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Isolation and Characterization of β-Galactosidase from Mangrove Soil derived Bacteria

Selvaraj Uthra[1]; Selvakumar Priyadharshini[2]; Ramachandiran Sivaramakrishnan[1]; Thirukumar Sangeshwari[1] and Muthuvel Arumugam[1]*

[*1]Faculty of Marine Sciences, Centre of Advanced Study in Marine Biology, Annamalai University, Tamil Nadu, India.
[2]AVC arts and science college, Mannampandal, Mayiladudurai, Tamilnadu, India

Abstract: Present study demonstrated the isolation of promising β-galactosidase producing bacterial strain from mangrove soil. 16s rRNA sequence analysis identified the bacterial strains as *Steptococcusthermophillus* and *Escherichia coli*. Upon growth studies *S. thermophilus* and *E. coli* entered the logarithmic phase at around 48 and 36 hours of incubation with an enzyme activity of 183.28% and 171.76%respectively at 37 °C. Role of pH, temperature and nutrient supplements were determined and the effective pH and temperature were found to be (40°C and pH 6) and (30°C and pH 7) respectively. Simultaneously, SDS-PAGE was subjected to identify the molecular weight of β-galactosidase as (116kDa).

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1.INTRODUCTION

Marine organisms represent a promising source for natural products of the future due to the incredible diversity of chemical compounds that were isolated. The oceans, which cover almost 70% of the earth's surface and over 90% of volume of its crust (Fenical, 1993; Whitehead, 1999), contain a variety of species, many of which have no terrestrial counterparts. 34 of the 36 phyla of life are represented in oceans in contrast to 17 phyla representing the terrestrial environment (Faulkner, 2002). The pioneers of marine microbiology, such as Claude Zobell, became active in delineating the vast numbers and diversity of true marine bacteria. One of the early isolations of secondary metabolites from marine sources was the isolation of cephalosporin in 1948 by Brotzu. Cephalosporin (cephalosporin C) was isolated from the fungus *Cephalosporium acremonium.* Burkholder and his co-workers had isolated the first marine metabolite from the bacterium *Pseudomonas bromoutilis*, the highly brominated pyrrole antibiotic pentabromopseudiline (Burkholder *et al.*, 1966).The systematic investigations of marine environment as sources of novel biologicallyactive agents began intensively in the mid-1970s. Among the many phyla found in the oceans, bacteria (including cyanobacteria), fungi, certain group of algae, sponges, coelenterates, seahares, bryozoans, tunicates and nudibranchs were the most studied organisms.

The search for new bioactive chemicals from marine organisms resulted in the isolationof about 10000 metabolites (Kelecom, 2002), many of which are potential biomedicals. These agents show a broad spectrum of biological activities. These microorganisms were isolated from seawater, sediments, algae and marineinvertebrates.

Polysaccharide specifc β-galactosidases include β-galactanases, which attack pectic polymers and β-galactosidases that attack xyloglucans. These enzymes have two main applications; the removal of lactose from milk products for lactose intolerant people and production of galactosylated products (Hsu, Yu, and Chou, 2005; Heyman, 2006; Neriet al., 2008). β-galactosidase is widely used in the food industry to improve sweetness, solubility, flavor and digestibility of dairy products (Richmond, Gray, and Stine, 1981; Grosova et al., 2008a).

β Galactosidases are found in microorganisms (bacteria, fungi, yeasts), plants especially in almonds, peaches, apricots, apples and animal organs (Nagy et al., 2001; Flood and Kondo, 2004; Haider and Husain, 2007a). The major industrial enzymes are obtained from *Aspergillus*sp. and *Kluyveromyces*sp. β Galactosidase of *Kluyveromyceslactis*is most commercially available enzymes (Zhou and Chen, 2001a; Jurado et al., 2002; Lee et al., 2003; Klewicki, 2007).Since β-galactosidase or lactase is an intracellular enzyme, one of the major hindrances in effective production of this enzyme is the release of enzymes in sufficient quantities from the cells (Panesar et al., 2006).

It has been established that the industrial application of β-gal is hampered by the difficulty and cost of extracting and purification of active enzyme in sufficient quantity from bacteria and yeast cells (Panesar et al., 2006). Thus, a major drawback in the use of whole cells as a source of β-gal is the poor permeability of the β-gal through the cell membrane. Different methods have been applied to increase lactase permeability through microbial cells (Panesar et al., 2006). It is important to evaluate the ideal method in terms of efficacy and enzyme yield so that the process could be scaled up to at commercial levels.This study investigated the production of β-Galactosidase (β-gal) from *streptococcus thermophilus* and *Escherchia coli*from marine source and optimizing its nutrient sources.

2. MATERIALS AND METHODS

2.1 Sample Collection and Strain Screening

The soil sample was collected from the Vellar estuary, Parangipettai in a sterile Falcon tube (30 ml) and brought to the laboratory immediately for further processing. Then 1g of the soil was diluted in 10ml of distilled water and serial diluted up to 11 concentrations from 10^{-2} to 10^{-12}. Aliquots (100µl) from the sample were plated on Nutrient Agar (NA) plates and incubated at 37 °C for 24 hours. The isolated bacteria were screened on MRS Agar plates [Constituents in g/l; Tryptone-15, Soytone-5, NaCl-5, Agar-15and 0.1% of 5-bromo-4-chloro-3-indolyl-β-d-galactopyranoside (X-gal)] for determining β-galactosidase activity (Kumar et al., 2017).

2.2 Characterization of β-galactosidase strain

2.2.1 Gram's Staining

The microorganisms were subjected to Gram's staining for determining the Gram nature. The Gram-positive would appear purple and Gram negative would appear red under the microscopic examination.

2.2.2 Catalase Activity

Most microorganisms that grow aerobically possess the enzyme, catalase. The isolated strains were checked for the production of the enzyme catalase. Colonies grown on MRS agar plates were mixed with 1 ml of 3% H_2O_2 solution and observed for effervescence formation. The effervescence is a result of free oxygen bubbles and indicates the presence of catalase (Liu et al., 2008).

2.2.3 16S rRNA Sequencing

Single isolated colony of the strain was taken from the agar plate and suspended in 50µl of lysis buffer (10mM Tris-HCl, pH 7.5; 10mM EDTA and 50µl/ml of proteinase K). The reaction mixture was then incubated at 55°C for 15min followed by proteinaseK inactivation at 80 °C for 10 min. The reaction mixture was then centrifuged at 15,000 rpm at 4 °C for 15 min. The supernatant that contains genomic DNA was directly used as template in PCR reaction. PCR amplification of almost full-length 16S rRNA gene was carried out with eubacteria specific primer set 16F27N (5′-CCAGAGTTTGATCMTGGCTCAG-3′) and 16R1525XP (5′-TTCTGCAGTCTAGAAGGAGGTGWTCCAGGC-3′) . A 25µl reaction volume PCR was performed using about 10ng of the genomic DNA, 1X reaction buffer (10mM Tris-HCL, pH 8.8 at 25°C, 1.5mM MgCl2, 50mM KCl and 0.1% Triton X-100), 0.4mM deoxynucleoside triphosphates (Invitrogen), 0.5U DNA Polymerase (New England Labs, UK). The PCR was performed in an automated Gene Amp PCR system 9700 thermal cyclers (Applied Bio systems, Foster City, USA) under the following conditions. The amplification conditions were as follows: 94 °C for 1 min (denaturation), 55 °C for 1 min (annealing), 72 °C for 1.30 min (elongation) at and 72 °C for 10 min final elongation. Expected PCR product of around 1.5 Kb was checked by electrophoresis with 5µl of the PCR product on 1% agarose gel in 1X TBE buffer and stained with ethidium bromide 0.5µl/ml. The PCR product was precipitated by PEGNaCl (20% PEG in 2.5 M NaCl) precipitation at 37°C for 30 min. The reaction mixture was centrifuged at 12,000 rpm for 30 min at room temperature. The supernatant was discarded and the pellet was washed twice with 70% ethanol. After drying the pellet was resuspended in 5µl of sterile nuclease-free water. One microliter (~ 50ng) of purified PCR product was sequenced as described earlier. The analysis of the sequence was done at NCBI server (http:// www.ncbi.nlm.nih.gov/BLAST) whereas the 42 alignment of the sequence was done using CLUSTALW programmed at European Bioinformatics site (http:// www.ebi.eic.uk/clustalw). Trees were constructed using the MEGA Software version 3.1.

2.3 Growth Studies

S. thermophilus and *E. coli* were used for the growth studies. The MRS medium was used for growth studies which includes calculation of cell dry weight, change in turbidity of medium and β-galactosidase activity of *E. coli and S. thermophilus*. For inoculum preparation, *E.coli and S. thermophilus* strain was subculture successively three times in MRS broth and then three times in modified MRS broth (Appendix A) for 14 h at 37^0 C. During the transfers, 200 µL of samples were transferred into 10 ml fresh medium.

Optical density (OD_{600}) of inoculum culture (10 ml) was measured before the inoculation into 600 ml modified MRS medium at pH 6.35. Inoculum culture was diluted with peptone water. Dilutions were carried out by mixing 10 ml of peptone water with 10 ml of inoculum culture. Blank solutions were prepared by mixing 10 ml of peptone water with 10 ml of sterile modified MRS medium. Readings were recorded against the blank solution. Optical density of diluted inoculum was measured at 600 nm. In parallel assays turbidity of inoculum culture was set to same optical density by appropriate dilution. Therefore in each assay the concentration of the culture kept constant.

From "Diluted inoculum culture", 6 ml of inoculum was transferred into 600 ml modifiedMRSmediumforgrowthcurveanalysis.Every twohours,30.4mlsample wastakenfromgrowthmedium;20mlforpHandβ-galactosidaseassay,2.4mlfor OD_{600} readings, 8 ml for cell dry weight (CDW) calculation. During the assay, readings were carried out as parallel readings in triplicate assay (Sumit et al, 2014).

Eightmillilitreofsamplefromthegrowthmediumwastransferredintotheependorf tubes (1.5 ml) with two hour periods. These samples were harvested by centrifugation at 6000 rpm for 15 min at room temperature. The pellet was washed with 8 ml distilled water one times. The pellet obtained from the second centrifugation was resuspended in 4 ml distilled water. Onemillilitreofsamplewaspouredonpre-weighedaluminiumpapers.Thesepapers were incubated at 100^0 C for 10 h. Then their weight was calculated and subtractedfromtheinitialweighttofindthedryweightof 1mlsample.

Protein content was determined according to the method of Lowry et al., 1951 using Bovine Serum Albumin (BSA) as standard. The samples were read at 600 nm using a double beam UV – visible spectrophotometer (model SL 164, Elico, Hyderabad, India). The sample analyzes were performed against respective blank solutions. Protein concentration readings were taken in triplicate and an average value was used for the calculation.

2.4 Effects of temperature and pH on enzymatic activity

The effect of temperature on β-galactosidase activity was examined by incubation of the enzyme and the substrate (10mM ONPG in 0.1 M sodium phosphate buffer pH 6.8) at temperatures ranging from 30 to 60 °C. Further, the effect of pH was determined on 45 °C by varying buffers used for substrate preparation. Sodium acetate buffer (pH 4.0–8) and were used for this purpose. The enzyme activity measurement was conducted in the previously described manner (Basha et al.,1990).

2.5 Role of Carbon and Nitrogen sources:

Carbon sources such as glucose, mannose, galactose, glucose with mannose and galactose with mannose were tested at 1% (w/v) Eightmilliliterofsamplefromthegrowthmediumwastransferredintotheependorf tubes (1.5 ml) with 2 h periods. These samples were harvested by centrifugation at 6000 rpm for 15 min at room temperature. The pellet was washed with 8 ml distilled water 1 time. The pellet obtained from the second centrifugation was resuspended in 4 ml distilled water. Onemillilitreofsamplewaspouredonpre-weightedaluminiumpapers.Thesepapers were incubated at 100^0 C for 10 hours. Then their weight was calculated and subtractedfromtheinitialweighttofindthedryweightof 1mlsample.Nitrogen sources like peptone, beef extract, sodium carbonate, ammonium sulphate and ammonium chloride were also tested at 1% (w/v) concentration.[Kazemi et al., 2004]

X.6 β-galactosidase Production Test.

X-gal (5-bromo-4-chloro-3-indoxyl-β-D-galactopyranoside) is a substrate, which has been used to screen β-galactosidase positive organisms. 50µl of X-gal (20mg in N-N dimethyl form amide) solution was poured over the MRS agar. This medium was incubated at 37 °C for 1 h. 50µl from each strain was poured over MRS agar. Medium were incubated for 24 h for color formation. X-gal forms blue color if the culture has β-galactosidase activity (Miller et al., 1972).

2.7Molecular weight determination of β-galactosidase from E.coli and S. thermophilus

The purity of enzymatically active pools from the various steps of purification and the crude preparation were analyzed by SDS-PAGE system using resolving gel (12%) and stacking gel (5%). After electrophoretic run, the proteins were stained by Coomassie brilliant blue and destained using methanol–water containing 10% acetic acid. Finally, the gel was observed in white illuminator for bands. A purple band on gel indicated the presence of enzyme. The molecular mass was estimated by comparing its mobility with those of the standard proteins.

3. RESULTS

3.1 Strain Screening

The soil sample from Vellar Estuary was investigated as a source of β-galactosidase producing bacteria. The 11 dilutions of sample yielded a consortium of microorganisms. Among which, 8 bacteria and 2 fungi samples were predominantly found in all these dilutions (Fig.1). From these, 2 bacterial strains were selected owing to visual morphological characteristics.

Fig.1 Bacterial strains isolated from Vellar estuary soil sample.

3.2 Characterization of β-galactosidase strain

The 2 bacterial strains were subjected to Gram staining and were identified as Gram positive and Gram-negative bacteria (Fig.2). The Gram-positive bacteria appeared as purple rods and Gram-negative bacteria appeared as pink rods under the oil immersion microscopy. Both the organisms were found to be catalase positive. The 2 selected strains also exhibited prominent blue colored colonies indicating the presence of β-galactosidase production (Fig.3).

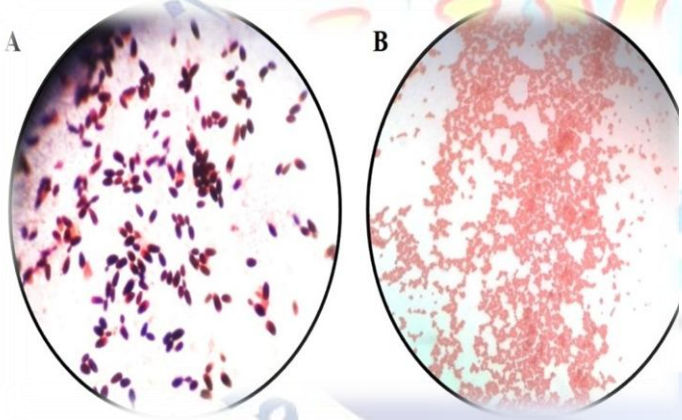


Fig.2 Gram positive bacteria (A); Gram negative bacteria (B)

Fig.3 Bacterial strains on MRS Agar indicating blue colony formation

The 16S rRNA molecular characterization of the bacterial strains revealed the presence of *S. thermophillus*Fig. 4aand *E. coli*Fig. 4b. The obtained gene sequence of the bacteria was as mentioned below:

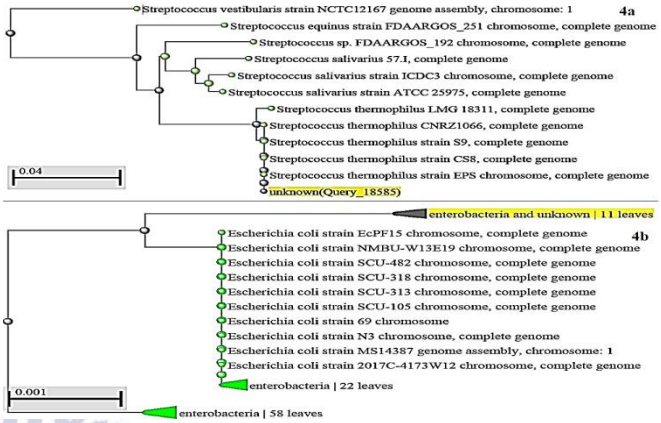
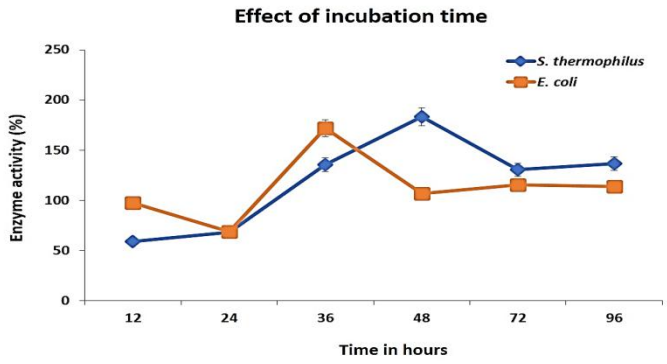


Figure 4a & 4b.Phylogenetic tree constructed for the S. thermophilus and E. coli using Neighbor joining method

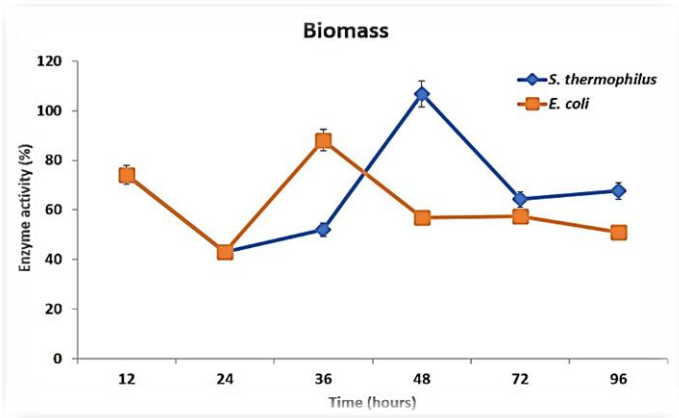
3.3 Growth Studies

Biomass and incubation time were optimized during the growth studies of *S. thermophilus* and *E. coli.* Graph 1 shows the incubation time of the selected strains observed for a period of 96 h. It was found that the strains, *S. thermophilus* and *E. coli* entered the logarithmic phase at around 48 and 36 hours of incubation with an enzyme activity of 183.28% and 171.76%respectively at 37 °C in 100 ml MRS broth.



Graph 1. Effect of incubation time on growth curves of *S. thermophilus* and *E. coli*

The correlation between biomass and enzyme activity is as illustrated in Graph 2. The strain, *S. thermophilus* exhibited maximum biomass and enzyme activity at 48 h, whereas *E. coli* attained maximum biomass and enzyme activity at 36 h. The enzyme activity for the isolates, *S. thermophilus* and *E. Coli* was found to be 106.74% and 88.04% respectively.

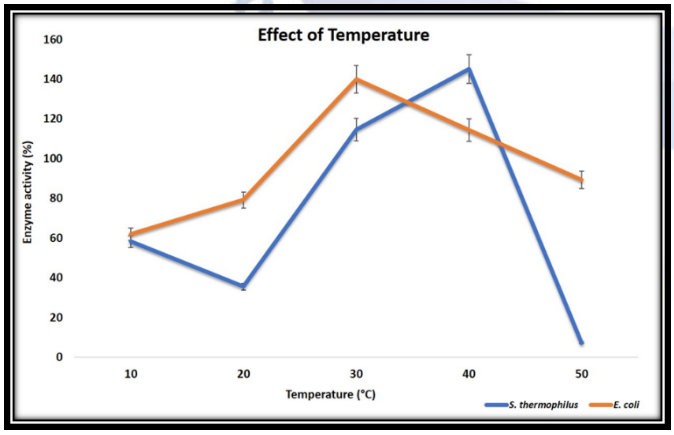


Graph 2. Effect of biomass on growth curves of *S. thermophilus* and *E. coli*

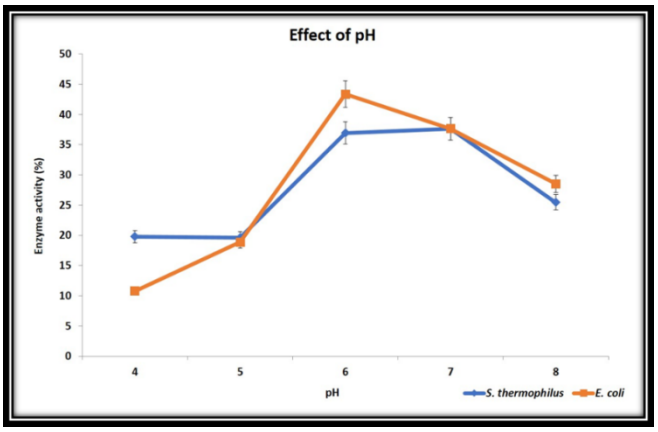
3.4 Effect of temperature and pH on enzymatic activity

The effect of temperature on the β-galactosidase enzyme activity was studied for the selected microorganisms. The optimal temperature for the β-galactosidase activity for *S. thermophilus* was determined to be 40 °C with an enzyme activity of 145.03%. The *E. coli*exhibited an optimal temperature of 30 °C for the β-galactosidase with 139.89% activity. The graphical representation of effect of temperature on β-galactosidase activity of both the strains was as depicted in Graph 3.

The enzymatic activity of β-galactosidase with respect to pH was studied and illustrated in Graph 4. The graph shows that pH 6 and 7 are the optimum pH for the enzymes from the isolates, *S. thermophilus* and *E. coli*with an enzyme activity of 37.64% and 43.36% respectively.



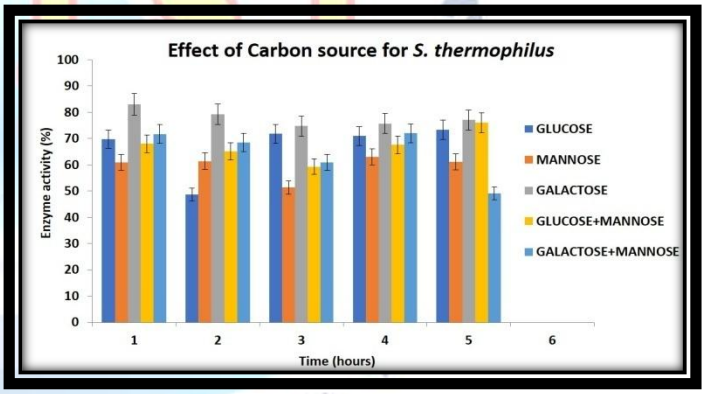
Graph 3. Effect of temperature on β-galactosidase activity of *S. thermophilus* and *E. coli*



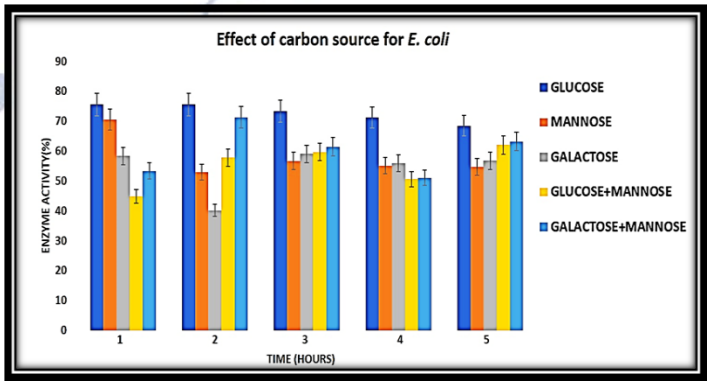
Graph 4. Effect of pH on β-galactosidase activity of *S. thermophilus* and *E. coli*

3.5 Role of Carbon and Nitrogen source

The effect of various carbon sources on β-galactosidase of selected strainswas studied. Glucose, mannose, galactose, glucose+mannose and galactose+mannose were the sugars used in this work. Galactose significantly influenced the β-galactosidase of *S. thermophilus* with a maximum activity as 82.97% at the 1st h (Graph 5). Whereas, β-galactosidase of *E. coli* recorded highest enzyme activity, 75.56% at the 1st h under the effect of sugar, glucose (Graph 6).



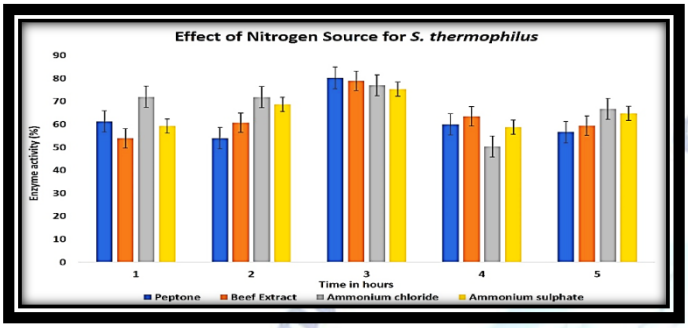
Graph 5. Effect of Carbon sources on β-galactosidase activity of *S. thermophilus*



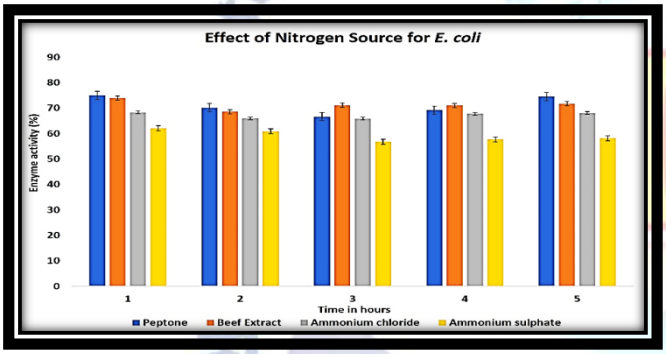
Graph 6. Effect of carbon source on β-galactosidase activity of *E. coli*

Various sources of Nitrogen were investigated in this study to observe their effect on β-galactosidase from

selected strains. Nitrogen sources like Peptone, beef extract, Ammonium chloride and Ammonium Sulphate were used in this study. Peptone was found to be the most influential source for the β-galactosidase of *S. thermophilus* with a maximum enzyme activity recorded at the 3rd hour as 80.24% (Graph 7). Whereas, β-galactosidase of *E. coli* recorded maximum enzyme activity, 74.54% at the 5th hour due to the effect of peptone (Graph 8).



Grap7. Effect of Nitrogen sources on β-galactosidase activity of *S. thermophilus*



Graph 8. Effect of Nitrogen sources on β-galactosidase activity of *E. coli*.

3.6. SDS-PAGE:

The molecular weight of β-galactosidase was determined in comparison with the commercially available β-galactosidase. Both the standard and sample had a molecular weight of 116 kDa (Fig. 5).

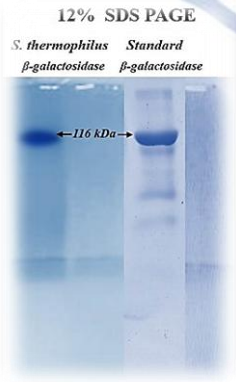


Fig.5. SDS-PAGE analysis of β-galactosidase from *Streptococcus thermophilus*

4. DISCUSSION

The estuarine soil is a rich source for microbes that produce novel bioactive compounds. Beta galactosidases have been obtained from microbes such as fungi, bacteria and yeasts; plants, animal's cells, and from recombinant sources. Lactose has a low relative sweetness and solubility, and excessive lactose in large intestine can lead to tissue dehydration due to osmotic effects, poor calcium absorption due to low acidity, and fermentation of the lactose by micro flora resulting in fermentative diarrhea, bloating, flatulence, blanching and cramps, and watery diarrhea (Shukla and Wierzbicki, 1975). Furthermore, lactose is a hygroscopic sugar and has a strong tendency to absorb flavors and odours and causes many defects in refrigerated foods such as crystallization in dairy foods, development of sandy or gritty texture, and deposit formation (Carrara and Rubiolo, 1994).

In this work, microbes were isolated from the soil of Vellar estuary, Parangipettai through the serial dilution-plating method and screened for the β-galactosidase activity. Among these, 10 bacteria and 2 fungi were predominantly present in all the dilutions. From the bacterial population, 2 bacteria were identified as potent β-galactosidase producers and characterized. The selected 2 bacterial strains were Gram positive and Gram negative rods in nature exhibiting purple and pink colonies respectively. Selected strains were also found to be catalase positive and showed dense and intense blue color colonies indicating high β-galactosidase enzyme production. In conglomeration with the present study, it has been stated in the literature that soil samples from Vellar estuary are rich habitat for microbial diversity (Dhanasekaran et al., 2008; Selvi and Kanthamani, 2017). Previously, Sharma and Singh, 2014 had reported Gram positive, *Lactobacillus delbrueckii*from curd sample with potential β-galase activity. Further, Gram negative and catalase positive, *Thermus thermophilus* KNOUC112 was isolated from hot springs of avolcanic area near Golden Springs in New Zealand with β-galase potential (Nam et al., 2004). Similar blue color colony formation was exhibited by Deep-sea bacterium *Alteromonas* sp. ML52 that was used for the production of novel Cold-adapted β-galactosidase from the deep-sea water, Egypt (Sun et al., 2018).

S. thermophilus and *E. coli* were the bacteria identified through the 16S rRNA sequencing of the selected strains. The use of 16S rRNA gene sequences to study bacterial phylogeny and taxonomy has been by far the most common housekeeping genetic marker used for a number of reasons (Janda and Abbott, 2007). *S. thermophilus* and *E. coli*have been previously reported to be potential producers of β-galactosidase and been isolated from various sources. Efficiency of different methods for disruption of *S. thermophilus* cells, isolated from different dairy products, to release β-galactosidase and synthesis of GOS by extracted enzyme using whey supplemented with different concentrations of lactose as a substrate was studied (Sangwan et al., 2015). Rao and Dutta, 1977 reported beta-D-Galactosidase extracted from *S. thermophilus* grown in deproteinized cheese whey.Overproduction of β-galactosidase enzyme from *E. coli* through genetic improvement was described by Khedr et al., 2013. Liu and Roffler (2006) have examined the expression of *E. coli* beta-galactosidase in muscle fibers and concluded that repeated intramuscular injections of beta-galactosidase can encourage strong immune responses in immune-competent animals and cause abolition of transduced muscle fibers by inflammatory cells.

Growth studies were performed to analyze the optimum parameters for the bacterial growth. The *S. thermophilus* and *E. coli* entered the late logarithmic phase at around 48 and 36 hours of incubation with an enzyme activity of 183.28% and 171.76%respectively at 37 °C in 100 ml MRS broth. Similar to this, *Lactobacillus plantarum* maximum total β-galactosidase activity corresponds to the early stationary phase for three of the strain (Kara, 2004). The decline in total enzyme activity could be considered as a result of inhibition of cellular functions due to high pH, depletion of a nutritional factor from the growth medium,deactivation of the enzyme due to low pH catabolite repression, or/and inducer exclusion. The optimal temperature and pH for the growth of the two bacteria were also standardized. *S. thermophilus* exhibited maximum enzymatic activity at 40 °C and pH 6. Likewise, *E. coli* possessed maximum β-galactosidase production at pH 7 and 30 °C. The *S. thermophilus* from yogurt samples exhibited maximum activity at pH 7.2 and temperature was 40ºC (Princey et al., 2013).

Various carbon and nitrogen sources were employed for determining the optimum ingredient for the enhanced β-galactosidase production. Galactose and glucose was determined to be the most influential carbon source for *S. thermophilus* and *E. coli* respectively. Likewise, peptone was found to be the most reliable source of nitrogen for bith the bacteria with regards to β-galactosidase activity. The present result was similar to the findings of Kamel et al., 2016, where they illustrated that Galactose, lactose, tryptone and yeast extract were the best carbon and nitrogen sources for enzyme production*by Bacillus megaterium* NM56 Isolated from raw milk. Likewise, the optimal production of α- and β-galactosidases was obtained with soybean oligosaccharides as a carbon source andprotease peptone no. 3 as a nitrogen sourcefor*Bifid bacterium longum subsp. longum* RD47 (Han et al., 2014).

The molecular weight of β-galactosidase was determined in comparison with the commercially available β-galactosidase by the SDS-PAGE analysis. Both the standard and sample had a molecular weight of 116 kDa. Similar molecular weight was expressed by the 116 kDa protein of β-galactosidase activity produced by the recombinant viruses (Kim et al., 2000). The presence of individual band near the molecular weight of 70 kDa indicates the presence of β-galactosidase (Kumar et al., 2012) which is lower than the molecular weight of *S. thermophilus* β-galactosidase.

5. CONCLUSION:

The use of enzymes in industrial sector promisingly increased due to exhaustive industrialization. The present findings demonstrated the isolation characterization, identification and optimization of β-galactosidase production by the bacterial strains *Streptococcus thermophilus* and *Escherichia coli.* To overcome this complicationslike cost and time, we have optimized the culture parameters for optimal production of enzymes.

6. ACKNOWLEDGMENTS

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Earth Science (MoES), [G4(2)/14748/2016] Under Drugs from the Sea] and the authorities of Annamalai University for providing necessary facilities.

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