sequence of *B. licheniformis* penicillinase has revealed that exopenicillinase contains approximately 271 amino acids, with both the N and C termini being lysine. Membrane-bound penicillinase, on the other hand, contains 293 amino acids with the C-terminus also being lysine, similar to that observed in exopenicillinase.

Various studies have applied different amino acid sequencing methods to the N-terminal unit, but none have identified any specific amino acids in the region. Further investigation using protein staining after electrophoresis with oil red O or amid black as well as lipid-degrading enzymes have demonstrated that the N-terminal unit of membrane penicillinase is bound to exopenicillinase, contains 24 additional amino acids, is attached to a phospholipid at the end, and is embedded within the bacterial membrane via phospholipids. Membrane-bound penicillinase is an integral protein with a large polar hydrophilic portion and a small hydrophobic domain, located within the lipid bilayer membrane. Its amphiphilic properties as compared to the hydrophilic properties of exopenicillinase are mainly attributed to the presence of phosphatidylserine in the N-terminal. Attempts to obtain selective inhibitors for clinical applications have not been very successful, though some substrate analogs have been used as competitive and non-competitive inhibitors.

Attempts to obtain selective inhibitors for clinical applications have not been very successful. Some substrate analogs are used as competitive and non-competitive inhibitors. Compounds such as Hg (+2), certain alcohols, alkyl sulfates, rhodamine, alpha-heterosilicylates, boronic acid derivatives, acyl-phosphates, I_2, and certain antibodies inhibit the penicillinase enzyme to varying degrees. Some compounds such as penicillinase substrate analogs created by altering the side chain of beta-lactam agents to inhibit penicillinase include clavulanic acid and its derivatives, sulbactam and its sulfonate derivatives, gentamicin and its analogs, carbapenem and its derivatives, halopenicillinate and its analogues, halopenicillinate, isotonic anhydrides, and bronchic acid. Some of these compounds act as substrates for the penicillinase enzyme. Four chemical compounds citrate, diethyl-malate, malic anhydride, and malic acid can also act as penicillinase inhibitors due to their chemical similarity to compounds used to inhibit penicillinase [11]. Generally, the inhibitory effects of chemical compounds on ecological efficiency are complex, and different industries show significant differences in different life cycle stages. This study used MIC and MBC values to determine the inhibitory effects of the four chemical compounds on *B. licheniformis* and eco-efficiency then determined the inhibitory effects of the four compounds in the presence of penicillin G. on *B. licheniformis* according to protocol.

Materials and Methods

Determining MIC and MBC of Antibiotic against *Bacillus licheniformis* on Eco-Efficiency with and without Inhibitor

The optimum growth conditions (30°C, a Shaker incubator at 200 RPM [Bionexus], and pH 6.5) required for the production of penicillinase enzyme by bacterial strain *B. licheniformis* PTCC 1320 (NCIB6346) were provided according to our former study [12-16]. Then the revived ampoule of *B. licheniformis* PTCC 1320 (NCIB6346) bacterial strain was prepared

and maintained as stock after preparation [17, 18]. Therefore, the stock culture of the bacterial strain in the tryptic soy broth (TSB) liquid culture medium was adjusted to 0.5 McFarland turbidity standard (4 h culture) [19, 20]. Mueller-Hinton broth (MHB) was used to adjust the turbidity. A dilution of 1:200 was then prepared from the resulting solution (by adding 0.1 mL of the resulting suspension to 19.9 mL of Mueller-Hinton broth) [21-23]. The prepared solution contained 105-106 microorganisms/mL and should be used up to 30 min after preparation. For MIC determination, the TSB medium was prepared (30 g/L) and added to serial tubes (1 mL per tube) before being autoclaved [24, 25]. The desired antibiotic was added to the tubes after cooling under sterile conditions. Finally, the prepared bacterial suspension was added to the tubes (1 mL per tube). One tube containing only TSB culture medium (2 mL) and one tube containing TSB culture medium plus antibiotic (2 mL) were considered as culture medium and antibiotic controls, respectively [26-28]. To add each inhibitor, the culture medium (0.5 mL) with a double concentration (60 g/L) was mixed with the desired inhibitor at a concentration of 8 mg/mL (0.5 mL) in the presence of the desired antibiotic, so that the concentration of the medium was again 30 g/L. Due to the acidic nature of some inhibitors, especially malic acid, a stock solution with a concentration of 8 mg/mL was prepared before adding them to the TSB medium, and the pH was adjusted to 7-7.2 using 1 normal sodium hydroxide (NaOH).

After determining the MIC values of the antibiotic in the presence of inhibitors, 100 µL of all the tubes that showed no bacterial growth were seeded on Mueller-Hinton agar (MHA) plates and incubated at 37ºC for 24 h. When 99.9% of the bacterial population is killed at the lowest concentration of an antimicrobial agent, it is called the MBC endpoint. The control contained only inoculated broth, which was also incubated at 37ºC for 24 h.

The experiments were repeated three times to ensure the accuracy of the results obtained for the MIC and MBC of benzylpenicillin in the presence of each inhibitory compound.

Molecular Docking

Using modeling, the correlation between the practical data related to the inhibition percentage, and the data obtained from molecular docking could be evaluated. The high correlation between the practical and theoretical data proved the validity of these experiments. Penicillinase protein with PDB (Protein Data Bank) code 350I was downloaded from the Protein Data Bank (www.rcsb.org). Polar hydrogens were added to this protein, and SYBYL software was used to optimize the initial structure [29-31]. The resulting structure was used for molecular modeling and docking studies. The structures of four compounds, namely citrate, diethyl maleate, malic anhydride, and malic acid, were drawn by Hyper Chem software and then minimized using the semi-empirical AM1 method (Austin Model 1). Using Molsoft software, each inhibitor in combination with penicillinase protein (PDB code 350I) was considered separately, and the inhibitor affinity with the protein was obtained in kcal/mol [32-34].

Then for each compound, the conformation with the lowest ΔG was considered as the dominant conformation. This experiment was repeated four times for each inhibitor, and the obtained data were analyzed using SPSS software Version 20 (SPSS Inc., Chicago, Ill., USA) to find statistically significant data.

Results and Discussion

Results of MIC and MBC Determination

The MIC and MBC values of benzylpenicillin alone (as detailed in Table 1) and in combination with other inhibitors (citrate, diethyl maleate, malic anhydride**,** and malic acid) against *B. licheniformis* are summarized in Table 2.

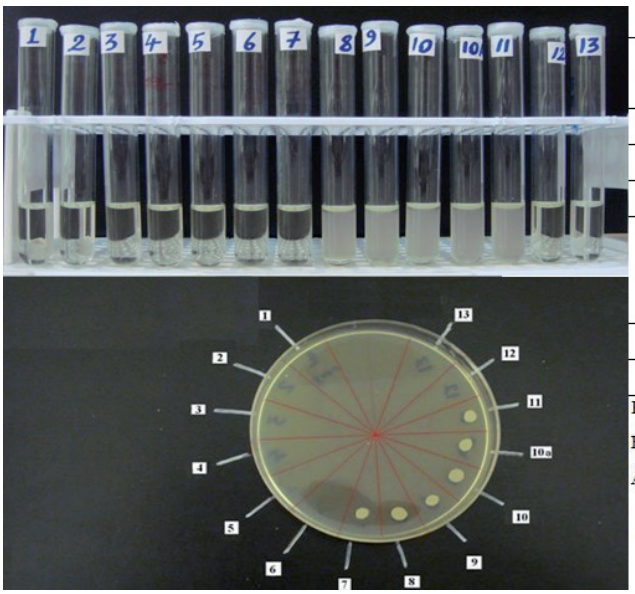
The MIC and MBC values of benzylpenicillin against *B. licheniformis* were determined to be 2 µg/mL and 4 µg/mL, respectively. These two factors were then evaluated in the presence of benzylpenicillin and citrate. The observations showed that turbidity started from tube No. 8, and the tested bacillus was able to grow from tube No. 8 onwards (Fig. 1). Therefore, the MIC of benzylpenicillin in the presence of citrate was 0.125 µg/mL; similarly, its MBC value was 0.25 µg/mL according to the culture tubes on the MHA medium. Also, due to the onset of turbidity and bacterial growth on the MHA culture medium from tube No. 7, the MIC

Table 1. MIC and MBC of *B. Licheniformis* in the presence of Benzylpenicillin (Pen.G).

Tube	µg/ml	MIC	MBC
1	8	-	-
2	4	-	-
3	2	-	+
4	1	+	+
5	0.5	+	
6	0.25	+	
7	0.125	+	
8	0.0625	+	
9	0.0312	+	
10	0.0156	+	
Inoculum control		+	
Broth control		-	
Antibiotic control		-	

Table 2. MIC and MBC values (µg/mL) of different inhibitors against *B. licheniformis.*

	Benzylpenicillin [µg/ml]	Benzylpenicillin and citrate [µg/ml]	Benzylpenicillin and diethyl maleate [µg/ml]	Benzylpenicillin and malic anhydride [µg/ml]	Benzylpenicillin and malic acid [µg/ml]
MIC	2	0.125	0.25	0.5	1
MBC	4	0.25	0.25	0.5	2



Tube	µg/ml	MIC	MBC
1	8	-	-
2	4	-	-
3	2	-	-
4	1	-	-
5	0.5	-	-
6	0.25	-	-
7	0.125	-	+
8	0.0625	+	+
9	0.0312	+	+
10	0.0156	+	+
Inoculum control		+	+
Broth control		-	-
Antibiotic control		-	-

Fig. 1. The turbidity of the test tubes in the presence of the antibiotic benzylpenicillin and citrate inhibitor started from tube number 8 and the tested bacillus from tube 8 was able to grow. Consequently, the MIC of benzylpenicillin in the presence of citrate reached 0.125 µg/ml, and similarly, its MBC level was obtained according to the culture of the tubes on the agar culture medium (MHA) at 0.25 µg/ml.

and MBC values of benzylpenicillin in the presence of diethyl malate were both 0.25 µg/mL. In the presence of malic anhydride, the MIC and MBC values of benzylpenicillin were both 0.5 µg/mL, and turbidity started from tube No. 5. In determining the MIC and MBC values of benzylpenicillin in the presence of malic acid, turbidity was started from tube No. 5. Thus, the MIC was determined in tube No. 4 where no turbidity was visible with 1 µg/mL antibiotic concentration. Also, bacterial growth in tube No. 3 was completely inhibited with a 2 µg/mL antibiotic concentration. Therefore, this concentration was considered the MBC value. According to the results, citrate was found to be the most effective compound in inhibiting the growth of bacteria, followed by diethyl malate, malic anhydride, and malic acid, respectively, in terms of inhibiting the growth of the desired bacterium. Statistical analysis performed using ANOVA and Tukey's post hoc test to determine MBC values among different concentrations tested against *B. licheniformis* showed significant growth inhibition for concentrations of 0.625, 1.25, 2.5, and 5 mg/mL compared to 0.156 and 0.312 mg/mL, and the MIC was determined to be 0.625 mg/mL (complementary file).

Modeling

Fig. 2, 3, and 4 show the molecular interaction of citrate as the most active inhibitor, and this inhibitor showed the highest inhibitory power on the penicillinase enzyme based on the practical data (MIC and MBC). Fig. 2 shows the three-dimensional (3D) structure of the penicillinase enzyme in the presence of citrate as an inhibitor.

Fig. 3 shows the active site of the penicillinase enzyme in the presence of citrate, with nitrogen atoms in bold blue, carbon atoms in gray, hydrogen atoms in white, and oxygen atoms in red. The image displays the electron cloud density for each atom. The carboxyl and hydroxyl groups in citrate can react with the amino acids in this active site, thus inhibiting the enzyme.

Fig. 4 shows the interaction of citrate with the amino acids of the penicillinase active site from another angle. Lysine 73 has a distance of 2.93 Å (through the nitrogen of its side chain) with the citrate hydroxyl group, which seems suitable for hydrogen bond formation. The side chain oxygen of serine 70 could also interact with the citrate carboxyl group. Furthermore, asparagine 170 could form hydrogen bonds at a distance of 3.24 Å with the citrate carboxyl group.

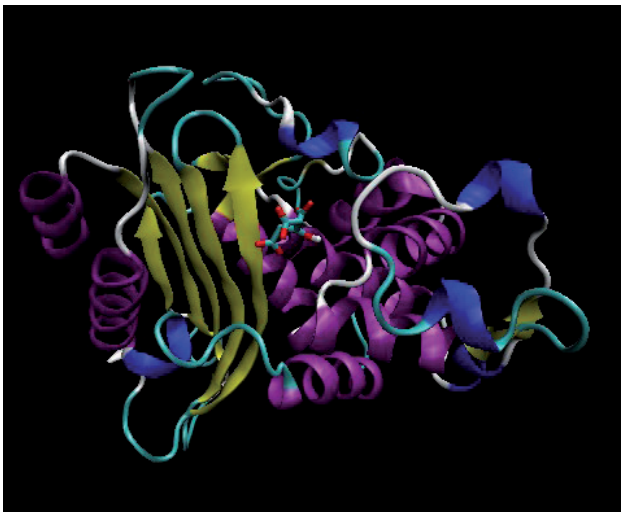


Fig. 2. 3D structure of penicillinase in the presence of citrate.

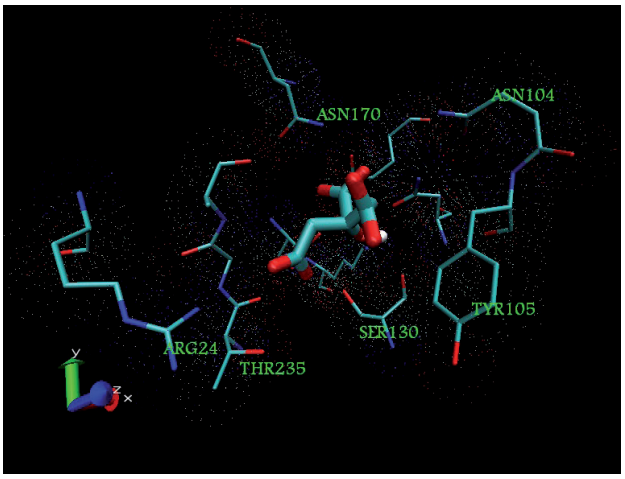


Fig. 3. The active site of penicillinase in the presence of citrate.

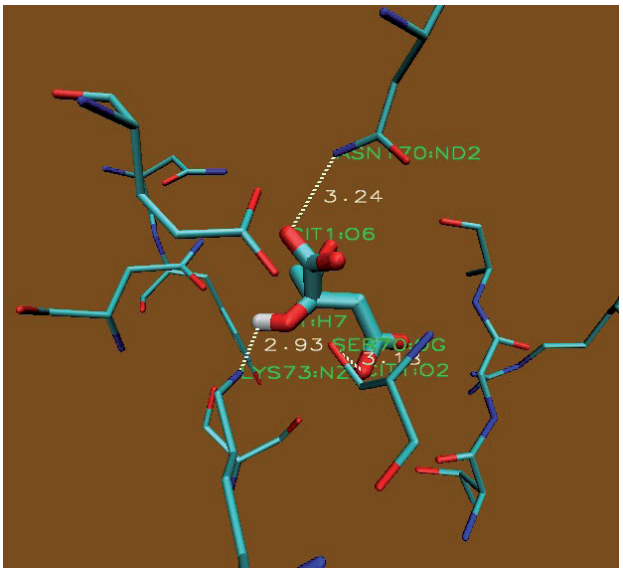


Fig. 4. Interaction of citrate with penicillinase active site amino acids.

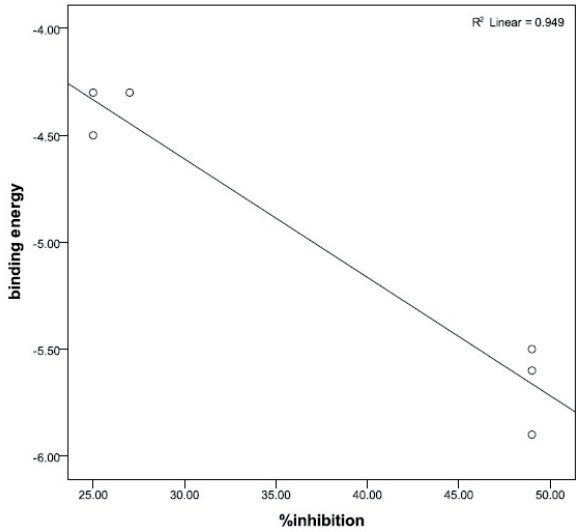


Fig. 5. Degree of correlation between inhibition percentage and molecular docking data.

Fig. 5 shows the degree of correlation between the experimental data related to the inhibition percentage and the data obtained from molecular docking. The R^2 or correlation coefficient between these two variables was 0.94, indicating that there is more than 94% correlation between the practical and theoretical data, thus proving the validity of molecular modeling and docking studies.

In this investigation, the values of MIC and MBC of Bacillus licheniformis for the benzylpenicillin antibiotic were determined using a TSB medium. The next step was to evaluate the effect of four compounds: citrate, diethyl malate, malic anhydride, and malic acid, as penicillinase inhibitors in the presence of penicillin G. The MIC and MBC values for *B. licheniformis* in benzylpenicillin were obtained at 2 µg/ml and 4 µg/ml, respectively. Subsequently, these two factors were evaluated in the presence of benzylpenicillin and citrate. The MIC of benzylpenicillin in the presence of citrate reached 0.125 µg/ml, and similarly, its MBC value was 0.25 µg/ml according to the culture tubes on agar medium (MHA).

Bacillus licheniformis is known to cause septicemia, eye infections, gastrointestinal inflammation, blood poisoning, and food poisoning [37-40].

The penicillinase enzyme in *Bacillus licheniformis* is a class A serine hydrolase. This medium-sized monomer protein has a secondary structure consisting of 11 α-helixes and 5 anti-parallel β-sheets, making up 45% and 25% of the enzyme structure, respectively. The β-sheets form a flat anti-parallel plate surrounded in the anterior part by the N-terminal α1 and the C-terminal α11 helixes. To the left and close to the β-sheets are nine remaining helixes and a single helix at position 90 [13].

The 3D structure of penicillinase shows that several sequences are conserved among most penicillinases. These conserved sequences include a Ser-x-x-Lys tetrad,

a Ser-x-Lys triad, a D/E peptide fragment containing two carboxylic amino acids, and a Lys-Thr-Ser triad. The SxxK sequence is located at the N-terminal of the α2 helix and occupies the central position of the cavity: the SxK sequence connects the helices α4 and α5, while one side of the central cavity forms the ligand binding site. Additionally, the KTS sequence is located at the cavity entrance [13, 14]. Some amino acids such as Ser 70, Lys 73, Ser 130, Glu 166, Leu 169, and Lys 234 are involved in the substrate binding site, which is in the β3-sheet, and the active site is in the α2 helix. SxxK is the conserved sequence of the active site [13, 14]

According to a study by Simons in 1978, the membrane-bound penicillinase enzyme is not merely a surface membrane protein but rather is both a surface and integral protein covalently attached to the cytoplasmic membrane. Therefore, to determine the percentage of penicillinase enzyme active sites and to achieve a higher percentage of the membrane-attached enzyme, Triton X-100 was first used to separate the enzyme from the cytoplasmic membrane; however, only small amounts of this enzyme were obtained. Lysozyme (100 mg/mL) and mild sonication for 30 seconds were then used to disrupt the membrane, and then Triton X-100 was used to release the remaining penicillinase enzyme, which showed better results than the methods used in other studies. This demonstrates the importance of Triton X-100 in both the release of the remaining enzyme and in the stability of the enzyme [15, 16]. After cell disruption, the suspension was centrifuged at 10,000 RPM for 1 hour at 4°C. In the supernatant, the enzyme activity in the presence of benzylpenicillin was measured by spectrophotometry, and then the decrease in the enzyme activity in the presence of inhibitory compounds was investigated.

The molecular docking results showed that the active site of penicillinase had an anionic cavity where amino acid anions were present, stabilizing the intermediates formed in this site by inducing electrostatic interactions. Based on the data obtained from molecular modeling and their comparison with those of practical methods, citrate could competitively attach to this site. Thr 235, Arg 24, Ser 70, Ser 130, Tyr 135, Asn 170, and Asn 104 amino acids were present in this site (Fig. 3).

Fig. 4 shows the interaction of citrate with the amino acids of the penicillinase active site from another angle. Lysine 73 had a distance of 2.93 Å with the citrate hydroxyl group through the nitrogen of its side chain, which seemed suitable for forming hydrogen bonds. The side chain serine 70 oxygen could also interact with the citrate carboxyl group. Asparagine 170 could also form hydrogen bonds at a distance of 3.24 Å with the citrate carboxyl group. Thus, this inhibitor could inhibit the penicillinase enzyme by inducing these interactions [34-36]. Three other compounds considered as inhibitors in this research (diethyl malate, malic anhydride, and malic acid) exhibit the same binding pattern as citrate. However, they are smaller in size and have fewer functional groups. Consequently, they are less active and have fewer and weaker interactions compared to citrate. This reduction in interaction diminishes their inhibitory power [37-40].

Conclusions

In this research, we assayed the inhibitory effects of four chemical compounds on the activity of *B. licheniformis* PTCC 1320 (NCIB6346) penicillinase enzyme, which positively affects the improvement of ecological efficiency level. The MIC and MBC values of penicillin G antibiotic against *B. licheniformis* were determined with and without penicillinase inhibitors (diethyl malate, malic anhydride, malic acid, and citrate). Then, the results were confirmed by molecular modeling data. In the presence of citrate, the MIC and MBC values of benzylpenicillin reached 0.125 and 0.25 μg/mL, respectively, which had the highest reduction of MIC and MBC compared to other inhibitors. The active site of the penicillinase enzyme contains an anion cavity that holds amino acid anions, which helps stabilize the intermediates formed at this site through electrostatic interactions. Data obtained from molecular modeling, as well as comparisons with practical methods such as determining MIC and MBC, suggest that the citrate inhibitor can competitively bind to this position and reduce the activity of the penicillinase enzyme. The R^2 or correlation coefficient between these two variables was 0.94, thus proving the validity of molecular modeling and docking studies. Therefore, citrate is the best inhibitory compound for *B. licheniformis* penicillinase and positively affects ecological efficiency. Relevant government departments need to improve resource allocation efficiency for science and technology and accurately allocate resources to the chemical compound and green development. They should also strengthen the construction of environmental protection facilities in chemical compound local areas, reduce resource consumption intensity in green technology processes, standardize pollution emission treatment, reduce environmental burden, and accelerate the transformation of ecological efficiency externalities of green technology.

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Conflict of Interest

The authors declare no conflicts of interest.

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