Yeni İzole Edilen *Bacillus licheniformis* KG9'dan β-Galaktozidazın Üretimi, Kısmi Saflaştırılması ve Karakterizasyonu

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Özet

Batman'da (Taşlıdere, Türkiye) kaplıca sularından izole edilen, *Bacillus licheniformis* KG9 yeni bir ekstrasellüler β-galaktozidazın üretimi için büyük bir potansiyele sahiptir. Kültür koşulları ve ortam optimizasyonu NB (Nutrienth broth) β-galaktozidaz enziminin üretimi için gerekli olduğunu ortaya çıkarmıştır. Termostabil ekstrasellüler bir enzim olan β-galaktozidaz amonyum sülfat çöktürmesi ve diyaliz ile kısmen saflaştırılmıştır. Enzimin spesifik aktivitesi 1631 U/mg proteinden 19030.45 U/mg proteine yükseltilmiştir. Kısmi saflaştırılmış enzimin moleküler ağırlığının non-denatüre poliakrilamid jel elektroforezi ile 116 kDa'dan az olduğu tespit edilmiştir. Laktoz içeren ve içermeyen ortamlarda, çalkalama şişelerinde büyümenin daha sonraki aşamalarında (96 saat) enzim aktivitesinde önemli bir üretim seviyesi belirlenmiştir, bunlar sırasıyla (2.45 U/mg protein) ve (3.25 U/mg protein)'dir. Enzim için optimum pH ve sıcaklık 8.0 ile 55 °C'dir. Kullanılan çeşitli karbon ve azot kaynakları arasında, karbon kaynaklarının enzim üretimini arttırmadığı ancak laktozun araştırılan diğer karbon kaynaklarından daha yüksek aktivite gösterdiği belirlenmiştir oysa, azot kaynaklarından glisin ve amonyum sülfat enzim üretimini hafif bir şekilde yükseltmiştir.

Anahtar Kelimeler: *Bacillus licheniformis* KG9, β-galaktozidaz, Enzim üretimi ve karakterizasyonu

Abstract

Bacillus licheniformis KG9 isolated from the waters of the hot spring (Taşlıdere, Turkey) in Batman, has a great potential for the production of a novel extracellular β-galactosidase. Optimization of culture conditions and media revealed that NB (Nutrienth broth) is essential for the production of β-galactosidase. An extracellular thermostable β-galactosidase has been partially purified by ammonium sulphate precipitation and dialysis. The specific activity of the enzyme was increased from 1631 U/mg protein to 19030.45 U/mg protein. The molecular mass of the partially purified enzyme as determined by non-denaturing polyacrylamide gel electrophoresis was an approximately less than 116 kDa. In the lactose-absence medium and lactose-presence medium, production of a significant level of enzyme activity was determined (2.45 U/mg protein) and (3.25 U/mg protein) next stages of growth (96h) in a shake flasks, respectively. The pH and temperature optima for the enzyme are 8.0 and 55°C. Among the various carbon and nitrogen sources used, carbon sources didn't increase the enzyme production but lactose showed higher activity than the other investigated carbon sources, whereas the nitrogen sources glycine and ammonium sulphate slightly increased enzyme production.

Key words: *Bacillus licheniformis* KG9, β–Galactosidase, Enzyme production and characterization

1.Introduction

Glycosidases (EC 3.2.1, EC 3.2.2, and EC 3.2.3) hydrolyze the bond(s) between two or more carbohydrates or the bond between a carbohydrate moiety and a noncarbohydrate moiety [19]. Glycosidases play crucial roles in biology, and increasingly in biotechnology, through the cleavage of glycosidic bonds under mild conditions. A very large number of such enzymes are now known, consistent with the many different naturally occurring glycosidic linkages that must be cleaved, and these enzymes have been classified into families on the basis of amino acid sequence similarities. Amongst the more biotechnologically interesting glycosidases are the β -galactosidases, which have found particular application in the generation of lactose-free

products. β -galactosidases are found in four principal carbohydrate-active enzymes (CAZY) families to date, GH1, GH2, GH35 and GH42, all of which belong to Clan GH-A [18].

Lactose is the main sugar in mammal milk, constituting about 3±8% (w/v) [21]. β-galactosidase (EC 3.2.2.23), a commercially important enzyme, catalyses the hydrolysis of β-D-galactopyranosides such as lactose to glucose and galactose. Because of this property, β-galactosidase is frequently employed in the food industry to alleviate the problems associated with lactose crystallization in frozen concentrated desserts, whey disposal and milk consumption by lactose-intolerant individuals. The enzyme is also used for the reduction of water pollution caused by whey from cheese and for treatment of milk for lactose-intolerance [3-11] . β-Galactosidases have been used as tools in molecular biology, and could be useful in the dairy industry [8]. In addition to catalyzing the conversion of lactose to glucose and galactose, β-galactosidase also catalyzes transgalactosylation reaction; lactose serves as galactosyl donor and an acceptor to form di-, tri-, or higher galactooligosaccharides (GOS) [9]. This, however, creates a potential market for the application of β-galactosidase [22]. β-Galactosidase is a widespread enzyme in nature. It has been found in numerous microbial, plant and animal tissues exerting a variety of physiological roles. Enzymes from these microorganisms have different properties. Therefore, they can be used in different applications [6]. β-Galactosidases obtained from mould have pH optima in the range 3–5 with an optimum temperature range of 55–60 °C with the limitations to high acid product applications and pharmaceutical preparations . However, yeast β-galactosidases, recognised for their higher activities, are characterised by their neutral pH optima [23]. More recently, thermophilic bacteria have become an object of interest for the commercial production of β-galactosidase [24]. *Bacillus licheniformis* KG9 isolated from Taşlıdere hot spring was rod-shaped, Gram-positive, facultatively anaerobic, motile and endospores are oval at subterminal location. KG9 grows at a temperature of 30- 55 °C (optimum 50 °C), which has been considered as moderatively thermophilic strain [25].

In the present study, we have studied some properties of a novel thermostable extracellular β-galactosidase from the thermophilic *Bacillus licheniformis* KG9.

2. Materials and Methods

2.1. *Cell Cultivation*

Bacillus licheniformis KG9 isolated by Dr. Reyhan Gül-Güven in Batman, (Taşlıdere, Turkey). The strain was grown in 100 ml Erlenmeyer flasks containing 25ml of liquid medium (NB; Nutrient Broth). The flasks were inoculated with 1 ml of a cell suspension and stirred in a water bath at 120 rpm at 50 °C for 48 h. After the desired growth time, the cells were harvested by centrifugation at 10.000 rpm for 10 min. The supernatant served as the enzyme source.

2.2. *Enzyme Assay and Definition of Units*

β-Galactosidase activity was determined by release of o-nitrophenol from a 60 mM solution (18 mg/ml stock solution) of O-nitrophenyl-β-D-galactopyranoside (ONPG; Sigma) in 0.1 M sodium phosphate buffer (pH 8.0) at 55 °C. The substrate (50µl) was pre-incubated at the required temperature and then the reaction was initiated by the addition of known amount of enzyme solutions. After 10 min of incubation at desired temperature, the reaction was stopped using 500 µl 1 M sodium carbonate solution. Absorbances at 405 nm were converted to o-nitrophenol concentration using a millimolar extinction coefficient ($Є_{mM405}$) of 4.3834 mM^{-1} cm^{-1} for ONPG.

2.3. *Partial Purification of β-Galactosidase*

The crude extract with β-galactosidase activity was precipitated by ammonium sulphate added slowly over period of time on ice with a constant stirring up to a final concentration of 70 % (w/v). The centrifuged precipitate (15.000 rpm 20min, 4°C.) was redissolved in a small volume of 0.1 M pH 8.0 sodium phosphate buffer, dialysed twice at 4 °C against 1lt volume of the same buffer overnight.

2.4. *Enzyme Production in Presence and Absence of Lactose Over a Period of Time*

The cultures were grown in 250 ml Erlenmeyer flasks containing 100 ml of liquid medium in the absence and presence of %1lactose. The flasks were inoculated

with 4ml of a *Bacillus licheniformis* KG9 cell suspension and stirred in a water bath at 120 rpm at 55 °C for 6–96 h. β-Galactosidase enzyme activity was carried out and protein was assayed according to the Lowry method [15].

2.5. *Effect of Temperature and pH on Enzyme Activity*

The effect of pH on β-galactosidase activity was performed at 55 ^{o}C in 0.1 M Citric acid buffer (pH 4.0-5.5), sodium-phosphate buffer (pH 6.0--8.0), Tris-HCl buffer (pH 8.5-9.0), Glycine-NaOH buffer (pH 9.5-10.0) for 10 min. respectively. The effect of temperature for activity was determinated by assaying activity between 30 and 90 ^{o}C for 10 min. All buffers were preheated at desired temperatures before adding the enzyme into reaction mixture.

2.6. *Effect of Various Carbon Sources on Enzyme Production*

In order to determine the effect of various carbon sources on enzyme production, 1 ml from obtained isolate was inoculated into liquid medium containing 1% concentration of various carbon sources (galactose, glucose, lactose and soluble starch) in 100 ml erlenmeyers and it was cultivated in optimum temperature and pH. β-galactosidase enzyme activity was carried out and protein was assayed according to the Lowry method.

2.7. *Effect of Various Nitrogen Sources on Enzyme Production*

In order to determine the effect of various nitrogen sources on enzyme production, 1 ml from obtained isolate was inoculated into liquid medium containing %1 concentration of various nitrogen sources (yeast extract, glycine, ammonium sulphate, peptone, tryptone and beef extract) in 100 ml erlenmeyers and it was cultivated in optimum temperature and pH. β-galactosidase enzyme activity was carried out and protein was assayed according to the Lowry method.

2.8. *Electrophoretic Analyses*

Non-denaturing polyacrylamide gel electrophoresis was performed on according Laemmli et.al [14]. The molecular weight was estimated by comparison with marker

proteins, 155kDa non-denaturing *Alicyclobacillus acidocaldarius* ssp. *rittmannii* (strain MR1) [7] , 116kDa *E.coli*'s prestained weight marker. β-Galactosidase enzyme activities determined over a period of time (12–96 h) in cells in the presence and absence of lactose **(Fig.1.)** have been also confirmed by non-denaturing polyacrylamide gel electrophoresis, demonstrated by activity gels staining with BNG **(Fig.2.)**

3. Results and Discussion

As shown in Fig.1. we have tested the time course experiments of β-galactosidase synthesis in *Bacillus licheniformis* KG9, which seems to be constitutive (basic enzyme) and increases by increasing time up to 96 h of cultivation. It was observed that enzyme activity in lactose-containing medium continuously increased, strating at 48 h (1.39 U/mg) up to 96 h (3.25 U/mg) whereas in lactose-free medium continuously increased, starting at 36 h (1.38 U/mg) up to 96 h (2.45 U/mg), but the increasing in this medium was less than the increasing in lactose containing medium. Used in the growth rate of bacteria effect the incubation time and enzyme producing property.

Ref [7] reported that the β-galactosidase synthesis in thermoacidophilic *Alicyclobacillus acidocaldarius* ssp. *rittmannii* (strain MR1) seems to be constutive and increases by increasing time up to 40 h of cultivation whereas the highest β-galactosidase activity in *A. acidocaldarius*subsp. *rittmannii* cells was achieved at 24 h of cultivation at 65 °C in the medium containing 1% lactose. [3] reported that the most appropriate incubation determined as the duration of 16h β-galactosidase synthesis in *Bacillus sp*. MTCC 3088. [4] reported that in *Aspergillus nidulans*, β-galactosidase synthesis increase when the presence of lactose.

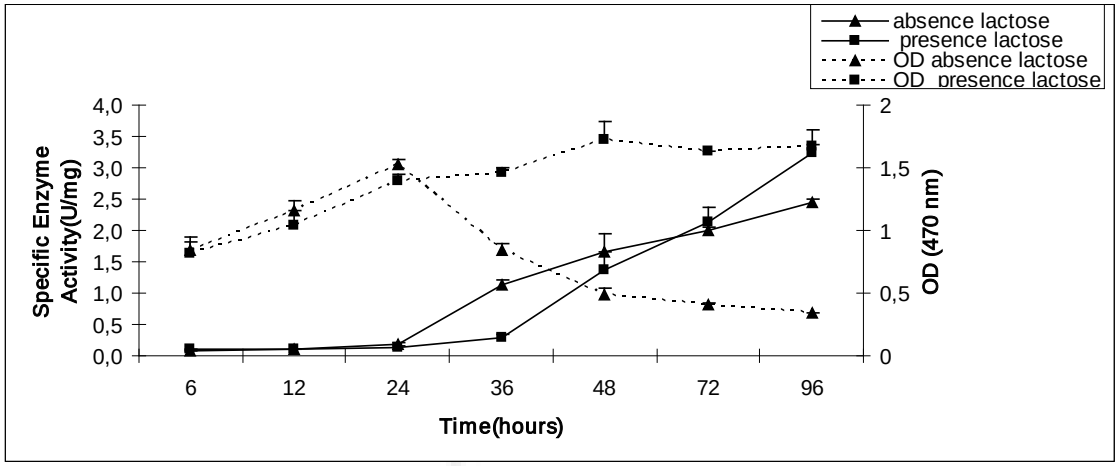

Fig.1. The time course experiments of β-galactosidase synthesis in the cells show constitutive expression of enzyme, starting from 36 h (with specific activity of 1.38 U/mg) up to 96 h (2.45 U/mg) for lactose-free samples, whereas the enzyme production starts at 48 h (1.39 U/mg) for lactose-containing samples, which increases sharply by increasing time up to 96 h (3.25 U/mg) of cultivation at 50°C.

Enzymatic hydrolysis of lactose is one of the most important biotechnological processes in the food industry because of the potentially beneficial effects on the assimilation of foods containing lactose, as well as the possible technological and environmental advantages of industrial application [10]. The enzymatic hydrolysis of lactose into its component monosaccharides-glucose and galactose-is of interest from both the nutritional and technological viewpoints. The resulting sugars are sweeter, more readily fermented and are absorbed directly from the intestine. This has led to the development of low-lactose milk, the production of sweeteners from hydrolyzed-lactose whey and the incorporation of both these products into other foods [16]. The thermophilic enzyme from *K. fragilis* was competitively inhibited by galactose ,however it is much strongly than its mesophilic counterpart (*Thermus sp*) [13].

β-galactosidase enzyme activities determined over a period of time (12–96 h) in cells in absence of lactose have been also confirmed by non-denaturing polyacrylamide gel electrophoresis, demonstrated by activity gels staining with BNG **(Fig.2.)**

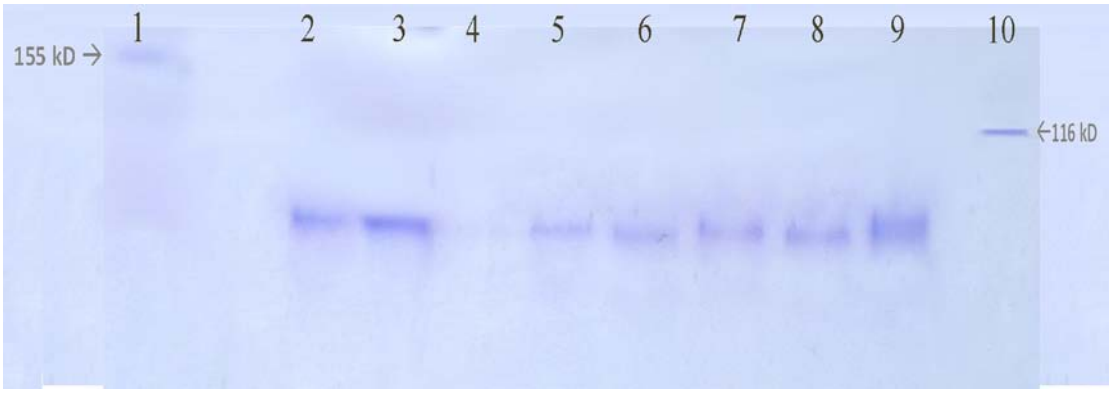

Fig.2. Shows the non-denaturing polyacrylamide gel electrophoresis of β-galactosidase expressed constitutively over a period of time (12- 96 hours) in NB medium. Lane 1, 155kDa non-denaturing *Alicyclobacillus acidocaldarius* ssp. *rittmannii* (strain MR1); Lane 2, crude extract; Lane 3, partially purified enzyme; Lane 4,12h; Lane 5, 24h; Lane 6, 36h; Lane 7, 48h; Lane 8, 72h; Lane 9, 96h;Lane 10, 116kDa *E.coli*'s prestained weight marker. As it shown that in fig2. the molecular mass of the enzyme was an approximately less than 116kDa.

Enzyme was partially purified by using ammonium sulphate precipitation and dialysis **(Table 1)**. In crude extract specific activity value was 1631 U/mg, efficiency %100, purification coefficient was 1, after dialysis the specific activity value was 19030.45 U/mg, efficiency 15.7 % and purification coefficient was change 11.66 respectively.

Table 1. Partially purified by using ammonium sulphate precipitation and dialysis

Purification Steps	**Total Activity (U)**	**Total Protein (mg)**	**Specific Activity (U/mg)**	**Purification (fold)**	**Yield (%)**
Crude extract	1810	11.10	1631	1	100
Ammonium sulphate precipitation and dialysis	2845	0.14	19030.45	11.66	15.7

We have determined the effects of pH and temperature on β-galactosidase activity. The optimal values were found to be pH 8.0 and 55 °C. **Figure 3 (a) and Figure 3 (b)**. Enzyme shows pH 6.0-8.0 intervals near the optimum range of activity ,therefore it has potential application in diary product industries. Moreover in milk and diary industries

requiers high temperatures for reducing the microbial risk contamination in operations, so the β-galactosidase shows the optimum activity at 55 °C, this is advantages for all application. β-galactosidase from *Streptococcus thermophilus* [20] showed the similar results for the pH and temperature. Moreover β-galactosidase from *Bacillus sp*. [3] *Bacillus coagulans* [1] optimum pH and temperature were found 8-60 °C ,6-7-65°C respectively.

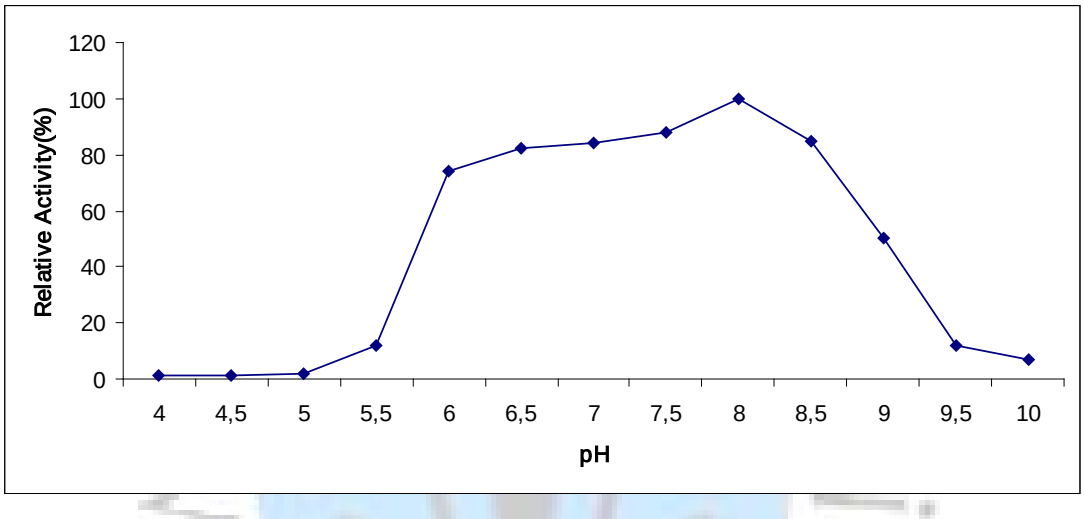

Fig.3(a) Effect of pH on the Activity of the β-galactosidase

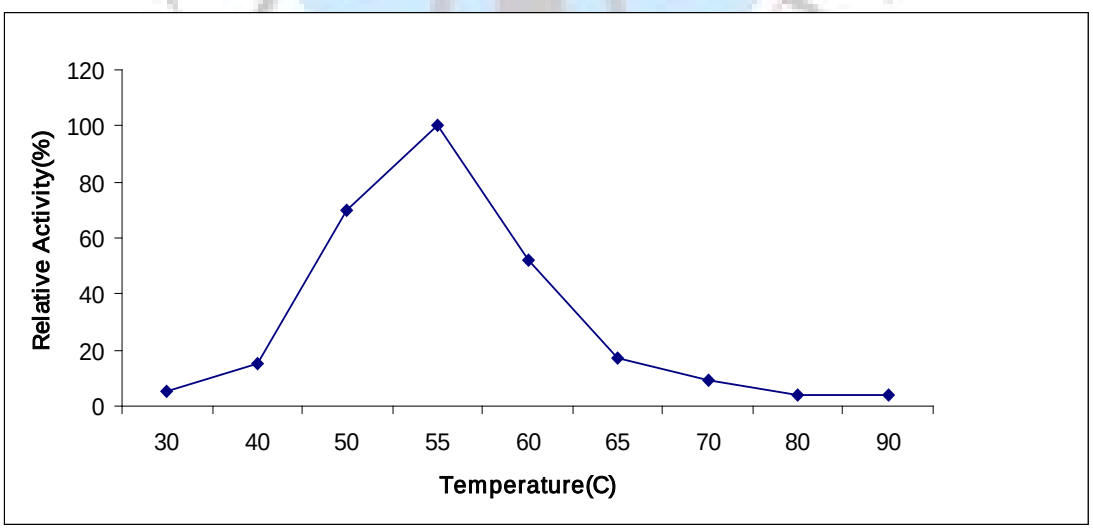

Fig.3.(b) Effect of Temperature on the Activity of the β-galactosidase

We have determined effect of different nitrogen and carbon sources in 1 % ratios on the production of β-galactosidase by *Bacillus licheniformis* KG9. It is clear form the figure

that carbon sources didn't increase the enzyme production **Fig.4.** *B. licheniformis* KG9 produced a low amount of β-galactosidase in the media containing soluble starch, glucose or galactose, in comparison to lactose. It seems that galactose, glucose and soluble starch substantially inhibited extracellular enzyme production by means of negative effect on the mechanism of the synthesis β-galactosidase in *Bacillus licheniformis* KG9. β-galactosidase from *Bifidobacterium longum* CCRC 15708, *Kluyveromyces fragilis, Rhizomucor sp***.** showed that highest level activity was produced with lactose [9-5-17]. [2] also reported a higher level of β-galactosidase activity by *Kluyveromyces marxianus* IMB3, when 2 % (w/v) lactose was used in the fermentation media. Lactose was the most effective carbon sources for β-galactosidase synthesis, giving a maximum activity of 0.93 U/mg.

As shown in **Fig.5.** amount of the various nitrogen sources glycine and ammonium sulphate slightly increased the synthesis of β-galactosidase in *B.licheniformis* KG9 . Maximum enzyme activity of 2.7 U/mg and 2.8 U/mg were obtained when glycine and ammonium sulphate were used in the medium, respectively. Yeast extract, beef extract, pepton and trypton didn't increase enzyme synthesis. β-galactosidase from *Bifidobacterium longum* CCRC 15708, *Kluyveromyces fragilis, Bacillus subtilis* showed that highest level activity was produced with yeast extract [9-5-12]. Moreover [12] reported that glycine slightly increased the synthesis of β-galactosidase in *Bacillus subtilis.*

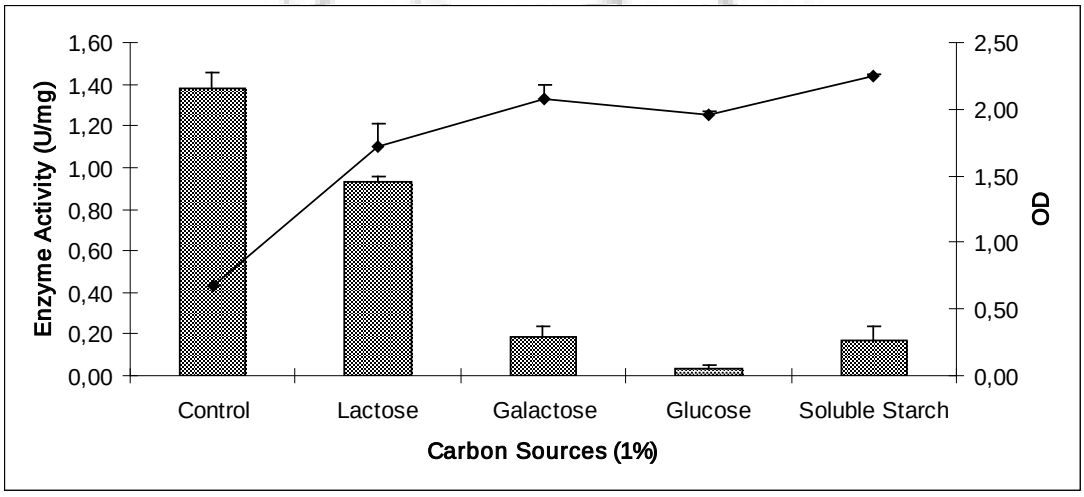

Fig.4. Effect of Various Carbon Sources on β-galactosidase Production (1%)

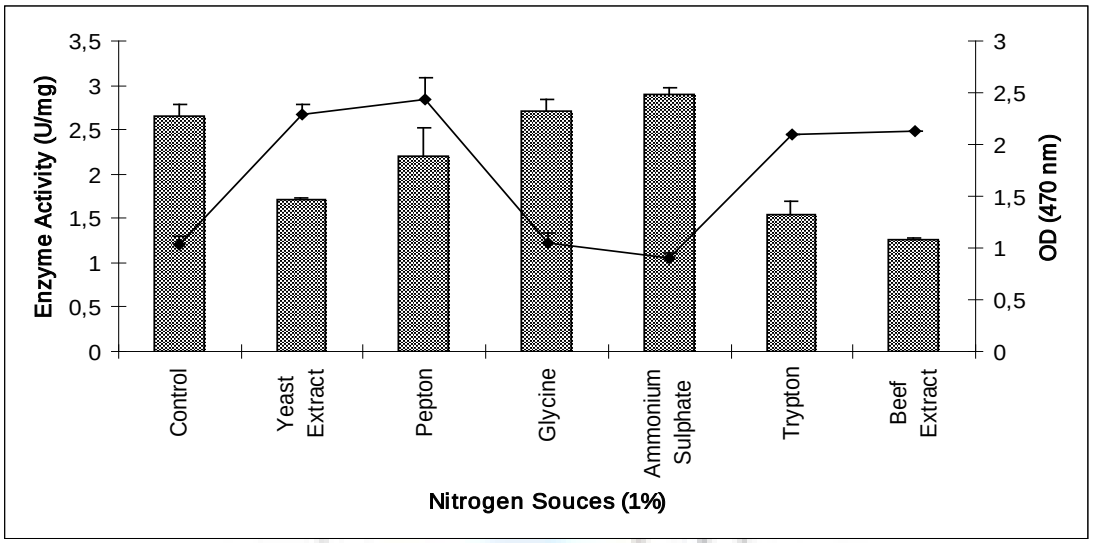

Fig.5. Effect of Various Nitrogen Sources on β-galactosidase Production (1%)

In this study, the thermophilic bacteria *B. licheniformis* KG9 produces a high amount of an extracellular β-galactosidase with high activity. Based on the relatively good features of thermostability and the negligible product inhibition by glucose are likely to render, this enzyme a good candidate for biotechnological use in the treatment of milk and related products requiring lactose hydrolysis.

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References:

[1] Batra, N., Shıng, J., Banerjee, U.C., Patnaık, P.R., Sobti, R.C., 2002. Production and characterization of a thermostable β-galactosidase from *Bacillus coagulans* RCS3, *Biotechnology and Applied Biochemistry*, 36, 1- 6.

[2] Brady, D., Marchant, R. , McHale, L. , McHale, A.P., 1995. Isolation and partial characterization of β-galactosidase activity produced by a thermotolerant strain of *Kluyveromyces marxianus* during growth on lactose-containing medium, *Enzyme and Microbial Technologies* ,17pp,696-699.

[3] Chakraborti, S., Sani, R. K., Banerjee, U.C and Sobti, R.C., 2000. Purification and Characterization of a Novel β-Galactosidase from *Bacillus* sp. MTCC 3088, *Journal of Industrial Microbiology & Biotechnology*, vol:24, pp: 58–63.

[4] Fantes P. A. and Roberts, C. F., 1973. β-Galactosidase Activity and Lactose Utilization in *Aspergillus nidulans*, *Journal of General Microbiology*, 77,47 I -486.

[5] Fiedurek, J., Szczodrak, J., 1994. Selection of strain, culture conditions and extraction procedures for optimum production of β-galactosidase from *Kluyveromyces fragilis. Acta Microbiologica Polonica* ,43, 57– 65.

[6] Gaulas, T., Goulas, A., Tzortzis, G., Gibson, R. G., 2009. Comparative analysis of four β-galactosidases from Bifidobacterium bifidum NCIMB41171: purification and biochemical characterisation, *Applied and Environmental Microbiology*, 82,1079–1088.

[7] Gül-Güven, R., Güven, K., Poli, A., Nicolaus, B., 2007. Purification and Some Properties of a β-Galactosidase from the Thermoacidophilic *Alicyclobacillus acidocaldarius subsp. Rittmannii* Isolated from Antarctica, *Enzyme and Microbial Technology*, vol: 40 pp: 1570–1577.

[8] Hidaka, M., Fushinobu, S., Ohtsu, N., Motoshima, H., Matsuzawa, H., Shoun, H., Wakagi. T.,2002. Trimeric Crystal Structure of the Glycoside Hydrolase Family 42 β-Galactosidase from *Thermus thermophilus* A4 and the Structure of its Complex with Galactose, *Journal of Molecular Biology*, 322, 79–91.

[9] Hsu, C. A., Yu, R. C., Chou, C. C., 2005. Production of β-galactosidase by *Bifidobacteria* as influenced by various culture conditions, *International Journal of Food Microbiology*, 104, 197–206.

[10] Jurado E., Camacho, F., Luzón, G., Vicaria, J.M., 2002. A new kinetic model proposed for enzymatic hydrolysis of lactose by a β-galactosidase from *Kluyveromyces fragilis* ,*Enzyme and Microbial Technology*, 31, 300–309.

[11] Kim, W.J., Rajagopal, S.N, 2000. Isolation and Characterization of β-galactosidases from *Lactobacillus crispatus*, *Folia Microbiology*, 45(1), 29-34.

[12] Konsoula, Z., Liakopoulou-Kyriakides, M.,2007. Co-production of a-amylase and β-galactosidase by Bacillus subtilis in complex organic substrates, *Bioresource Technology* , 98, 150–157.

[13] Ladero, M., Santos, A., Garcı´a L.J. F., Garcı´a-Ochoa, F., 2002. Studies on the Activity and the Stability of β-Galactosidases from *Thermus sp* strain *T2* and from *Kluyveromyces fragilis*, *Enzyme and Microbial Technology*, 30, 392-405.

[14] Laemmli U.K. Nature, 1977., vol.227, p. 680–685.

[15] Lowry, O.H., Rosebrough. N.J., Farr, A.L., 1951. Protein measurement with the folin phenol reagent, *Journal of Biological. Chemistry* , 193, 265- 275.

[16] Mahoney, R.R., 1998. Galactosyl-oligasaccharide Formation During Lactose Hydrolysis: A Review. *Food Chemistry*, **,** 63 (2): 147–154.

[17] Shaikh, S.A., Khire, J.M., Khan, M.I., 1997. Production of β—galactosidase from thermophilic fungus *Rhizomucor sp.*, *Journal of Industrial Microbiology and Biotechnology* , 19, 239–245.

[18] Shaikh-Aidha, F., Müllegger, J., He, S., Withers, G. S., 2007. Identification of the catalytic nucleophile in Family 42 β-galactosidases by intermediate trapping and peptide mapping: YesZ from *Bacillus subtilis*, *FEBS Letters*, 581,2441–2446.

[19] Sheridan, P. Peter and Brenchly, E. Jean.,2000. Characterization of a Salt-Tolerant Family 42 β-Galactosidase from a Psychrophilic Antarctic *Planococcus* Isolate, *Applied and Environmental Microbiology*, , p. 2438–2444.

[20] Somkuti,G.A., Steinberg, D.H.,1979. Beta-D- Galactoside galactohydrolase of *Streptococcus thermophilus:* induction, purification, and properties, *Journal of Applied Biochemistry* , 1,357-368.

[21] Speer, E., 1998. Milk and dairy product technology. New York: Marcel Dekker.

[22] Tari, C., Ustok, F. I., & Harsa, S., 2009. Optimization of the associative growth of novel yoghurt cultures in the production of biomass, β-galactosidase and lactic acid using response surface methodology. *International Dairy Journal*, 19, 236–243.

[23] Ustok, F. I., Tari, C& Harsa, S., 2010. Biochemical and thermal properties of b-galactosidase enzymes produced by artisanal yoghurt cultures, *Food Chemistry* , 119 1114–1120.

[24] Vasiljevic, T., Jelen, P., 2001.Production of β-galactosidase for lactose hydrolysis in milk and dairy products using thermophilic lactic acid bacteria. *Innovative Food Science & Emerging Technologies*, Vol. 2, p. 75–85.

[25] Gül-Güven,R., 2007. Sıcak su kaynaklarından bakteri izolasyonu, tanımlanması ve *Alicyclobacillus acidocaldarius* subsp. *Rittmanii*'nin β-galaktozidaz enziminin saflaştırılması. Doktora Tezi. Dicle Üniversitesi Fen Bilimleri Enstitüsü, Diyarbakır, syf 6.

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