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Characterization of intracellular β-galactosidase from *Bacillus subtilis* 4NK and *Bacillus paralicheniformis* 5NK isolated from a hot water spring and effects of various inhibitors on enzyme activity

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Abstract

In this study, the intracellular β-galactosidases of *Bacillus subtilis* 4NK and *Bacillus paralicheniformis* 5NK isolated from Bingöl Binkap hot spring was partially purified and characterized. As a result of purification, the yield of the enzyme for *B. subtilis* 4NK was 85.2% and the purification fold was 2.8. The yield for *B. paralicheniformis* 5NK was 76.8% and the purification fold was 2.0. The optimum temperature of the enzyme was determined as 45 °C for *B. subtilis* 4NK and 55 °C for *B. paralicheniformis* 5NK and the optimum pH was 6.0 for both. In addition, in the thermal stability experiments even at the end of 120 min both enzymes were stable at 50 °C. It was determined that the partially purified enzyme activity increased in the presence of iodoacetamide and phenylmethylsulfonylfluoride for *B. subtilis* 4NK, dithiothreitol, N-ethylenemaleimide and phenylmethylsulfonylfluoride for *B. paralicheniformis* 5NK. The metals were found to activate the enzyme at low concentrations of Co^{2+}, Cd^{2+} and Mn^{2+} for *B. subtilis* 4NK, Cu^{2+} and Cd^{2+} were found to inhibit the enzyme at high rates for *B. paralicheniformis* 5NK. K_m and V_{max} values for 4NK and 5NK, respectively; 23.80 mM, 1.978 µmol/min and 5.61 mM, 1.869 µmol/min.

Introduction

Thanks to the developing industrial enzyme technology and the development of fermentation methods, the production potential is increased in the production of enzymes of microbial origin, regardless of environmental conditions (Topal, 1998). Enzymes used in many fields in industry are generally obtained from microorganisms. Because microorganism-derived enzymes have higher catalytic activities compared to other sources, they do not form undesirable by-products (Kıran et al., 2006). Moreover, they can be produced through fermentation techniques in a cost-effective manner with less time and space requirement because of their high consistency, as well as easy process modification and optimization (Grung et al., 2013). In recent years, many studies have focused on thermophilic enzymes of microbial origin. Since thermostable enzymes purified from thermophilic ones maintain their stability even at temperatures exceeding the temperature at which the microorganism grows, these enzymes are more preferred (D'Auria et al., 1999). There are many advantages of thermophiles related to industrial use due to high growth rates accelerating processes of fermentation two to three times compared to those with mesophilic producers, less unwanted microbial contamination, as well as higher diffusion rate and mass turnover. Enzymes from thermophiles are well known to be more resistant to proteolysis and chemical denaturation. Thus, the process of enzyme "aging" is slowed down because of the stability of these molecules, which is preferred in commercial preparations allowing their storage at room temperature with a longer half-life (Kambourova, 2018). *B. paralicheniformis* is used in the biotechnology

industry to produce enzymes, antibiotics, biochemical and consumer products (Rey et al., 2004; Dunlap et al., 2015; Du et al., 2019).

β-galactosidase has been obtained from different microorganisms in the studies conducted so far. β-galactosidase (β-D-galactohydrolases, EC 3.2.1.23) enzymatically hydrolyses the β-glycosidic bond in lactose and produces glucose and galactose, which are sweeter than lactose and have higher solubility (Panesar, 2006). β-galactosidases are used for a variety of applications, including the production of dairy products, low-lactose milk and probiotics, biosynthesis of different transgalactose products, improvement of lactose tolerance, and various analytical approaches (Loveland et al., 1994; Neri et al., 2008) and they are also used in the production of other industrially important products such as ethanol and biosensors (Saqib et al., 2017).

As it is known, β-galactosidase is used in biotechnologically beneficial and important areas in the production of prebiotic food, in the processes of wastewater that occurs in the developing milk and dairy industry, production studies and post-production, and in the process of eliminating the problems caused by the milk products consumed by humans. In this study, isolation, partial purification and characterization of β-galactosidase from *B. subtilis* 4NK and *B. paralicheniformis* 5NK bacteria were aimed.

Materials and Methods

Biological Materials and Chemicals

In this study, *B. subtilis* 4NK and *B. paralicheniformis* 5NK were isolated and identified from Binkap hot springs in Ilısu district of Bingöl province (Aslan, 2018). Nutrient Broth (NB) and Agar were obtained from Merck Darmstatd, *o*-nitrophenyl-beta-D-galactoside (*o*NPG), dithiothreitol (DTT), N-ethylenemaleimide (NEM), iodoacetamide (IAA), phenylmethylsulfonylfluoride (PMSF) and *p*-chloromercuribenzoate (PCMB) were obtained from Sigma, ethylenediamintetraeceticacide (EDTA), $CaCl_2$, $CuCl_2$, $MnCl_2$, $CoCl_2$, $NiCl_2$ and $CdCl_2$ were obtained from Merck Darmstatd, $MgCl_2$ was obtained from Kimetsan and $ZnCl_2$ from LACHEMA.

Cultivation of bacteria

B. subtilis 4NK and *B. paralicheniformis* 5NK bacteria were prepared using 1 mL inoculum each and incubated into 100 mL flasks with 25 mL NB, and incubated at 120 rpm at the optimum temperature for bacteria growth, using a shaker. The liquid culture was centrifuged at 10.000 rpm for 10 min and the pellet was sonicated by ultrasonication. The samples were centrifuged again under the same centrifuge conditions and the supernatant was used for β-galactosidase activity determination.

Determination of β-galactosidase activity

Enzyme activity was measured spectrophotometrically using *o*NPG determination of β-galactosidase activity. 60 mM *o*NPG was prepared by dissolving in 0.1 M Tris-HCl (pH 7.0) buffer in 10 mL and used as substrate. Enzyme source was obtained as a result of sonication of bacteria and added to the substrate. The mixture was incubated at 45 °C and 55 °C for 10 min. After the incubation, 1 M Na_2CO_3 was added in order to stop the reaction and measurement was made at 420 nm by spectrophotometer. Protein amount was determined using the Folin Reaction (Lowry et al., 1951). Unit enzyme is defined as the amount of enzyme that causes the release of *o*-nitrophenol in 1 min from 1 µM of *o*NPG substrate under certain conditions. The results are represented as the mean ± SD of at least 3 experiments.

Time and lactose dependent enzyme production

One mL of bacteria was inoculated separately into 250 mL of NB with 1% lactose and lactose-free NB (pH 7.0), and incubated for 48 hours and the bacteria reproduced at 6, 12, 24, 36, 48 hours. The culture was measured in the spectrophotometer at 600 nm. In addition, samples were taken from cultures centrifuged at 10.000 rpm for 10 min. After being centrifuged and sonicated, it was centrifuged again under the same centrifuge conditions and the supernatant was used for β-galactosidase activity determination and protein quantification.

Partial purification of β-galactosidase

Separate cultivation of bacteria was made on NB medium and incubated in shaker at 45 °C at 120 rpm for 24 hours. After incubation, samples were centrifuged at 10.000 rpm for 10 min. After the sonication process, the upper liquids were removed and used as crude extract. The ammonium sulphate was gradually added (70% for *B. subtilis* 4NK and 80% for *B. paralicheniformis* 5NK) into crude extracts and precipitation was performed in cold environment. The samples obtained after precipitation were centrifuged at 10.000 rpm for 25 min. The pellets were dissolved in 4 mL of 0.1 M Tris-HCl buffer (pH 7.0). Samples were dialyzed in 0.1 M Tris-HCl (pH 7.0) buffer at +4 °C overnight (approximately 18 hours) to remove ammonium sulphate from it using dialysis tubing. The final volume of each sample was calculated after dialysis. In order to concentrate the proteins in the solution more, the concentration process was carried out with the help of nitrogen gas using a stirred ultrafiltration system. The volume at the end of ultrafiltration was measured and transferred to a 1 mL sterile microcentrifuge tube and left at -20 °C. The specific activity, yield and purification fold of the enzyme were calculated by measuring the β-galactosidase activity and the protein amount of the samples was obtained from the crude extract and after dialysis.

Determination of optimum temperature and thermal stability

In order to determine the effect of temperature on the β-galactosidase activity, the partially purified enzyme activity was measured by incubating at different temperatures (25-65 °C). In order to determine the thermal stability of β-galactosidase activity, the enzyme activity was then measured in the temperature range of 45-60 °C for 10-120 min. For all these experiments, 50 u/mg of enzyme was used to investigate temperature effects and stability. The remaining activities after temperature exposures were calculated from the control (unheated enzyme) taking the activity as 100%.

Determination of optimum pH

To determine the effect of pH on the partially purified β-galactosidase activity, enzyme activities were determined using the prepared substrates in different buffers. The buffers were 0.1 M citric acid (pH 4.0-6.0), 0.1 M Tris-HCl (pH 7.0-8.0) and 0.1 M glycine-NaOH (pH 9.0- 11.0).

Effect of some chemicals and metals on enzyme activity

To determine the effect of some chemicals (DTT, PMSF, IAA, NEM, PCMB and EDTA) and metals (Mg^{2+}, Zn^{2+}, Ca^{2+}, Mn^{2+}, Co^{2+}, Ni^{2+}, Cd^{2+} and Cu^{2+}) on the activity of enzymes, the partially purified enzyme was prepared separately with chemicals (NEM and PMSF dissolved in ethanol) and metals prepared in 0.1 M citric acid pH 6.0 buffer at different concentrations (1-10 mM for chemicals, 1-20 mM for metals and EDTA). It was left to pre-incubate for 15 min. Then, the enzyme activity was measured under optimum conditions determined for each enzyme by adding substrate. Samples with no added chemicals or metals were used as controls. Divalent metals were used in chloride form. For all these experiments, 50 u/mg of enzyme was used to investigate inhibition effects of chemicals tested. The remaining activities after chemical exposures were calculated from the control (untreated enzyme with any chemicals) taking the activity as 100 %.

Enzyme kinetics

In order to determine the substrate specificity, the partially purified enzyme was incubated at the optimum pH and temperature using *o*NPG (at concentrations of 1-10 mM) as substrate and the β-galactosidase enzyme activity was measured. *Km* and *Vmax* values of *o*NPG were calculated using Linewear-Burk equation.

Results and Discussion

In recent years, studies have focused on the identification of bacteria isolated from extreme conditions such as hot springs and their important products (Poli et al., 2006). Organisms have developed cellular cell membranes and cellular metabolites (such as enzymes and proteins) and some functional adaptations in order to live and reproduce in extreme conditions (Haki & Rakshit, 2003; Reed et al., 2013). The most important feature of thermophilic bacteria isolated from hot water springs is that they have enzymes that are resistant to extreme conditions, which makes them biotechnologically important (Demirjian et al., 2001). In this study, it was determined that *B. subtilis* 4NK and *B. paralicheniformis* 5NK bacteria isolated and identified from Binkap hot water spring in Bingöl province have a biotechnologically important β-galactosidase production potential. In addition, this study is the first regarding β-galactosidase studies on *B. paralicheniformis*.

Time and lactose dependent enzyme production

Time-course experiments between 6-48 hours were carried out to investigate lactose effects on enzyme production, in comparison to lactose-free controls. The highest enzyme activity of *B. subtilis* 4NK was obtained at 24 hours in lactose and lactose-free medium, and at 36 hours (22.62 U/mg) in lactose medium (Figure 1).

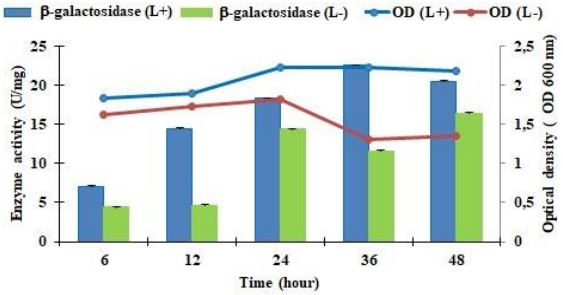

Figure 1. Time and lactose dependent enzyme production for *B. subtilis* 4NK (using NB with 1% lactose and lactose-free NB (pH 7.0), incubated at 40 °C for 48 h).

For *B. paralicheniformis* 5NK, the highest bacterial growth was obtained at 12th hour in lactose and lactose-free medium within 6-48 hours and the best enzyme activity was obtained at 48th hour (91.17 U/mg) in lactose-free medium (Figure 2).

For both bacteria, the enzyme production increased due to the increased lactose concentrations, indicating that their synthesis is constitutive (essential enzyme). Hirata et al. (1985), in their study, showed that with *B. subtilis* the synthesis of the enzyme was increased by inducing it in the presence of lactose and that the enzyme was synthesized constitutively.

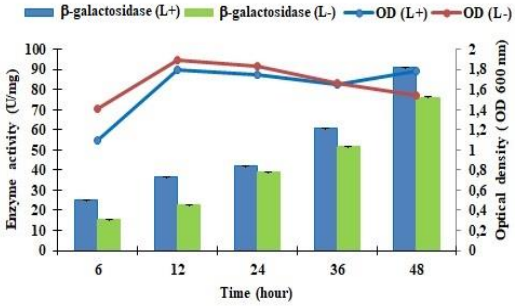

Figure 2. Time and lactose dependent enzyme production for *B. paralicheniformis* 5NK (using NB with 1% lactose and lactose-free NB (pH 7.0), incubated at 45 °C for 48 h).

Partial purification of β-galactosidase

The enzyme was partially purified by ammonium sulphate precipitation and dialysis. As a result of this purification, the purification fold was 2.8 and the yield was 85% for *B. subtilis* 4NK, the purification fold was 2 and the yield was 76.8% for *B. paralicheniformis* 5NK (Table 1). Literature data reveal some strategies for purifying β-galactosidases from microorganisms. Isobe et al. (2013a, b) purified β-galactosidase from *Teratosphaeria acidotherma* reaching a purification factor and yield of 375 and 2.9%, respectively. Martarello et al. (2019) purified β-galactosidase from fungi reaching a purification factor of 8.665 and a yield of 17.33%.

Optimum temperature and thermal stability

As seen in Figure 3, the effect of the temperature between 25-65 °C on the enzyme activity was investigated and β-galactosidase was active between 35-55 °C and the optimum temperature was 45 °C for *B. subtilis* 4NK. It was determined that the enzyme was active between 50-60 °C and the optimum temperature was 55 °C for *B. paralicheniformis* 5NK. With this feature, the enzyme shows that it can be used in processes such as the hydrolysis of lactose and whey processes, especially in the dairy industry, and it will also provide an advantage in reducing the risk of microbial contamination in processes that require high temperatures in these industries. In previous studies related to β-galactosidase obtained from various microorganisms, maximum β-galactosidase activity was obtained from *Streptococcus thermophilus* (Somkuti & Steinberg, 1979), *Lactobacillus kefiranofaciens* K-12

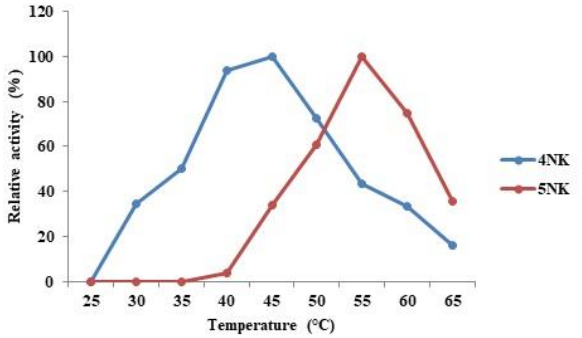

Figure 3. Effect of temperature on β-galactosidase (Optimum temperature was determined under standard assay conditions using *o*NPG at temperatures ranging from 25 to 65 °C).

(Itoh et al., 1993), *B. subtilis* (Torres and Lee, 1995), *B. circulans* (Vetere & Paoletti, 1998), *Bacillus* sp. MTCC 3088 (Chakraborti et al., 2000), *B. coagulans* RCS3 (Batra et al., 2002), *A. acidocaldarius* (Di Laura et al., 2008), *Anoxybacillus* sp. KP1 (Matpan Bekler et al., 2017) and *Anoxybacillus* sp. FMB1 (Yalaz et al., 2019) at 55 °C, 50 °C, 50 °C, 44 °C, 60 °C, 65 °C, 60 °C, respectively.

In Figure 4, the thermal stability of β-galactosidase enzyme activity was examined at 40-50 °C in the range of 10-120 min for *B. subtilis* 4NK. The enzyme was stable at 45 °C for 30 min. for *B. subtilis* 4NK. It was determined that the enzyme preserved its activity at a rate of 99% up to 100% for 120 min, and maintained its activity at a rate of 87% up to 120 min at 40 and 50 °C.

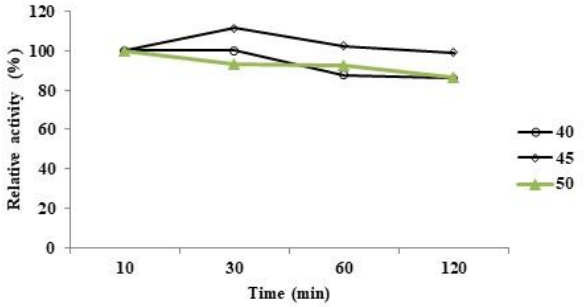

Figure 4. Thermal stability of the 4NK β-galactosidase (The enzyme was incubated at 40-50 °C for different time-course experiments (10-120 min). The unheated purified enzyme was taken as 100%. The remaining β-galactosidase activity was measured under standard assay conditions).

In Figure 5, the thermal stability of β-galactosidase enzyme activity was examined at 45-60 °C in the range of 10-120 min for *B. paralicheniformis* 5NK and the thermal stability of the enzyme was observed at 45-50 °C for 30 minutes. 65% residual enzyme activity was maintained after 120 min at 55 °C. However, enzyme activity was very low at 60 °C Chakraborti et al. (2003), in their study on the β-galactosidase enzyme of thermophilic *Bacillus polymxia*, found that the enzyme preserved its thermal stability at 50 °C, but could not maintain thermal stability at higher temperatures. Also, Levin & Mahoney (1981), as a result of their research on the β-galactosidase enzyme of *Bacillus coagulans* L4, found that this enzyme lost 70% of its enzyme activity in the 30th min at 55 °C.

Table 1. Purification steps of β-Galactosidase

Bacteria	Purification steps	Total Protein (mg/ml)	Total Activity (U)	Specific activity (U/mg)	Purification fold	Yield (%)
***B. subtilis* 4NK**	Crude extract	73.3	5328.1	72.7	1	100
	Ammonium sulphate precipitation and dialysis	22.7	4540.1	200.1	2.8	85.2
***B. paralicheniformis* 5NK**	Crude extract	184.7	11399.9	61.7	1	100
	Ammonium sulphate precipitation and dialysis	22.7	8756.9	121.0	2.0	76.8

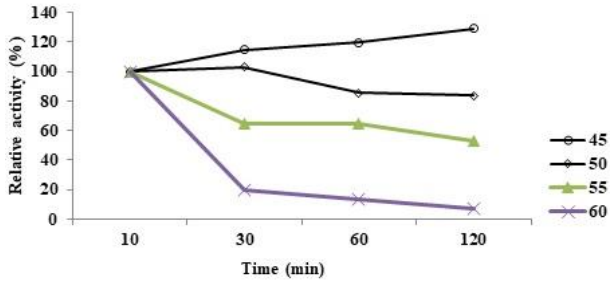

Figure 5. Thermal stability of *the* 5NK β-galactosidase (The enzyme was incubated at 45-60 °C for different time-course experiments (10-120 min). The unheated purified enzyme was taken as 100%. The remaining β-galactosidase activity was measured under standard assay conditions).

Determination of optimum pH

The effect of pH varying between 4.0-11.0 on β-galactosidase activity was investigated. As seen in Figure 6, the optimum pH value was found to be 6.0 for *B. subtilis* 4NK and *B. paralicheniformis* 5NK. Both enzymes from 4NK and 5NK strains were found to be stable at pH 6.0 up to 120 minutes at 45 and 50 °C (data not shown). The optimum pH value of β-galactosidase was found to be 6.0 for *B. subtilis* (Torres and Lee, 1995), *B. circulans* (Vetere & Paoletti, 1998), *B. licheniformis* (Phan Trân et al., 1998), *A. acidocaldarius* subsp. rittmannii (Gul Guven et al., 2007) and *B. licheniformis* KG9 (Matpan Bekler et al., 2015) and it was similar to our study. The pH result shows that its products can be used in milk and milk processes such as processing and lactose hydrolysis.

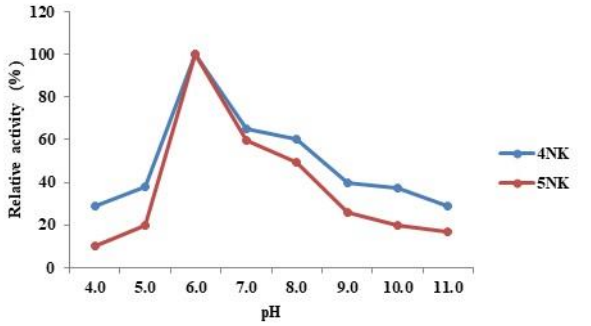

Figure 6. Effect of pH on β-galactosidase (Optimum pH was determined under standard assay conditions using *o*NPG (pH 4.0–10.0)).

Effect of some chemicals and metals on enzyme activity

As can be seen in Table 2, the effects of some chemicals on β-galactosidase activity were examined. It was determined that partially purified enzyme activity increased 13% with 1 mM IAA and 2% with 5 mM PMSF, and increasing concentrations of DTT, NEM and PCMB decreased the enzyme activity for *B. subtilis* 4NK. β-galactosidase activity increased 18% in the presence of 10 mM DTT, 3% with 1 mM NEM and 1% with 5 mM PMSF, and increasing concentrations of IAA, PMSF, NEM and PCMB caused high inhibition for *B. paralicheniformis* 5NK. It is known that the cysteine (Cys) residue is modified by PMSF and NEM and IAA is the SH group alkylating reagent. It can be said that the active center of the enzyme contains Cys and SH group for *B. subtilis* 4NK and *B. paralicheniformis* 5NK since increasing concentrations of PMSF, NEM and IAA inhibit the enzyme. PCMB is an inhibitor of the Cys amino acid and for both bacteria all concentrations of PCMB inhibited the enzyme activity. Considering this information, it is thought that PCMB blocks the sulfhydryl group in the enzyme's active site. In previous studies, Gul Guven et al. (2011), Matpan Bekler et al. (2018) and Shaikhan et al. (2020) stated that PCMB inhibit enzyme activity. Since DTT, which is a thiol marker, does not seriously affect the enzyme, it can be said that thiol groups are not determinant in enzyme activity. The study conducted with *Anoxybacillus* sp. FMB1 (Yalaz et al., 2019) showed similar results. It can be said that the Cys amino acid is present as the proton donor in the active center of the enzyme and it plays a role in breaking the glycosidic bond in the catalytic mechanism.

Table 2. Effect of some chemicals on enzyme activity (retained activity %)

Chemicals	Bacteria	1 mM	5 mM	10 mM
IAA	4NK	113	103	79
	5NK	73	25	14
DTT	4NK	89	72	68
	5NK	111	117	118
PMSF	4NK	88	102	78
	5NK	82	101	35
NEM	4NK	82	91	80
	5NK	103	64	61
PCMB	4NK	17	14	14
	5NK	9	8	7

As seen in Table 3, the activity of β-galactosidase enzyme was investigated on some metal and EDTA as a chelator. The enzyme activity increased in the presence of 1 mM Co^{2+}, 2.5 mM Cu^{2+} and Mn^{2+}, 5mM Mg^{2+}, Zn^{2+} and Cd^{2+}, 10 mM Ni^{2+} and 20 mM Ca^{2+} and EDTA while Zn^{2+}, Cu^{2+}, Mn^{2+} and Cd^{2+}inhibit the enzyme activity at 10 mM and 20 mM for *B. subtilis* 4NK. This table also shows that the enzyme activity increased in the presence of 1 mM Zn^{2+}, Co^{2+}, Ni^{2+} and EDTA, 2.5 mM Ca^{+2}, 5 mM Mg^{2+} and Mn^{2+} while increasing concentrations of Zn^{2+}, Cu^{2+} Ni^{2+} and Cd^{2+} inhibited the enzyme activity for *B. paralicheniformis* 5NK. It should also be taken into consideration that the divalent cations may bind to citrate used to adjust pH to the optimum, which in turn may change the metal sorption distribution coefficient (Kd) values reducing the concentrations of free ions (Lacal et al., 2010).

Generally, β-galactosidases can be activated or inhibited by metal ions or other reagents (Shaikh et al., 1999). In previous studies, Ladero et al. (2002), Shipkowski & Brenchley (2006) and Gul Guven et al. (2011) stated that Cu^{2+} inhibit enzyme activity. It is known that enzyme activity increases with Ca^{2+} and Mg^{2+} in most β-galactosidases (Berger et al., 1997; Ohtsu et al., 1998; Lu et al., 2007; Shaikhan et al., 2020). These

Table 3. Effect of Some Metals and EDTA on Enzyme Activity (retained activity %)

Chemicals	Bacteria	1 mM	2.5 mM	5 mM	10 mM	20 mM
Mg^{+2}	4NK	113	115	135	113	-
	5NK	122	119	136	129	112
Ca^{+2}	4NK	119	116	142	148	149
	5NK	119	126	104	103	-
Cu^{+2}	4NK	143	171	141	116	24
	5NK	15	14	13	15	-
Zn^{+2}	4NK	112	117	124	63	34
	5NK	128	106	110	92	60
Mn^{+2}	4NK	189	193	152	94	49
	5NK	150	160	165	162	123
Co^{+2}	4NK	156	151	154	144	162
	5NK	132	127	128	121	22
Ni^{+2}	4NK	123	148	170	179	165
	5NK	129	114	108	50	-
Cd^{+2}	4NK	-	-	109	39	29
	5NK	94	58	18	18	-
EDTA	4NK	143	153	-	151	162
	5NK	145	124	124	120	-

results support our study. EDTA has been observed to activate the enzyme at all concentrations. Therefore, it can be said that this enzyme is not a metallo enzyme.

Enzyme kinetics

In Figure 7, the *Km* value of the enzyme dependent on *o*NPG concentration for *B. subtilis* 4NK was calculated as 23.80, *Vmax* values as 1.978 µmol/min, and the *Km* value for *B. paralicheniformis* 5NK as 5.61, and *Vmax* values as 1.869 µmol/min. Levin & Mahoney (1981) found the *Km* value for this enzyme as 4.2-5.6 mM as a result of the research on β-galactosidase enzyme of *Bacillus coagulans* L4. *Km* value for *o*NPG counts were 1.32 mM (Shaikh et al., 1999). O'Connell & Walsh (2008) stated that *Km* and *Vmax* values for *o*NPG were 2.23 and 0.56 mM. In addition, O'Connell & Walsh (2010) reported *Km* value was 1.74 mM and V_{max} was 137 UI/mL for *o*NPG.

Conclusion

In this study, β-galactosidase was isolated and characterized from *B. subtilis* 4NK and *B. paralicheniformis* 5NK obtained from Binkap hot water spring in Bingöl province. It was determined that the enzyme was produced in a short time with low cost and high efficiency, and also the pH and temperatures of enzymes were suitable for milk and dairy industries and that they showed stability. In addition, β-galactosidase is the first in enzyme studies regarding *B. paralicheniformis* with this study.

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Author Contributions

ŞT: Conceptualization, Writing-review and editing; FMB: Data Curation, Formal Analysis, Investigation, Methodology, Visualization and Writing-original draft; KG: Funding Acquisition, Project Administration, Resources, Writing-review and editing.

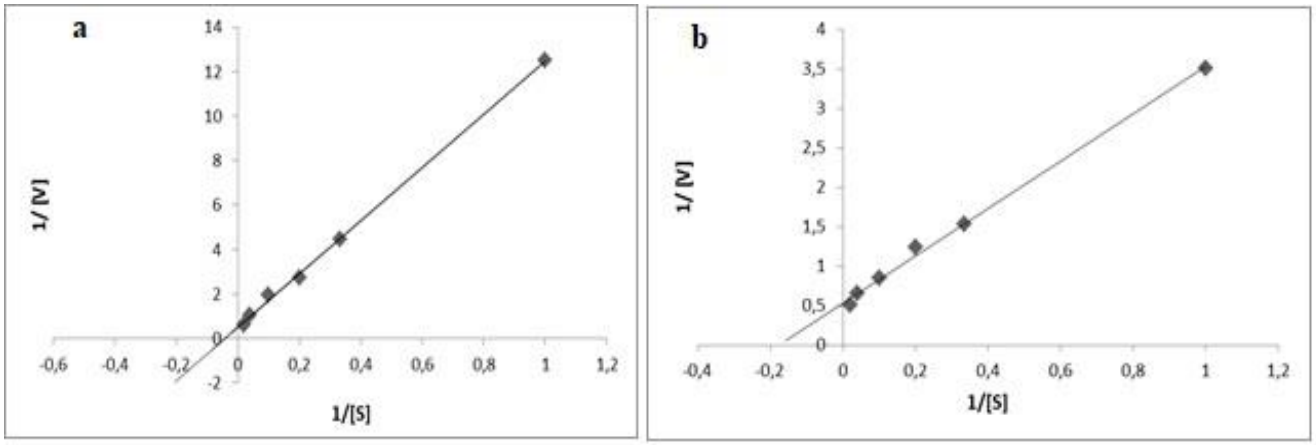

Figure 7. Linewear-Burk for **a)** *B. subtilis* 4NK and **b)** *B. paralicheniformis* 5NK.

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