WORLD JOURNAL OF PHARMACEUTICAL RESEARCH
SJIF Impact Factor 8.074
Volume 7, Issue 07, 996-1012. Research Article ISSN 2277–7105

PURIFICATION OF BETA GALACTOSIDASE ENZYME FROM DAIRY EFFLUENT *BACILLUS SPECIES.*

[1]V. Pavithra and [2]*Dr. J. Thirumagal

[1]M. Phil., Research Scholar., K.M.G. College of Arts and Science., Gudiyattam.

[2]Asst. Prof In Biochemistry., K.M.G. College of Arts and Science, Gudiyattam.

Article Received on
05 Feb. 2018,

Revised on 26 Feb. 2018,
Accepted on 18 March 2018

DOI: 10.20959/wjpr20187-11577

***Corresponding Author**
Dr. J. Thirumagal
Asst. Prof In Biochemistry.,
K.M.G. College of Arts and
Science, Gudiyattam.

ABSTRACT

Beta-galactosidases are the highly demanded enzymes in the diary industries for the hydrolysis of lactose into its constituent monosaccharides. Thermostable beta-galactosidases producing *Bacillus* sp D4 can be used as a probiotic food for lactose intolerant people. In the present study, the *Bacillus* sp was isolated from the diary industry effluent in Chennai and screened with X-Gal media. The *Bacillus* sp. strain D4 was characterized by biochemical test and identified based on 16S rDNA sequencing and their phylogenetic analysis was carried out. The strain D4 was assessed for its probiotic nature using antibiotic markers. The characterization of the enzyme and optimization of the production medium were very important in determining the maximum production and activity of beta-galactosidase. Maximum production of enzyme was obtained when the medium was incubated for 48 hours at 37°C and maintained at pH 7. The addition of various carbon, nitrogen, aminoacid sources, metal ions and natural substrates to the medium were studied at concentration 1% m/v. Xylose, Yeast extract, L-phenylalanine, Mg^{2+} ion, Mn^{2+} ion and wheat bran increased the production of beta galactosidase. The enzyme was partially purified by acetone and ammonium sulphate precipitation and characterized based on the temperature, pH. The enzyme showed highest activity at 55°C (0.350 U/ml) and at pH 7 (0.294 U/ml). The enzyme retained 100% of its activity at 45°C and retained 90% of its activity at pH 7.

KEYWORDS: *Bacillus* sp, probiotic, X-Gal, ONPG, beta-galactosidase.

INTRODUCTION

Enzymes work by lowering the activation energy for a reaction, thus increasing the rate of the reaction. As a result, products are formed faster and reactions reach their equilibrium state more rapidly. Most enzyme reaction rates are millions of times faster than those of comparable un-catalyzed reactions. As with all catalysts, enzymes are not consumed by the reactions they catalyze, nor do they alter the equilibrium of these reactions. However, enzymes do differ from most other catalysts by being much more specific. Enzymes are known to catalyze about 4,000 biochemical reactions. A few RNA molecules called ribozymes also catalyze reactions, with an important example being some parts of the ribosome.

β –GALACTOSIDASE

β-galactosidase (E.C 3.2.1.23) is a hydrolase enzyme that catalyses the hydrolysis of β-galactosides (e.g. lactose) into monosaccharides (e.g. galactose and glucose), where the glycosidic group on the non-reducing β-D-galactose residue is replaced by a hydroxyl group (an acceptor group). β-galactosidase is also known to catalyze the transglycosylation of sugars, i.e. when a sugar moiety is an acceptor instead of the water molecule. This then leads to the synthesis of new oligosaccharides (Yamamoto et al, 2004). β-galactosidase hydrolyses the β-1,4-D-galactosidic linkage of lactose, as well as those of related chromogens, o-nitro-phenyl-β-D-galactopyranoside (ONPG), p-nitrophenyl-β-Dgalactopyranoside (PNPG) and 6-bromo-2-naphthyl-galacto-pyranoside (BNG). This enzyme is widely distributed in nature, being found in various types of microorganisms, plant and animal tissues (Ahn and Kim, 1977).

RESULTS AND DISCUSSIONS

The species was isolated and conditions necessary for the optimization of the medium and the characterization of the enzyme were carried out and they are as follows.

Isolation and Screening

In this study, the bacterial strains were isolated from dairy industry effluent collected from the Heritage dairy industry, Ambattur, Chennai. After serial dilution and nutrient agar plating, 18 bacterial strains producing β-Galactosidase were isolated. Among these 18 strains, the best 8 strains were selected and plated on a new nutrient agar media and the best culture giving the better result were streaked to isolate the pure colonies as shown in Fig 1, 2, 3.

A pyschrophilic bacterial isolate, *Bacillus* sp. was isolated from blue green algal mats collected from Schirmacher Oasis of Antartica (Ram Kumar et al, 2004) was screened for β-galactosidase production. In contrast, thermophilic organisms isolated from Ta Pai hot spring, Maehongson, Thailand (Somyos and Phimchanok, 2010) produced β-galactosidase. Commercial lactases are produced from both yeasts, such as *Kluyveromyces lactis* and *Kluyveromyces fragilis*, and moulds such as *Aspergillus niger* and *Aspergillus oryzae* (Mahoney et al 1985. Even fungal cultures like *Aspergillus*, *Trichoderma*, *Penicillium*, *Rhizopus* and *Fusarium* sp. were known to produce β-galactosidase at promising level (Isil Seyis et al, 2004). The bacterial strain present in dairy industry effluent has been proved to be probiotic and has a greater ability to produce Beta-galactosidase (Sreekumar, 2010).

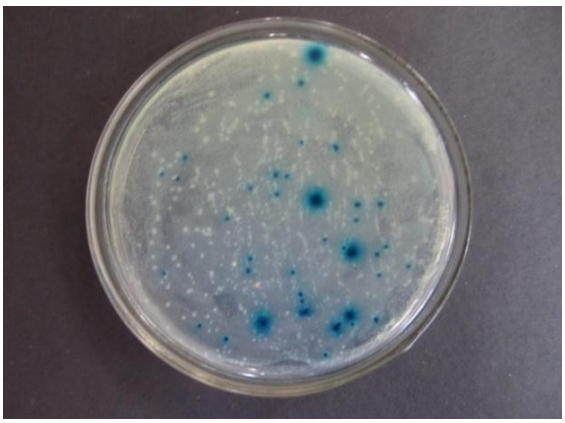

Fig 1: screening plate for beta-galactosidase infused with X-gal

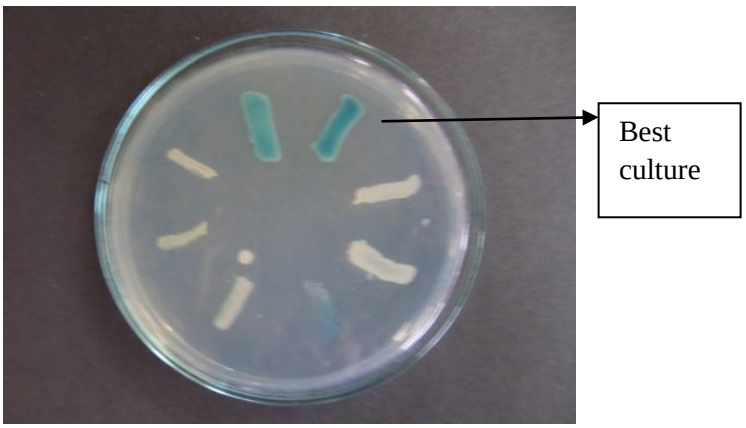

Fig 2: The best culture was selected.

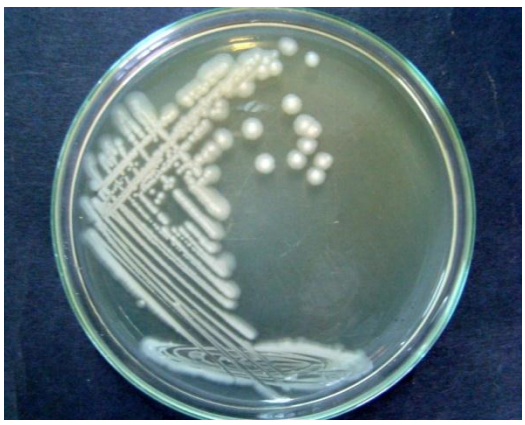

Fig 3: Pure colonies isolated after streaking.

Sporulation

The 8 best strains isolated were cultured in a sporulation media to detect the presence of spore forming bacteria. Six strains with spore forming ability were observed. Among the 6

strains, the best strain was selected and preceded for further procedure. These spores were then stained with Safranin using Schaeffer-Fulton Technique and the position of the spores was centralized (Fig: 4).

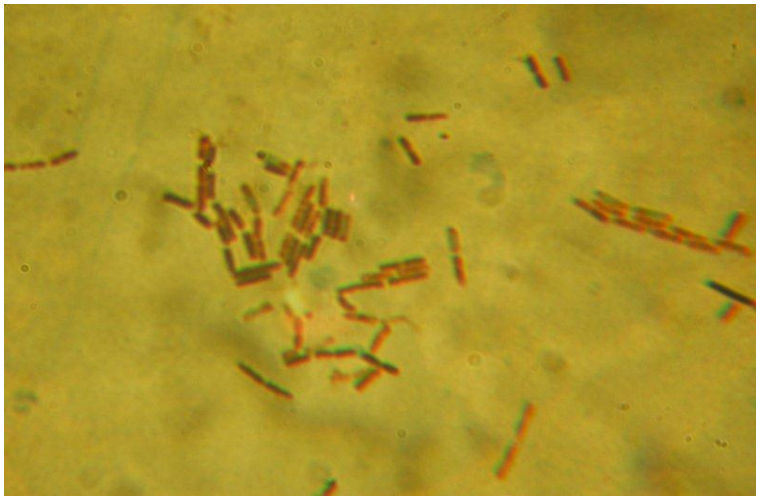

Fig 4: The presence of centralized spores in Bacillus sp.

In 2003, a report showed that out of 6 spore forming isolates, those spores which resisted the treatment of UV irradiation, γ radiation and desiccation were selected for further studies (Kasthuri Venkateshwaren et al, 2003).

Identification of the strain D4

The strain D4 was subjected to Gram Staining test, Catalase test, Oxidase test, Motility test and the strain was Gram positive, catalase positive, motile, rod shaped, and produces Cytochrome C. These characteristics confirmed that the strain D4 was identified as *Bacillus species.* Further confirmation was done by sequencing the 16S rRNA gene and compared with the Gen Bank databases using the BLASTN program. The 16S rRNA sequence of the isolate revealed a close relatedness to *Bacillus subtilis* with 99-100% similarity. Hence the strain was confirmed as *Bacillus subtilis.* The phylogenetic tree established the evolutionary relationship of the species.

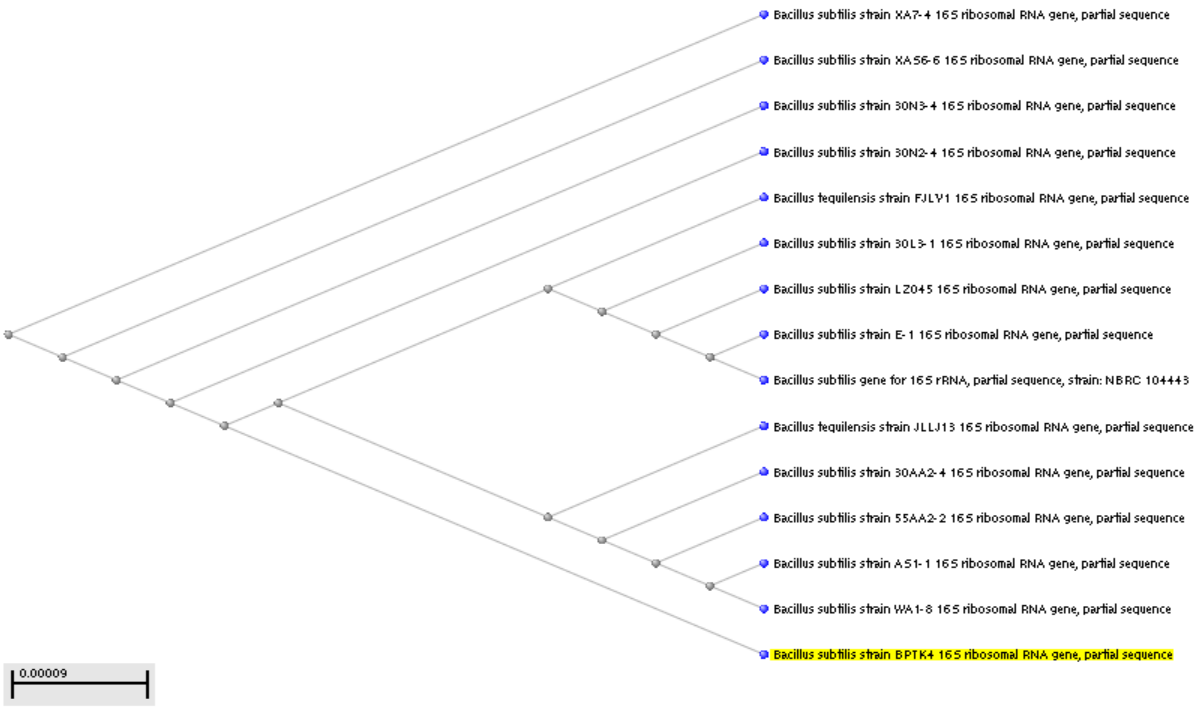

Fig 5: Phylogenetic relationship of the strain D4.

Antibiotic susceptibility of the *Bacillus* species

The isolate D4 was inhibited by most of the antibiotics like Amoxillin, Ciprofloxacin, Gentamycin, Cotrimoxazole, Chloramphenicol, Bacitracin, Tetracycline, Kanamycin, Erythromycin, Vancomycin, Ampicillin, Streptomycin, Clindamycin, Methicillin but showed resistance only to Penicillin. This makes the isolate to be used as a probiotic for lactose intolerance people and also in dairy industry. David et al, 1999 reported the different strains of *Bacillus* which are commercially available now like biosubtyl and Enterogemina as probiotic preparations by using antibiotic markers. Ammini et al, 2011 reported that *Bacillus pumilus* causes skin infections in immune compromised individuals. So, further studies would be performed to understand the effect of micro organisms on human health, their genetic basis and resistance to antimicrobials.

Table 1: the reaction of strain BPTK 4 towards a number of antibiotics of different concentration.

S.No	Antibiotics	Stimulatory	Resistance
1	Amoxillin	+	-
2	Ciprofloxacin	+	-
3	Gentamycin	+	-
4	Cotrimoxazole	+	-
5	Chloramphenicol	+	-
6	Bacitracin	+	-
7	Tetracycline	+	-
8	Kanamycin	+	-
9	Erythromycin	+	-
10	Vancomycin	+	-
11	Ampicillin	+	-
12	Streptomycin	+	-
13	Clindamycin	+	-
14	Methicillin	+	-
15	Penicillin	-	+

Optimization of cultural conditions for the maximum production of the enzyme

Effect of Incubation time

Incubation time depicts the characteristics of the culture and is also based on the growth rate and enzyme production.

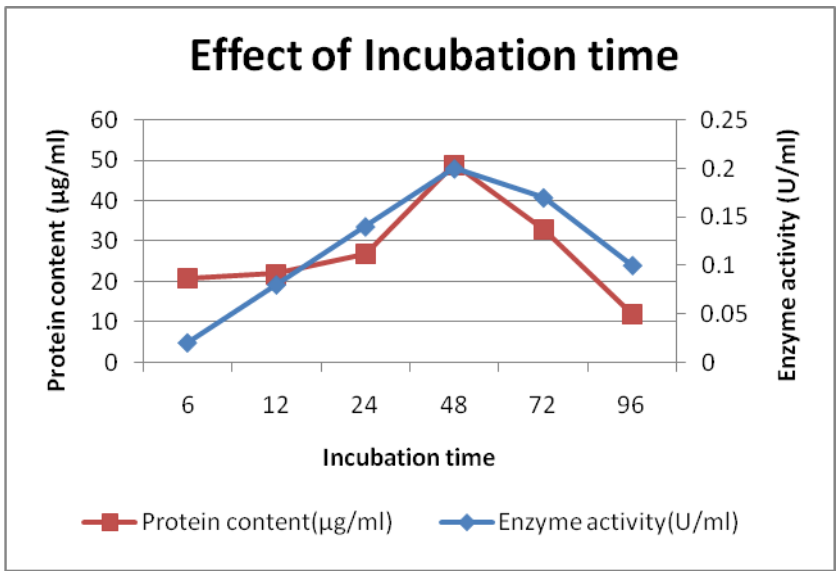

Fig 6: The effect of incubation time on Beta-galactosidase production.

It has been observed that till the 12th hour the organism should be in lag phase because we could not find considerable enzyme activity upto 12 hours of incubation, but, it was found that from 12th hour, gradual increase in the enzyme production occurred and there was a high activity in the enzyme production at 48 hours (0.20 U/ml) after which gradual drop in production occurred (Fig: 6). The decrease in the enzyme activity after 48 hours may be due to the decrease in the amount of nutrients in the medium or due to denaturation of the enzyme.

Similar results were obtained by Siham et al, 2010 while working on the production of Beta-galactosidase produced from *L. acidophilus*. It showed an increase in the level of enzyme activity after 48 hrs. Maximum Beta-galactosidse production was also reported at 48 hours by Nehad and Enas, 2011.

Effect of Incubation temperature

Incubation temperature is a very important parameter in determining the production of enzyme because at particular temperature optima the production will be high. Above or below the optima the production decreases.

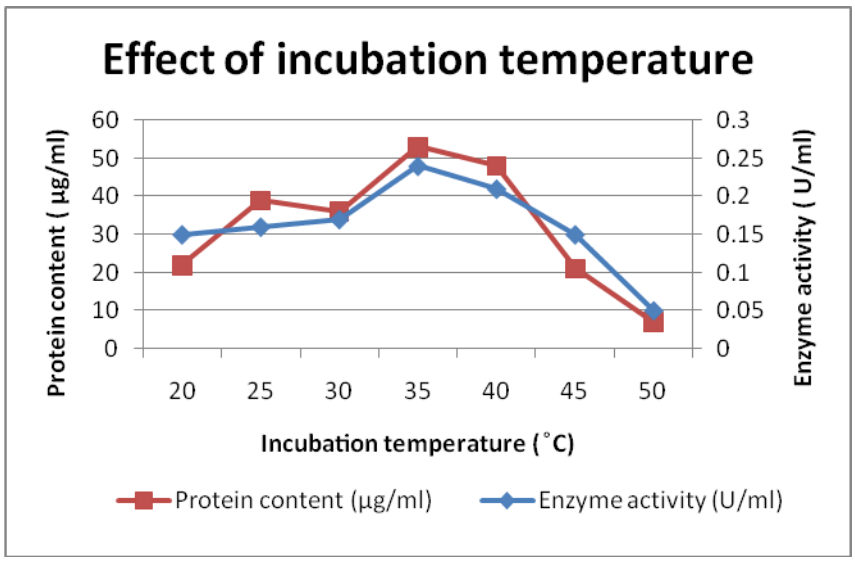

Fig 7: The effect of temperature on Beta-galactosidase production.

To optimize the temperature for better production, the medium was incubated at various temperatures (20-60°C). Like other mesophilic organisms, the isolated *Bacillus* strain also requires an optimum temperature for its growth and production. Enzyme production has been shown to maximum when incubated at 35˚C (Fig: 7).

Chakraborti et al, 2000 showed that maximum production of the enzyme was observed at 37°C. Shebawy et al, 2007 showed that the enzyme production was maximum at 37°C.

Effect of pH

The hydrogen ion concentration affects two aspects of the microbial cells: functioning of the cells and the transport of nutrients to the cells [Panesar et al, 2006]. So maintaining the pH value is very important.

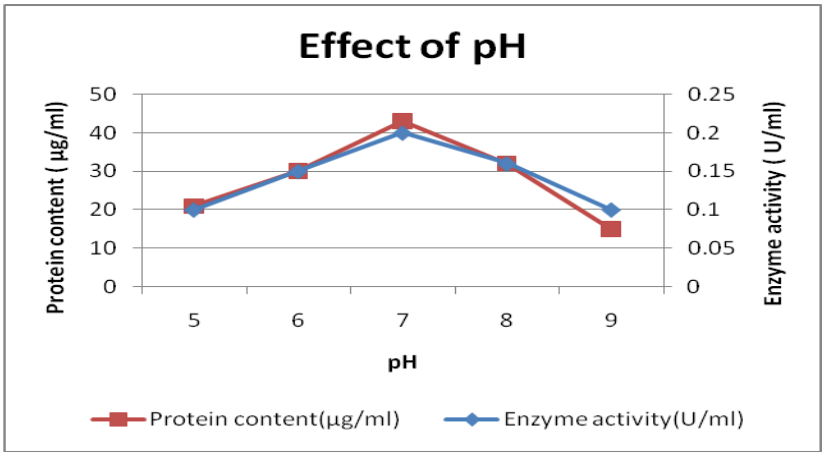

Fig 8: The effect of pH on Beta-galactosidase production.

At pH 7, the production of the enzyme was observed to be maximum (0.20 U/ml). At pH 5 and 9, the activity of Beta-galactosidase was 0.10 U/ml (Fig: 8).

Effect of various carbon sources

The addition of carbon source in the form of either Monosaccharide or Polysaccharide may influence the production of enzymes.

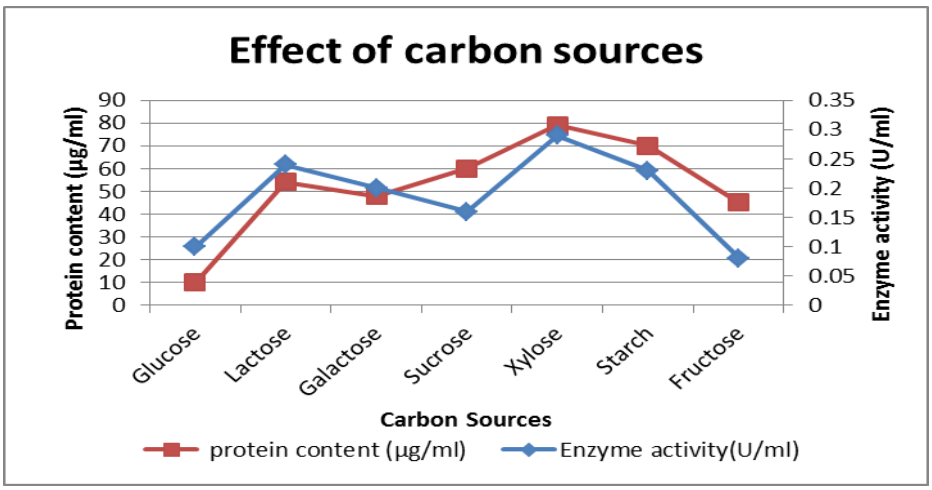

Fig 9: The effect of Various Carbon sources on Beta-galactosidase production.

Xylose (0.29 U/ml) was found to enhance the production of enzyme when compared with other substrates. Lactose (0.24 U/ml) and starch (0.23U/ml) also found to increase the enzyme production (Fig: 9).

This was correlated with the findings of Akcan, 2011. Galactose was shown to be the effective carbon source in the biosynthesis of α- galactosidase by *B. circulans* [Shebawy et al, 2007]. There are several reports supporting that the *Bacillus* sp produces high quantity of β-Galactosidase in lactose amended production medium [Mahoney et al, 1975]. In contrast, organisms like Halomonas produces high amount of β-Galactosidase in maltose amended medium collected 25 samples from dairy producing centers and from that they screened 30 different yeast isolates using Malt extract broth [Jime´nez-guzma et al, 1985; Nahvi and H.Moeini, 2004]

Effect of Various Nitrogen Sources : The nitrogen sources are of secondary energy sources for the organisms, which play an important role in the growth of the organism and the production of enzymes. The nature of the compound and the concentration which we are using may stimulate or down-regulate the production of enzymes.

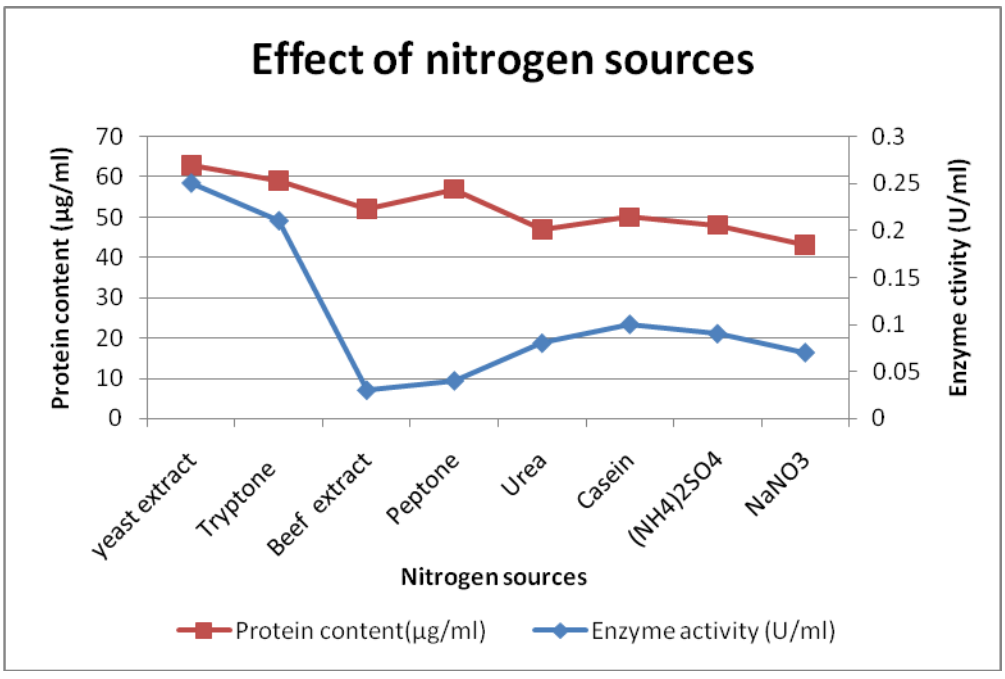

Fig 10: The effect of various nitrogen sources on Beta-galactosidase production.

The isolated *Bacillus* sp. has produced high rate of enzyme in the medium amended with Yeast extract (0.25 U/ml) and comparable amount of enzyme has been produced with the

medium amended with tryptone (0.21 U/ml). In contrast, negligible amount has been produced when the medium was amended with beef extract (0.03 U/ml) (Fig: 10).

These findings were related to Shebawy et al, 2007 who worked on the production of α-galactosidase from *B. circulans*. Siham et al, 2010 also showed that yeast extract had an influence on Beta-galactosidase activity. But the inorganic nitrogen sources such as ammonium sulfate (0.09 U/ml) and sodium nitrite (0.07 U/ml) did not contribute much to the good growth and the production when compared to organic sources. Similar results were shown by Shebawy et al, 2007, Siham et al, 2010.

Effect of aminoacid sources

Aminoacids being the building blocks of proteins are important in determining their influence on the beta-galactosidase production.

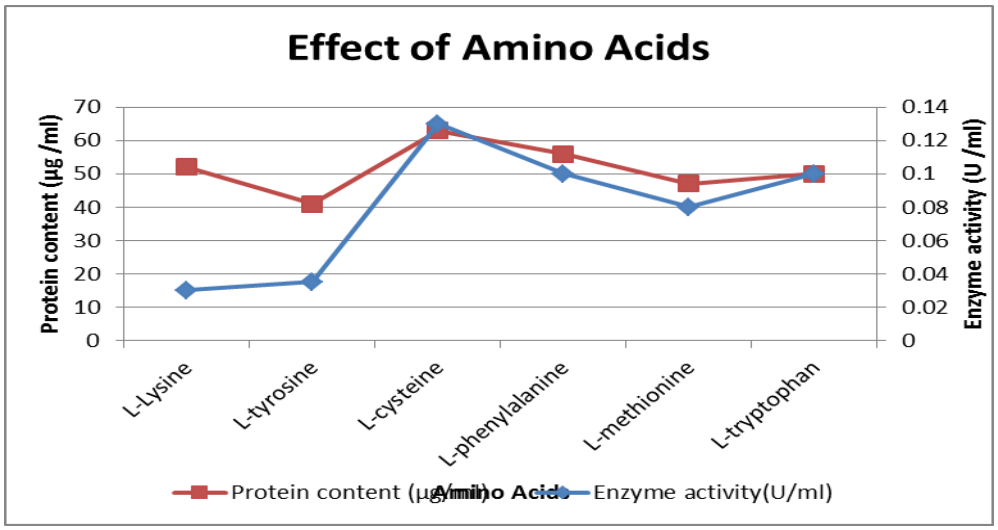

Fig 11: The effect of amino acids on Beta-galactosidase production.

Among the various amino acids used like L-lysine, L-tyrosine, L-cysteine, L-phenylalanine, L-methionine, L-tryptophan, it was observed that L-tryptophan and L-phenylalanine found equally to increase the enzyme activity (0.1 U/ml) (Fig: 11).

Akcan, 2011 reported that when the medium was supplemented with L-tryptophan, it increased the enzyme activity to upto 60%. Ashish et al, 2005 while working on the endoglucanase production from *Aspergillus terreus*, reported that methionine, tryptophan and asparagines increased the production of the enzyme.

Effect of metal ions

Metal ions either increases or decreases the production of enzyme by enhancing the metabolic rate of the enzyme.

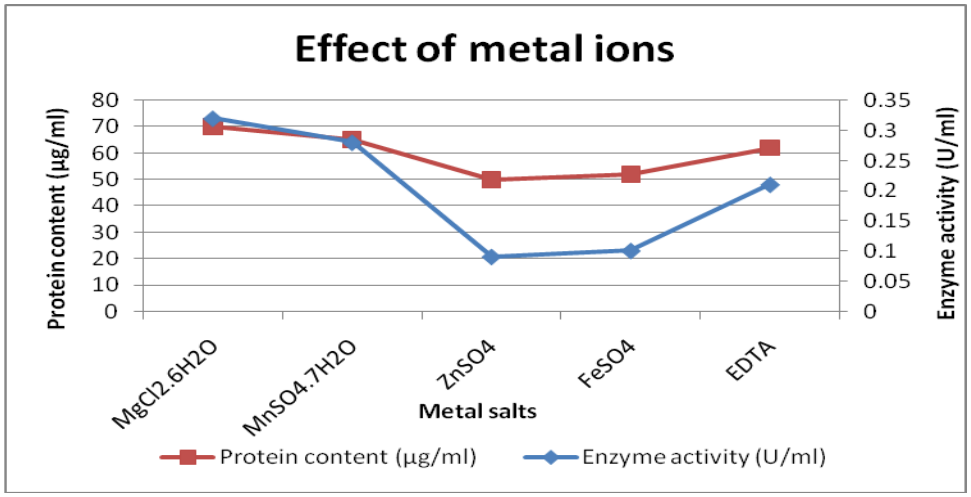

Fig 12: The effect of addition of different metal ions on Beta-glactosidase production.

The culture medium was supplemented with various metal ions such as $MgCl_2.6H_2O$, $MnSO_4.7H_2O$, $FeSO_4$, $ZnSO_4$ and EDTA. The addition of these ions to the medium had an influence over the beta-galctosidase production. In our study, it was observed that when the medium was supplemented with Mn^{2+} (0.28 U/ml) and Mg^{2+} (0.32 U/ml) at 1 mM concentration, an increase in the enzyme activity was observed (Fig: 12). This indicated that Mg^{2+} was required in the media for enzyme stabilization.

But different authors reported that Mn^{2+} and Mg^{2+} of different concentrations had an influence on the enzyme yield. Siham et al, 2010 reported that Mn^{2+} and Mg^{2+} of 0.02 M increased the enzyme yield. Shweta et al, 2011 worked on the production of Beta-galactosidase using yeast isolate from whey and reported that at the concentration of 0.05%, Mg^{2+} increased the enzyme production.

Effect of natural substrates

The addition of natural substrates to the medium found to increase the production of enzyme to a certain level, but has no major effect like synthetic substrates.

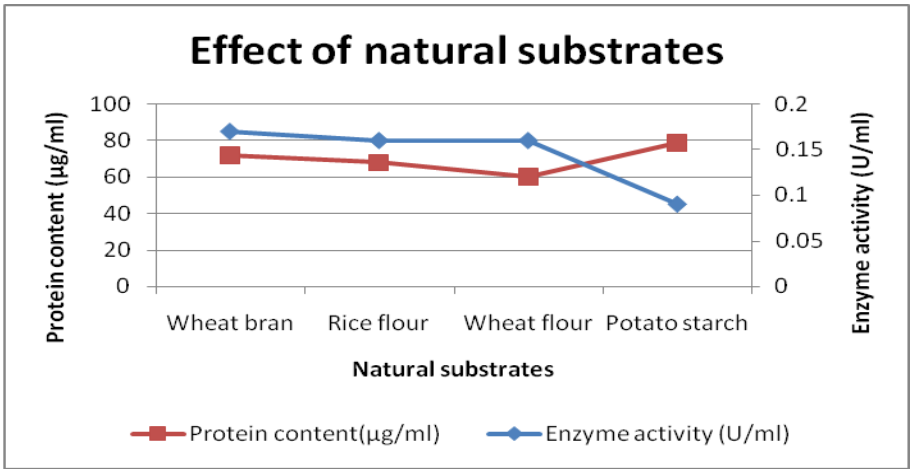

Fig 13: The effect of natural substrates on Beta-galactosidase production.

Among the natural substrates used, wheat bran showed a relatively high production of enzyme (0.17 U/ml). The production rate was slightly decreased by other substrates (Fig: 13).

Partial Purification of the enzyme

After the optimization of media, the enzyme was partially purified by acetone followed by ammonium sulfate precipitation.

Fig 14: precipitation of proteins by 65% acetone

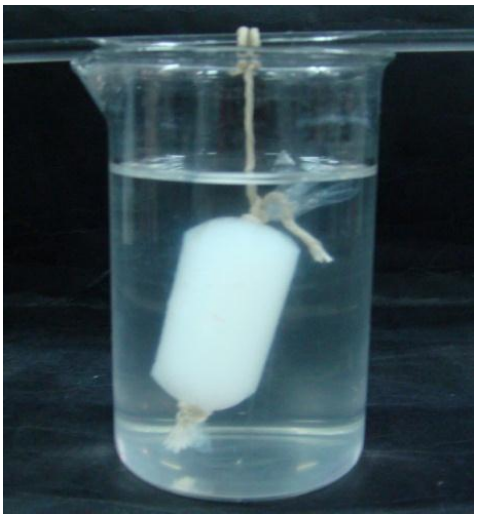

Fig 15: Precipitation of proteins in 60-80% ammonium sulfate followed by dialysis tubing.

SDS PAGE analysis

Finally, the protein profile was analyzed using SDS-PAGE; it showed the presence of multiple bands because in all the cases the total protein content of the samples is moderately

high. So other proteins (other than enzyme) may be present and form bands. But the presence of protein band nearing the molecular weight around 80-110 KDa confirms the presence of the enzyme β-Galactosidase (Fig: 16). Generally, the molecular weight of β-Galactosidase from *Bacillus sp.* is 70-90 kDa (Keller et al, 1993). The molecular weight of the denatured enzyme was estimated to be 86,000 by SDS-PAGE after the sample had been boiled in the presence of SDS and 2-mercaptoethanol (Onishi et al, 1995.) The *L. plantarum* β-galactosidase is a heterodimeric enzyme of a molecular mass of approximately 107 kDa, consisting of a larger subunit of approximately 72 kDa and a smaller subunit of ~35 kDa as estimated under denaturing conditions of SDS–PAGE (Dietmar Haltrich et al, 2010).

Activity of β-galactosidase was measured by incubating 20 µl of suitably diluted enzyme with 480 µl of 22 mM o-nitrophenyl-β-Dgalactopyranoside (ONPG) in phosphate buffer pH 6.5 at 40°C. After 15 min, 0.4 M Na_2CO_3 was added to the reaction mixture to stop the reaction (Phimchanok et al, 2008). β-phosphogalactosidase activity was routinely estimated at 37ºC in reaction mixtures containing 1 µmol of ONPG-6-P (Calbiochem), 50 µmol of sodium phosphate buffer, pH 7.0, 1 µmol of DTT (Calbiochem), and enzyme in a total volume of 1.0 ml. Reactions were terminated by the addition of 1.0 ml of 0.5 M Na2CO3 [McDonald et al,1974]. In this experiment, slight changes were made in terms of substrate concentration 20mM ONPG in phosphate buffer pH 7.0. 0.2 M of sodium carbonate solution was used to stop the reaction.

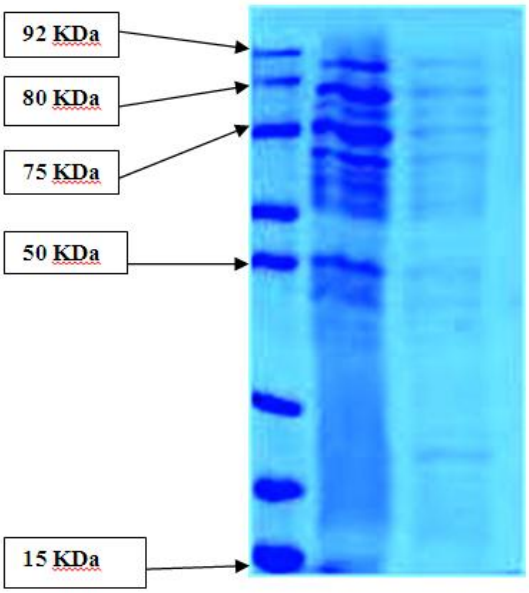

Fig 16: Various bands showed after each precipitation.

(Lane 1: Marker, Lane 2: Dialyzed sample)

Characterization of the partially purified enzyme

Both the temperature and pH has a profound influence on the activity of the enzyme.

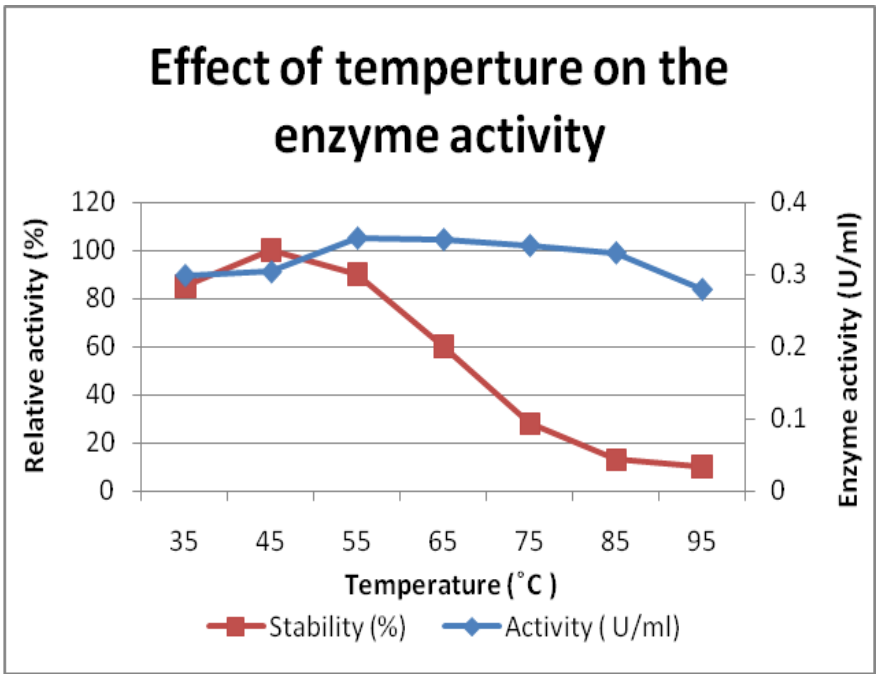

Fig 17: The effect of temperature on the activity and stability of partially purified enzyme.

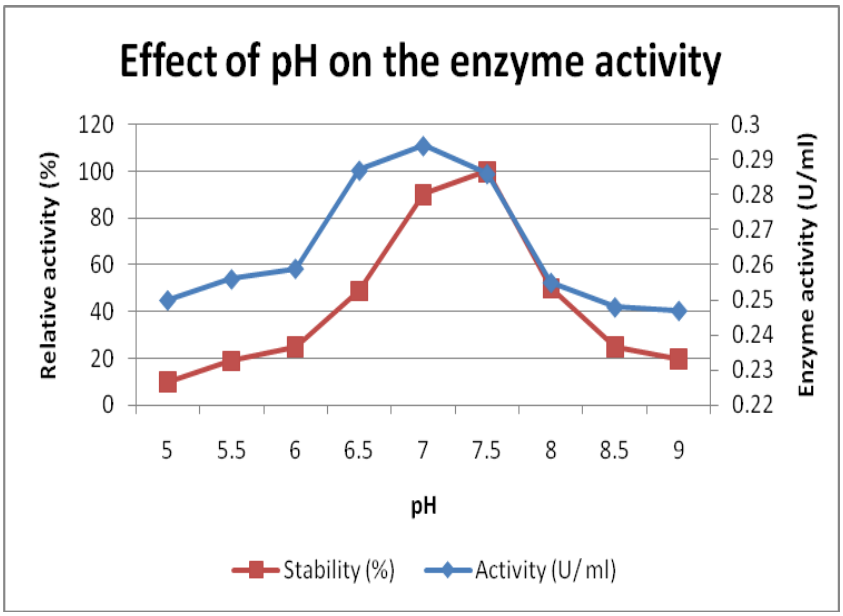

Fig 18: The effect of pH on the activity and stability of partially purified enzyme

In this method, the enzyme was incubated at different temperatures 35-95°C and maintained at different pH 5-9. The activity of the enzyme was found to be maximum at pH 7 (0.294 U/ml) and at 55°C (0.350 U/ml) (Fig: 17).

SUMMARY

The *Bacillus subtilis* strain D4 was isolated from dairy industry effluent collected from the Heritage food India Ltd, Ambattur, Chennai. The isolated strain was screened for the production of β-Galactosidase enzyme by using X-Gal as a substrate. The strain D4 was characterized based on Grams staining, Oxidase test, Motility Test and the presence of endospores. The genomic DNA extraction was performed for further use in 16Sr RNA Sequencing. The systematic position of bacterial culture was determined and BLAST homology analysis revealed that the sequence of *Bacillus subtilis* strain D4 showed 100% sequence identity with *Bacillus subtilis* from the database.

Furthermore, the strainD4 has been tested for its antibiotic susceptibility to determine the probiotic nature of the isolate which can be used in dairy industry for lactose intolerance people. The medium was optimized for the maximum production of β-Galactosidase based on various parameters like incubation time, temperature, pH and the effect of the suplementation of carbon, nitrogen, and aminoacid sources to the medium and the assay was performed using ONPG as substrate where the release of o-Nitro phenol produced a yellow colour read at 420 nm. Results showed that pH 7 and temperature 35ºC is an optimum environmental parameter for the growth of the strain and for its better production. In addition to this, xylose was found to be better carbon source and yeast extract as better nitrogen source for better production of β-Galactosidase.

The enzyme was purified by acetone and ammonium sulphate precipitation & dialysis. The protein profile in SDS-PAGE shows the presence of β-Galactosidase in partially purified culture crude by showing the suitable bands. Finally, the partially purified enzyme was characterized based on the temperature and pH using ONPG as a substrate. Results showed that the enzyme was active at pH 7 and showed 90% activity and when incubated at 55˚C retained 100% of its activity. The future work focuses on the formulation of β-galactosidase tablets to reduce the lactose in milk. This would definitely be a boon to lactose intolerant people in future.

CONCLUSION

The *Bacillus subtilis strain* D4 was isolated from the dairy industry effluent and exhibited an antagonist property which proved the probiotic nature of the isolated strain. The enzyme β-Galactosidase was isolated, screened and characterized. This shows that it is an ideal candidate for hydrolysis of lactose in milk which can be used for lactose intolerant people.

REFERENCES

1. Ahn, J.K. and Kim, H.U. Studies on the thermostable beta-galactosidase of *Bacillus coagulans* ATCC 8038 utilizable in dairy products. Korean J. An. Sci, 1977; 19(3): 220-226.
2. Ammini Parvathi, Kiran Krishna, Jiya Jose, Neetha Joseph and Santha Nair. Biochemical and molecular characterization of *Bacillus pumilus* isolated from coastal environment in Cochin, India. Braz. J. Microbiol, 2009; 40: 269-275.
3. Bhowmik, T. and Marth, E. H. β -galactosidase of Pediococcus species: induction. Purification, partial characterization. Appl. Microbiol. Biotechnol, 1990; 33: 317-323.
4. Brown. S., Sjoholm. C. and Kelly, R. Purification and characterization of a highly thermostable glucose isomerase produced by the extremely thermophilic eubacterium, *Thermotoga mantirna*. Biotechnol. Bioeng, 1993, 41: 878-886.
5. Chakraborti.S, R.K. Sani, D.K.Sahoo, U.C.Banerjee and R.C.Sobti. Production and Partial Characterization of a Novel beta Galactosidase from a newly isolated *Bacillus polymyxa*. Scientia Iranica, July 2003; 10(3): 279-286.
6. David. H. Green, Phil R. Wakeley, Anthony page, Andrew Barnes, Loredana baccigalupi, Ezio Ricca and Simon M. Cutting. Characterization of Two *Bacillus* Probiotics. Applied and Environmental Microbiology, 1990; 65(9): 4288-4291.
7. Fatma. Production of β-galactosidase using lactic acid bacteria and optimisation of fermentation parameters, 2007**.**
8. Firat Kara. Release and Characterization of Beta galactosidase from *Lactobacillus plantarum*, 2004.
9. Gekas, M. López-Leiva, Hydrolysis of lactose: A literature review. Process Biochem, 1985; 20: 1–12.
10. Holsinger, V. H. Applications of lactose-modified milk and whey. Food Technol, 1978; 3: 35-40.
11. Harley. S and M. Prescott. Laboratory exercises in microbiology, Mc Graw Hill, 2002; 58
12. John Wiley & Sons (Chichester, UK). Molecular biological methods for Bacillus, 1990.
13. Kang S.K. Three forms of thermostable lactose-hydrolase from *Thermus* sp. IB-21: Cloning, expression, and enzyme characterization. Journal of Biotechnology, 2005; 116: 337–346.
14. Liu W.Y., Shi Y.w., Wang X.q., Lou K. Isolation and identification of a strain producing cold adapted β-galactosidase, and purification and characterization of the enzyme. Czech J. Food Sci, 2008; 26: 284–290.

15. Mahoney, R. R. Modification of lactose and lactose-containing dairy products with β-galactosidase. In: Developments in Dairy Chemistry (P.F. Fox, ed.). Elsevier Applied Science Publishers, New York, 1985; 3: 69-110.
16. Miller, G., Jarvis, J. and McBean L.D. Lactose Intolerance. In: Handbook of Dairy Foods and Nutrition. CRC Press, Boca Raton. FL, 1995; 187-220.
17. Nicholson.W and P. Setlow. Molecular biological methods for *Bacillus*, 1990.
18. Panesar. R, P.S.Panesar, R.S.Singh, J.F. Kennedy, M.B.Bera. Process optimization of beta –D- Galactosidase Production using Yeast Culture. Journal of Biological Sciences, 2006; 6(1): 193-197.
19. Ram Kumar Dhaked, Syed Imtyaz Alam and Lokendra Singh. Characterization of beta-galactosidase from an Antarctic *Bacillus* sp. Indian Journal of Biotechnology, April 2005; 4: 227-231.
20. Robert Kranz, Kathleen Weston-Hafer, and Eric Richards. Identifying Unknown Bacteria Using Biochemical and Molecular Methods, 2006.
21. Somyos Osiriphun and Phimchanok Jaturapiree. Isolation and characterization of β-galactosidase from the thermophile B1.2. As. J. Food Ag-Ind, Feb 2010; 2(04): 135-143.
22. Sreekumar. G and Soundarajan Krishnan. Isolation and characterization of probiotic *Bacillus subtilis* SK09 from dairy effluent. Indian Journal of Science and Technology, August 2010; 3: 863-866.
23. Townley, R. R. Disaccharidase deficiency in infancy and childhood. Pediatrics, 1966; 38(1): 127-141.
24. Vijay Anand.S, J. Hemapriya, Joseph Selvin and Shegal Kiran. Production and Optimization of Haloalkaliphilic Protease by an Extremophile-Halobacterium Sp. Js1, Isolated from Thalassohaline Environment. Global Journal of Biotechnology & Biochemistry, 2010; 5(1): 44-49.
25. Yamamoto Y, Saito T and Ajisaka K. Study of the regioselectivity in the transglycosylation to D-galactose derivatives using β-galactosidases of various origins, 2004; 5: 125-137.
26. Zolta´n Nagy, Tunde Kiss, Attila Szentirmai, and Sandor Biro. β-Galactosidase of *Penicillium chrysogenum*: Production, Purification, and Characterization of the Enzyme. Protein Expression and Purification, September 2000; 21: 24–29.