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ISOLATION AND CHARACTERIZATION OF β-GALACTOSIDASE PRODUCING *BACILLUS SUBTILIS* FROM MILK

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ABSTRACT

The enzyme β-galactosidase has tremendous potential in research and application in various fields like food, bioremediation, biosensor, diagnosis and treatment of disorders. The sources of the β-galactosidase are microorganisms, plants and animals. In the present study β-galactosidase producing bacteria was isolated from milk and milk products. Bacteria were tested for their ability to hydrolyze 5-bromo-4-chloro-3-indolyl-β-D-galactosidase (X-Gal) and O-nitro phenyl-β-D-galactopyranoside (ONPG). Further, it was identified by conventional methods and 16S rRNA gene sequencing as *Bacillus subtilis*. The production of β-galactosidase by *Bacillus subtilis* was found to be optimum at temperature of 37°C and pH -7.0 after 48 hrs of incubation. The various carbon, nitrogen and metal ion sources used in the present study such as Xylose, Yeast extract and $MgSO_4.7H_2O$ ion increased the production of enzymes. Under optimal conditions, β-galactosidase enzyme produced by *Bacillus subtilis* was partially purified using ammonium sulphate precipitation, dialysis and SDS-PAGE in the molecular weight of the enzyme was found to be ~ 60 kDa.

Key words: β-Galactosidase, *Bacillus subtilis*, X-Gal, ONPG, Optimization, Purification.

INTRODUCTION

β-galactosidases enzyme has been used in the dairy industry for the improvement of lactose intolerance. It is used in the preparation of lactose hydrolyzed milk suitable for lactose intolerant people [1, 2].Lactose also known as β-galactosidase (E.C 3.2.1.23) is an enzyme

that hydrolyzes lactose to glucose and galactose, has a potential importance in the dairy industry [3].

Lactose intolerance is common in the majority of the adult world's populations because of the refuse of the intestinal β-galactosidase activity of the maturing intestine [4, 5] and is often associated with bowel symptoms [6]. The prevalence of lactose intolerance symptoms will be influenced by the population being seen, as adult Asians and Africans have a 90% prevalence [4], adults of northern and central Europe and Caucasians in North America and Australia have 5 to 15 % of lactose intolerance as well as more over 70% of adults worldwide are lactose malabsorbers [7]. These people tend to eliminate milk and dairy products from their diet and consequently their calcium intake may be compromised. Lactase activity is high at birth, decreases in childhood, adolescence and remains low in adulthood [7]. However, the activity of intestinal lactase in lactose intolerant individuals is usually less than 10% of childhood levels [8]. Therefore, lactose hydrolysis catalyzed by β-galactosidases is of great importance in the milk and dairy industries.

Increased industrial demand for β-galactosidase requires good cost-effective production methods to ensure the economic viability of lactose hydrolysis at commercial scale [9, 10]. The overall cost of enzyme production and downstream processing is the major obstacle against the successful application of any technology in the enzyme industry [11]. The optimization of fermentation conditions, particularly physical and chemical parameters, is important in the development of fermentation processes due to their impact on the economy and practicability of the process [12]. The growth and enzyme production of the organism are strongly influenced by the media composition thus optimization of media components and cultural parameters is the primary task in a biological process [13].

In the present study β-galactosidase producing bacteria was isolated from milk and curd. Bacteria were tested for their ability to hydrolyze 5-bromo-4-chloro-3-indolyl-β-D-galactosidase (X-Gal) and O-nitro phenyl-β-D-galactopyranoside (ONPG). Further, identified by conventional methods and 16S rRNA gene sequencing as *Bacillus subtilis*.The production of β-galactosidase was optimized by physical parameter such as the incubation period, temperature, pH and chemical parameter by using different carbon, nitrogen sources and metal ions. Under optimal conditions, a β-galactosidase enzyme produced by the *Bacillus subtilis* was partially purified using ammonium sulphate precipitation, dialyzed and SDS-PAGE was run for the molecular weight analysis.

MATERIALS AND METHODS

Sample collection

Milk samples were collected from the milk processing area in Dharwad. The organisms were isolated from cow's milk and curd.

Isolation and Screening of β-galactosidase producing bacteria

Using X-Gal substrate

For isolation of the microorganisms from milk and curd were serially diluted and plated on LuriyaBertani agar plates infused with 50 µl X-Gal (5-bromo-4-chloro-3-indole-β-D-galactopyranoside; 20mg/ml of DMSO) and 10 µl IPTG (iso-propyl-thio-β-D-galactopyranoside) as an inducer to select the colonies showing lactose fermenting ability. The plates were then incubated at 37°C for 24 to 48 hrs [14]. A plate which showed colony counts between 30-300 were selected for identification and the colonies thus obtained on isolation were counted using a colony counter, the number and types of organisms were recorded for each dilution as no of CFUs ×Dilution factor. Colonies showing β-galactosidase producing blue color was chosen and the streak plate method was used to obtain pure culture. These cultures were maintained on a nutrient agar slants at -20° C.

ONPG substrate for selection of high β-galactosidase producing bacterial isolates

Isolated bacteria were inoculated into 200 µl of 4 mg/ml ONPG in 0.1 M sodium phosphate buffer (pH-7.0) and peptone water (2 ml) was incubated at 37°C for 30 min. Then, the reaction was stopped by using 500 µL of 1 M Na_2CO_3 and the cells were precipitated by centrifugation at 7,000 rpm for 5 min. The released ONP was assessed by determining the absorbance at 420 nm in UV- spectrophotometer [15].

Characterization of isolated bacteria

β-galactosidase producing bacteria were identified based on conventional methods like gram staining, spore formation and Biochemical tests. The isolate (GS-6), which showed maximum β-galactosidase activity was further confirmed by molecular identification of 16S rRNA gene sequencing.

16S rRNA gene sequencing

The genomic DNA was isolated from the bacteria by the method described by [16]. The highly purified DNA was then amplified in a thermo cycler at conditions: 35 cycles of 94°C for 1 min, 55°C for 1 min and 72°C for 2 min using the primers.

Forward:63F-5' CAG GCC TAA CAC ATG CAA GTC 3'

Reverse: 1387R-5' GGG CGG AGT GTA CAA GGC 3'

DNA sequencing was performed in a highly automated gene sequence. These sequences were read in the Genbank databases (BLAST) and compared with the other sequences to analyze the bacterial class and its phylogeny.

Preparation of inoculums

For inoculum preparation, *Bacillus subtilis* strain was sub cultured successively three times in nutrient broth for 24 hrs at 37° C during the study.

Growth studies for *Bacillus subtilis*

6 ml of the inoculum was transferred into 600 ml of nutrient broth at pH 6.35 for growth curve analyses. At regular intervals of time, 10 ml of the inoculum was taken and the optical density was measured at 600 nm using UV- spectrophotometer against an uninoculated blank.

The β-galactosidase production medium

In a 250 ml Erlenmeyer flask containing 100 ml of the liquid production medium (g/L): lactose-10g, yeast extract-1g, peptone-1.5g, KH_2PO_4-1g, $NH_4H_2PO_4$-7g, $MgSO_4.7H_2O$-1g and $CaCl_2$-0.03g [17] 1ml of 18 hrs broth culture of the organism was inoculated and incubated for 48 hrs at 37°C under 100 ppm in rotary shaker condition [18]. Crude enzyme preparation was obtained from the supernatant after centrifugation of the broth culture.

Enzyme assay

The ONP enzyme assay was done as per Miller[15].

Protein estimation by Bradford method

The protein concentration was determined by the Bradford method [19] by using bovine serum albumin as a standard.

Optimization of various parameters for β-galactosidase enzyme production

The effect of various physical and chemical parameters in the production of β-galactosidase enzyme was checked. Physical characters like incubation time, temperature and pH etc, one at a time and combination of different parameter using standard production media with variations in the carbon, nitrogen and metal ions was performed.

Effect of incubation period on β-galactosidase production

The effect of incubation period was determined by incubating production medium for different incubation periods (12, 24, 36, 48, 60, 72 and 96 hrs) at 37°C taking other conditions into consideration [20].

Effect of temperatures on β-galactosidase production

The standard production medium was inoculated and incubated at temperatures ranging from 20°C, 25°C, 30°C, 37°C, 40°C, 45°C, 50°C and 55°C to test for their effect on β-galactosidase production and the optimum temperature for maximum β-galactosidase production by *Bacillus subtilis*[20].

Effect of pH on β-galactosidase production

The standard production medium was adjusted to different pH ranging from 5.0, 5.5, 6.0, 6.5, 7.0 7.5, 8.0, 8.5 and 9.0 using 1 N Potassium chloride and Hydrochloric acid buffers and inoculated with individual test organisms was checked for the optimum pH and its effect on β-galactosidase production [21].

Effect of various carbon sources on β-galactosidase production

The isolate was grown in the production medium containing various carbon sources (1% m/v) including glucose, fructose, sucrose, xylose, lactose, maltose and galactose to study their effect on enzyme production [20].

Effect of various nitrogen sources on β-galactosidase production

The production medium was supplemented with different nitrogen source (0.1% m/v) including organic nitrogen sources such as yeast extract, tryptone, beef extract, peptone, casein and the inorganic nitrogen sources such as $(NH_4)_2$ SO_4, NH_4PO_4 to investigate their effect on enzyme production [22].

Effect of various metal ion sources on β-galactosidase production

The effect of metal salts on β-galactosidase production is determined by adding different metal salts in the fermentation medium. The metal salts selected for the present study were EDTA, $MgCl_2.6H_2O$, $MgSO_4.7H_2O$, $FeSO_4.7H_2O$, $CuSO_4.5H_2O$ and $MnSO_4.7H_2O$ (at 1mM concentration) added to the culture medium in enzyme production was determined [20,21]

Enzyme Purification of Ammonium sulphate precipitation

Protein was concentrated from the culture by salting out method using ammonium sulphate

precipitation and further purified by dialysis[23].

SDS-polyacrylamide slab gel electrophoresis (SDS-PAGE)

To determine the molecular weight of β-galactosidase enzyme, SDS–PAGE was carried out according to the procedure of Laemmli [24].

RESULTS

Isolation and characterization of β-galactosidase producing bacteria

From the total 76 bacterial strains having β-galactosidase activity were isolated from milk and curd. The present results showed that out of total 76 isolates, 43 strains showed β-galactosidase production (Fig:1) containing 2% X-gal and 100 mM IPTG. The isolate that produced maximum β-galactosidase was chosen and analyzed for further studies.

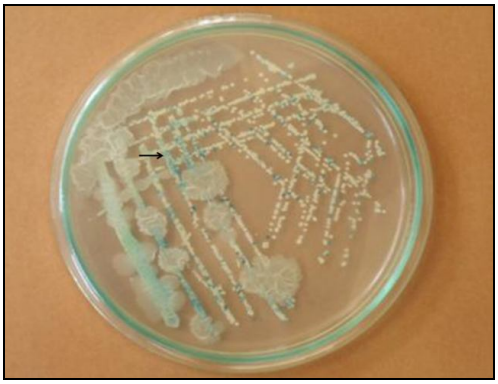

Fig:1 Colonies of *Bacillus subtilis* on X- gal plate.

The results recorded in isolation from different milk and curd samples showed bacteria in the range of 1 to 12×10^{-5} CFU/gm or ml for different samples. Seven isolates were obtained from milk sample and morphological characterizations were as further studies in GS-4 maximum number of colonies (10 CFUs)(Table:1) and Nine different isolates were obtained from curd and their morphology characterization revealed that GS-10 showed the maximum number of colonies (12 CFUs) and GS- minimum number of colonies (1 CFUs) were shown in (Table:2).

Table:1Morphological characteristics of isolates from milk.

Isolate No	Color	No. of Colonies	Whole colony appear	Margin	Elevation	Opacity	Gram's reaction
GS-1	Off white	8	Irregular	Lobate	Flat	Opaque	Gram +ve rods in group
GS-2	Yellowish	2	Irregular	Undulate	Flat	Opaque	Gram +veCocci
GS-3	Off white	5	Circular	Entire	Raised	Translucent	Gram -ve short rods

GS-4	Off white	10	Irregular	Lobate	Flat	Opaque	Gram +ve rods in chain
GS-5	Pinkish	2	Circular	Entire	Convex	Opaque	Gram -ve Rods
GS-6	Off white	2	Circular	Undulate	Flat	Translucent	Gram +ve rods in chain
GS-7	Creamish	5	Circular	Undulate	Convex	Opaque	Gram +ve short rods in chain

Table:2 Morphological characteristics of isolates from the curd.

Isolate No	Color	No. of Colonies	Whole colony appear	Margin	Elevation	Opacity	Gram's reaction
GS-8	Off white	8	Circular	Entire	Flat	Opaque	Gram -ve rods
GS-9	Off white	2	Irregular	Lobate	Raised	Opaque	Gram +ve rods in single
GS-10	Off white	12	Irregular	Lobate	Flat	Opaque	Gram +ve rods in chain
GS-11	Off white	3	Irregular	Undulate	Umbonate	Opaque	Gram +ve rods in group
GS-12	Creamish	8	Circular	Entire	Convex	Opaque	Gram +veCocci
GS-13	Off white	2	Circular	Undulate	Flat	Opaque	Gram -ve rods in group
GS-14	Orangish	3	Irregular	Undulate	Flat	Opaque	Gram +veCocci
GS-	Off white Creamish	1	Irregular	Entire	Convex	Translucent	Gram +veCocci in clusters
GS-16	Off white	2	Circular	Entire	Convex	Opaque	Gram +veCocci in clusters

ONPG substrate forselection of high β-galactosidase producing bacteria

A total of 43 isolates showed the yellow color indicating the production of β-galactosidase. However, the isolate GS-6 *Bacillus subtilis* showed a highest concentration of 1.67 U/ml ONP compared to all the other isolates.

Morphological and Biochemical characterization of isolated strain

From the morphological colony characterization of all isolated β-galactosidase producing strains were done on the basis of their colony shape, margin, surface, elevation, opacity and consistency. Out of total 76 isolates microorganisms from different samples which produce the maximum enzyme activity ONPG assay were selected for further screening out of 43 isolates, one isolate was selected for further characterization on the basis of morphology tests.

Identification through various morphological, biochemical characterizations was done as per Bergey's manual of determinative bacteriology was identified as *Bacillus* sp. (GS-6). Biochemical characterization was done only for the isolate GS-6, which was found to have characteristics of Bacillus *species*. These isolates showed positive fermentation test for Arabinose, Raffinose, Trehalose and Glucose. The isolate GS-6 was negative for pigmentation, Lysine utilization, Ornithine utilization, Urease, Phenylalanine deamination, no H_2S production results were shown (Table:3).

Table:3Morphological and Biochemical characteristics of the isolate.

Characteristics	**Results**
Colony Morphology	Circular spreading to flat
Pigmentation	-
Cell shape	Rods in chains
Gram's reaction	+
Endospore	+
ONPG	+
X-gal	+
Catalase	+
Lysine utilization	-
Ornithine utilization	-
Urease	-
Phenylalanine deamination	-
Nitrate reduction	+
H_2S production	-
Citrate utilization	+
VogesProskauer's	+
Methyl red	-
Indole	-
Malonate utilization	-
Esculin hydrolysis	-
Fermentation of carbohydrates	
Arabinose	+
Xylose	-
Adonitol	-
Rhamnose	-
Cellobiose	-
Melibiose	-
Saccharose	-
Raffinose	+
Trehalose	+
Glucose	+
Lactose	-
Oxidase	-

16S rRNA gene sequencing

To confirm the conventional identification results, 16S rRNA gene investigation was performed. The PCR product of the 16S rRNA gene (about 1500 bp) was amplified (Fig:2) and compared to the sequence with the database in Gen Bank (http://www.ncbi.nlm.nih.gov) by BLAST program (Fig:3). The alignment of 16S rRNA gene sequence of isolated GS-6 was identical *Bacillus subtilis***(accession no.NZCM000487.1)** with 98-100 % similarity.

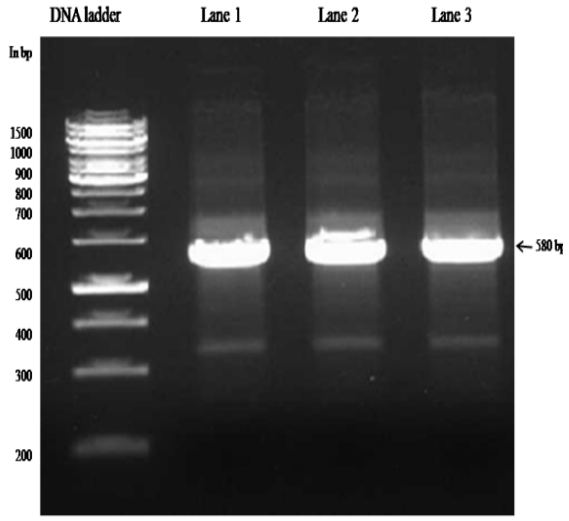

Fig:2 Photographic representation of PCR amplified product in agarose gel.

DNA ladder - 100bp ladder, Lane 1, 2 and 3. PCR amplified sample.

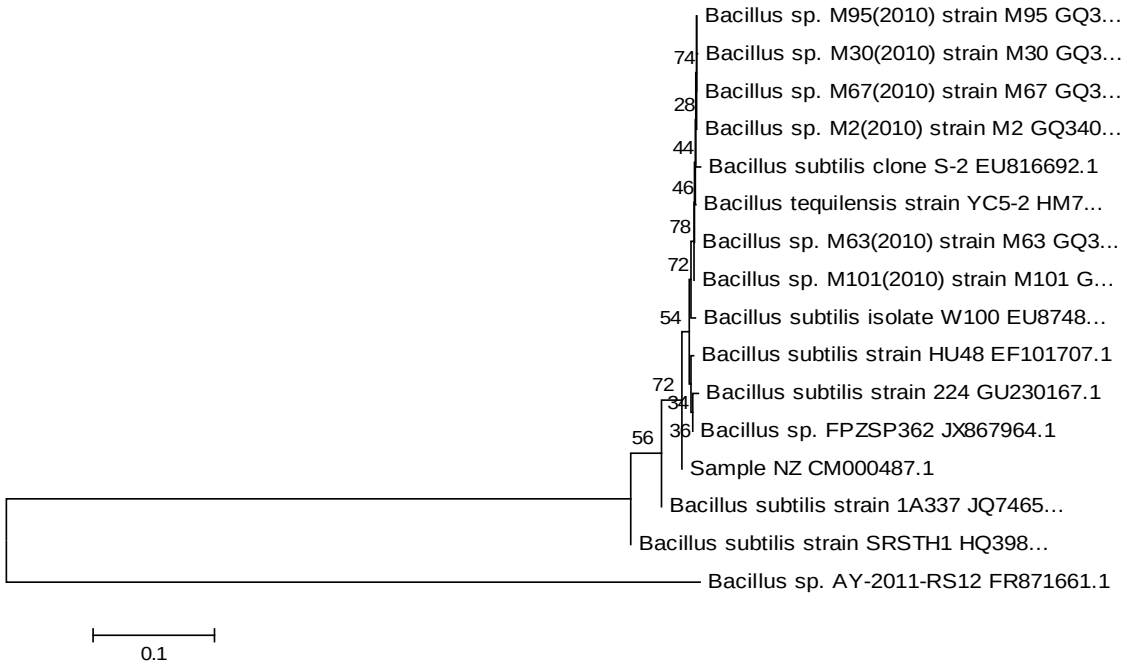

Fig:3 Phylogenetic tree of isolated *Bacillus subtilis* based on 16s rRNA gene sequence.

Growth curve of *Bacillus subtilis*

The results of the effect of incubation time on the growth of *Bacillus subtilis* is shown in (Fig:4). The duration of lag phase of about 12-30 hrs was observed for *Bacillus subtilis*, with minimum growth with OD at 600 nm 0.011 Log phase was observed in 36-60 hrs with maximum growth and OD of 1.594 was observed after 48 hrs of incubation.

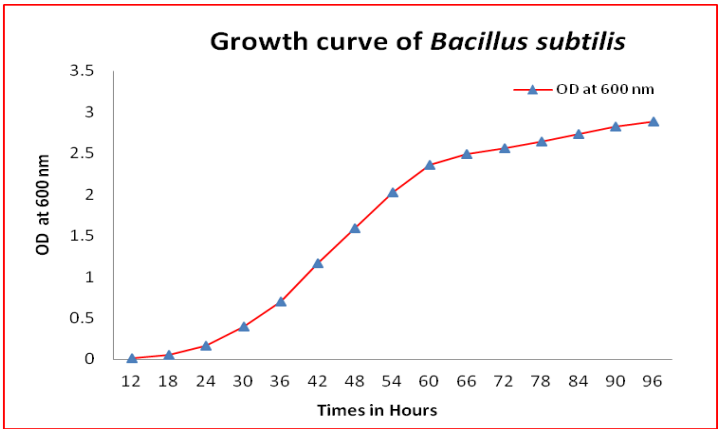

Fig:4 Growth curves of *Bacillus subtilis.*

Effect of incubation period on β-galactosidase production

Bacillus subtilis showed maximum activity of 0.065 U/ml at 12 hrs, 0.118 U/ml at 24 hrs, 0.8 U/ml at 36 hrs, 0.271 U/ml at 48 hrs, 0.235 U/ml at 60 hrs, 0.191 U/ml at 72 hrs and 0.098 U/ml at 96 hrs. Therefore, the present studies revealed that the enzyme activity increased up to 48 hrs and further increase in the incubation period decreased the enzyme activity. After 48 hrs of incubation maximum β-galactosidase activity of 0.271 U/ml was observed and minimum β-galactosidase activity of 0.065 U/ml was observed after 12 hrs of incubation. Thus, 48 hrs were considered as the optimum incubation period for the production of β-galactosidase by *Bacillus subtilis* (Fig:5).

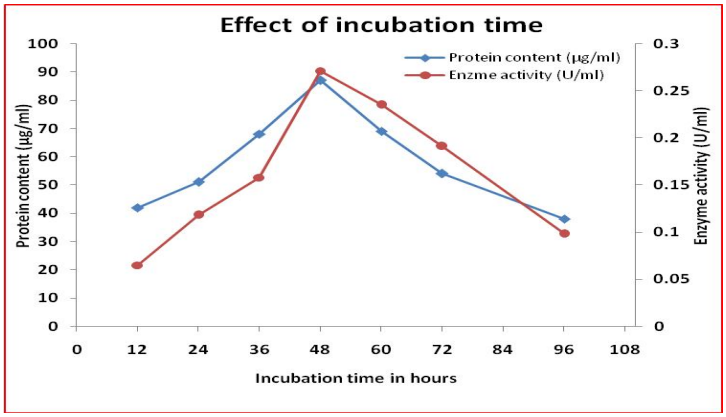

Fig:5 Effect of incubation time on β-galactosidase production.

Effect of temperature on the production of β-galactosidase

In order to find out the optimum temperature of the fermentation, *Bacillus subtilis* was incubated at various temperature ranges such as 20°C to 55°C keeping other conditions at their optimum levels. Highest activity was observed at 37°C which showed 0.253 U/ml at 48 hrs of incubation period followed by 30°C with activity of 0.214 U/ml, 40°C with activity of 0.182 U/ml, 25°C with activity of 0.174 U/ml, 20°C with activity of 0.7 U/ml and 45°C with activity of 0.2 U/ml whereas lowest enzyme was produced at 55°C with activity of 0.08 U/ml respectively at 48 hrs. The highest β-galactosidase activity of 0.253U/ml were observed in consortia at 37°C and minimum β-galactosidase activity 0.08 U/ml was observed at 55°C by (Fig:6). Thus, from the present study it was found that there was an increase in the enzyme activity up to 37°C and further increase in the temperature will decrease the enzyme activity. Hence, 37°C was considered as the optimum temperature for the production of β-galactosidase.

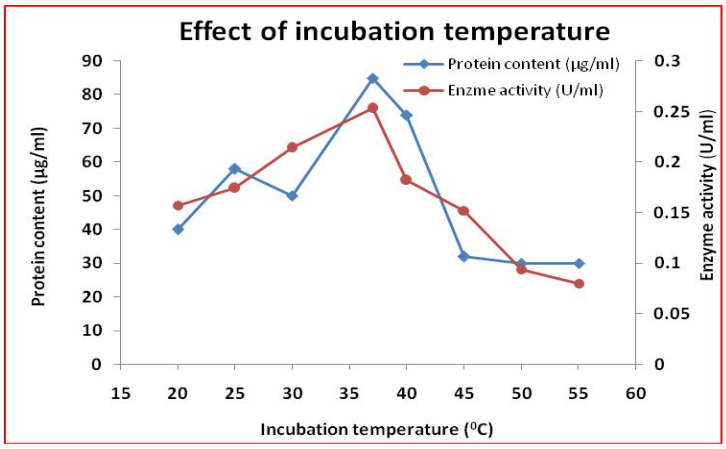

Fig:6 Effect of temperature on β-galactosidase production

Effect of pH on the production of β-galactosidase

Effect of different pH ranging values pH-5.0 to 9.0, on growth and the β-galactosidase activity of *Bacillus subtilis* was measured from the initial pH level of substrate plays crucial role in the successful β-galactosidase production. The fermentative medium pH was adjusted accordingly with 1N HCl/NaOH. Production of β-galactosidase at 48 hrs was found to be maximum at pH-7.0 with the activity of 0.243 U/ml. It was followed by pH-7.5 with maximum activity of 0.203 U/ml, pH-8.0 with maximum activity of 0.187 U/ml, pH 6.0 and pH 8.5 with activity of 0.176 U/ml, pH 5.5 with 0.165 U/ml, pH 5.0 with 0.132 U/ml and the least activity was observed at pH 9.0 with 0.111 U/ml at 48 hrs incubation period (Fig:7). The present results suggested that enzyme activity increased with an increase in the pH and

maximum activity for β-galactosidase production was observed at pH-7.0 after 48 hrs of incubation period.

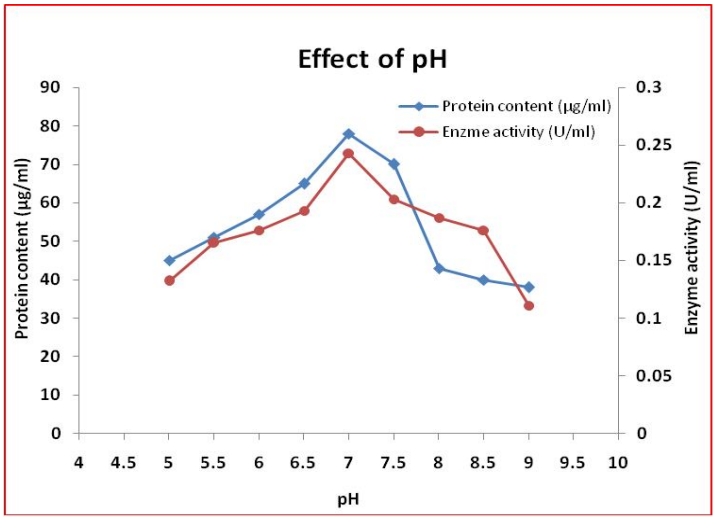

Fig:7 Effect of pH on β-galactosidase production.

Effect of various carbon sources on β-galactosidase production

Production of β-galactosidase by *Bacillus subtilis* was found to be maximum in 48 hrs. Maximum β-galactosidase activity 0.305 U/ml was observed with xylose, lactose with activity of 0.274 U/ml, galactose with 0.21 U/ml, maltose with 0.186 U/ml, sucrose with lesser activity of 0.163 U/ml, fructose with activity of 0.142 U/ml and glucose with the least activity of 0.103 U/ml respectively at 48 hrs of incubation period and minimum β-galactosidase activity with glucose. The present results suggested that optimum β-galactosidase activity was observed with xylose as a carbon source (Fig:8)

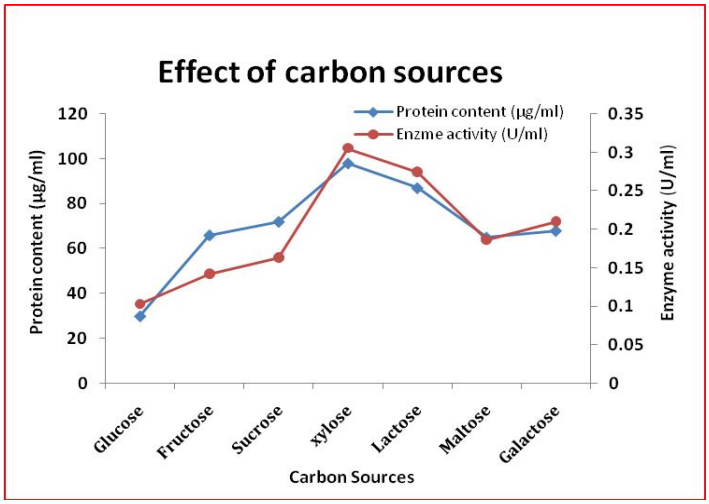

Fig:8 Effect of carbon sources on β-galactosidase production.

Effect of various nitrogen sources on β-galactosidase production

β-galactosidase activity was calculated for culture grown in different nitrogen source. Production of β-galactosidase was found to be maximum in yeast extract showing activity of 0.243 U/ml, NH_4PO_4 showed an activity of 0.204 U/ml, tryptone with activity of 0.202 U/ml, peptone with activity of 0.136 U/ml, beef extract activity of 0.123 U/ml, casein with 0.078 U/ml and $(NH_4)_2.SO_4$ minimum activity of 0.044 U/ml respectively in 48 hours of incubation period. Medium containing yeast extract showed maximum activity 0.243 U/ml and minimum activity 0.044 U/ml was observed with medium containing $(NH_4)_2.SO_4$ in *Bacillus subtilis*. Thus, among all the organic nitrogen sources, yeast extract emerged as the best organic nitrogen source for the β-galactosidase production (Fig:9).

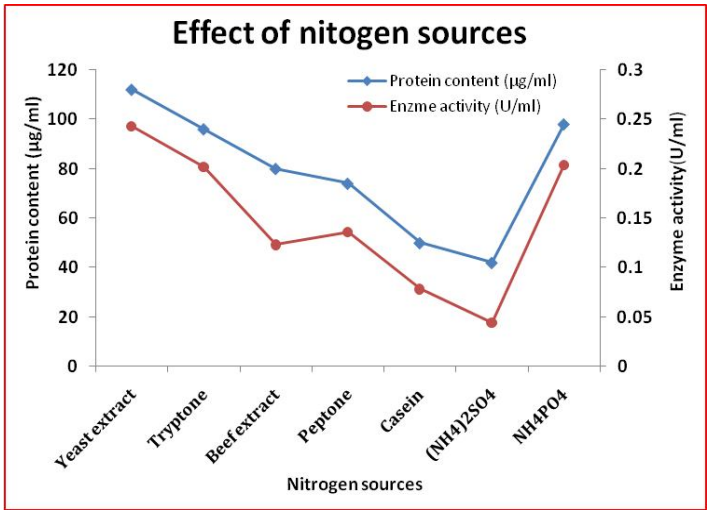

Fig:9 Effect of various nitrogen sources on β-galactosidase production.

Effect of various metal ions on β-galactosidase production

The effect of various metal ions on the β-galactosidase activity of *Bacillus subtilis* is given in (Fig:10) culture grown with $MgSO_4.7H_2O$ showed maximum β-galactosidase activity of 0.3 U/ml, $MgCl_2.6H_2O$ with activity of 0.301 U/ml, $MnSO_4.7H_2O$ with the least activity of 0.278 U/ml, EDTA with 0.223 U/ml, $FeS0_4.7H_2O$ with 0.185 U/ml and $(CuSO_4).5H_2O$ with minimum activity of 0. U/ml respectively at 48 hrs of incubation period.Medium containing $MgSO_4.7H_2O$ showed maximum activity (0.3 U/ml and minimum activity 0.044 U/ml was observed with medium containing $(CuSO_4)$. $5H_2O$ in *Bacillus subtilis*. Thus, among all the metal ions, $MgSO_4.7H_2O$ emerged as the best metal ions for the β-galactosidase production.

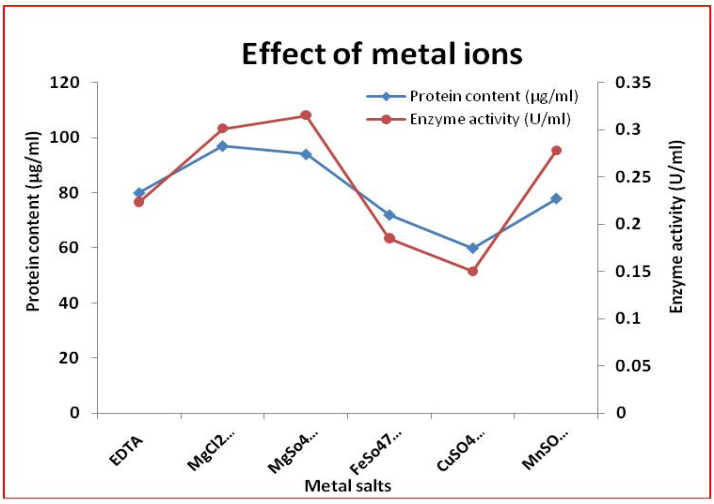

Fig:10 Effect of different metal ions on β-galactosidase production.

Purification of β-galactosidase by Ammonium sulphate precipitation

The protein purification was carried by ammonium sulphate precipitation. The crude extract showed 80 µg/ml protein and 0.280 U/ml of β-galactosidase activity. The enzyme was precipitated at 85% saturation. The perusal of the data indicated that the ammonium sulphate precipitation method showed 19.8 µg/ml protein and 0.203 U/ml. This clearly indicated that there is 2.8 fold in the protein purification with 72.5% of recovery of protein by the ammonium sulphate method. After dialysis, the total protein obtained was 16 µg/ml with the β-galactosidase activity of 0.186 U/ml. Through this step the enzymes were purified by 3.3 folds with a recovery of 66.4%. The purified enzyme was further analyzed by SDS-PAGE. (Table:4)

Table:4 Purification of β-Galactosidase enzyme.

Purification steps	Total volume (ml)	Enzyme activity (U/ml/mg)	Protein activity (U/ml/µg)	Specific activity (U/ml/µg)	Purification fold	Yield %
Crude extract	10	0.280	80	0.0035	1	100
Ammonium sulphate precipitation	4	0.203	19.8	0.010	2.8	72.5
Dialysis	2	0.186	16	0.011	3.3	66.4

Molecular weight determination of β-galactosidase activityby SDS-PAGE

The purified β-galactosidase activity was subjected to SDS-PAGE to determine the molecular weight (Fig:11). The crude enzyme was precipitated by ammonium sulphate and after dialyses purified enzyme proteins molecular weight markers were run on SDS-PAGE. Thick

protein bands were observed in the crude enzyme (Lane-2 and 3), one protein bands were observed in (Lane-1 and 4) for dialyses β-galactosidase. The standard protein marker used was in the range of 29 kDa to 205 kDa. In comparison with standard protein markers (Lane-M) the apparent molecular weight of *Bacillus subtilis* β-galactosidase was shown at ~ 60 kDa.

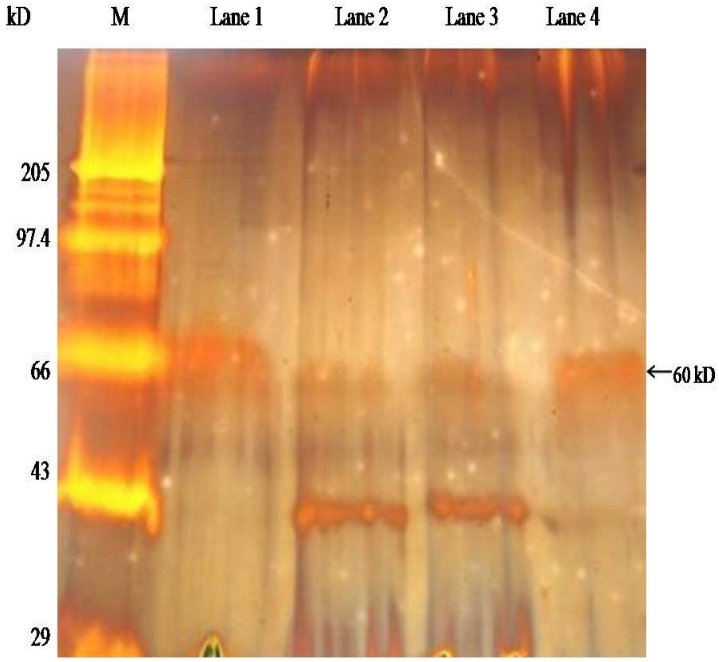

Fig:11 Photographic representation of SDS-PAGE gel Lane- M. Marker protein, Lane -2 and 3. Showing crude extract, Lane-1 and 4.β-galactosidase single band after dialyses.

DISCUSSION

The enzyme β-galactosidase has main biotechnological applications in milk and dairy industries, in the removal of lactose from milk for lactose-intolerant people and the production of galacto oligosaccharides (GOS) for use in probiotic food [25].The enzyme β-galactosidase has been used to hydrolyze lactose in milk to glucose and galactose [26]. which is beneficial for the lactose intolerant people. This has led to the study of the characterization of β-galactosidase enzyme from various microorganisms; *Kluveromyces marxianus, Bacillus subtilis, Lactobacillus plantarum*and *L. sake, L. reuteri*etc in order to improve processes for dairy products from this enzyme. A large number of bacteria can produce β-galactosidases and only a few were reported as safer sources [20].

The present results showed that out of total 76 isolates, 43 strains showed β-galactosidase production indicating the blue colonies on the Luria Bertaini agar plate containing 2% X-gal

and 100 mM IPTG. The isolate that produced maximum β-galactosidase was chosen and analyzed for further studies. The isolate GS-6 from milk sample produced highest β-galactosidase using ONPG substrate of 1.67 U/ml OD at 420 nm was further analyzed for enzyme optimization. The substrate diffuses into the cell. If the organisms possess β-galactosidase, the enzyme will split the β-galactosidase bond releasing o-nitrophenol a yellow colored is formed. Lactose hydrolyzes ONPG to release galactose and ONP under alkaline condition ONP is yellow colored.

Alignment of the 16S rRNA gene sequence of the isolated strain with the sequences available in the GenBank database showed the organism to have 98% identity with various *Bacillus* Sp. **(accession no. NZCM000487.1)** Hence the strain was confirmed as *Bacillus Subtilis.*

Effect of incubation time on β-galactosidase activity of the *Bacillus subtilis* after 48 hrs of incubation maximum β-galactosidase activity 0.271 U/ml was observed and minimum β-galactosidase activity of 0.065 U/ml was observed after 12 hrs of incubation. Thus, 48 hrs were considered as the optimum incubation period for the production of β-galactosidase.The decrease in the enzyme activity after 48 hrs might be due to the decrease in the amount of nutrients in the medium or due to denaturation of the enzyme. Mesophilic bacteria, *Bacillus* sp. producing β-galactosidase was best grown when incubated at 35°C (0.24 U/ml) [27].

The highest β-galactosidase activity 0.253 U/ml was observed in 37ºC and minimum β-galactosidase activity 0.08 U/ml was observed at 55°C. Therefore, 37°C was considered as optimum temperature for the production of β-galactosidase by *Bacillus subtilis* these observations agree with [28]. who found that the optimum activity was at 37°C for *Bifido bacteria* β-galactosidase production.

The effect of different initial pH in the β-galactosidase production was analyzed. β-galactosidase production increase concentration up to a range of 7.0 and decrease in enzyme production with respect to increase in pH was observed, the pH 7.0 has been observed as optimum for the β-galactosidase production by *Bacillus subtilis.* These observations agree with [29] have reported that the optimum initial pH was 7.0 to get to the maximum enzymatic activity for *L. acidophilus* NRRL-4495. [17] Have stated that the optimal initial pH of the medium for β-galactosidase production was 6.0 while the cells of the isolated bacteria strain grew best at initial pH7.0. The results also showed that when the initial pH was higher or lower than 6.0, β-galactosidase activity decreased sharply, indicating that β-galactosidase

production by the *Bacillus licheniformis* E66 strain was very sensitive to the change in initial pH. While [30] stated that initial pH was optimized at 6.5 which giving higher enzymatic activity and productivity for *Bifido bacterium longum* CCRC 708. Also[31] stated that *Thermu thermophilus* KNOUC114 β-galactosidase showed good stability at neutral pH of 6.3 to 7.0 and at the pH lower than 6.3 and higher than 7.0 the stability decreased.

In different carbon sources (1% m/v) the production medium was used to study their effect on enzyme production of β-galactosidase at 48 hrs maximum activity of 0.305 U/ml was observed with xylose, at 48 hrs of fermentation period and minimum β-galactosidase activity with glucose in *Bacillus subtilis*. The present results suggested that optimum β-galactosidase activity was observed with xylose as carbon source. This result stated that it was almost similar to Hsu *et al*., [30] who found that the final viable population of *B. longum* CCRC 15708 was higher in cultures containing either lactose as the sole carbon source with the highest β-galactosidase activity detected with lactose followed by galactose and the lowest activity with glucose as the carbon source.

In this results yeast extract showed maximum activity 0.243 U/ml and minimum activity 0.044 U/ml was observed with medium containing $(NH_4)_2.SO_4$ in *Bacillus subtilis*. Thus, among all the organic nitrogen sources, yeast extract emerged as the best organic nitrogen source for the β-galactosidase production. This result was almost similar with Hsu *et al.*, [30] who have reported that yeast extract is necessary for β-galactosidase production, while casein, peptone and beef extract repressed β-galactosidase formation. However, other works reported that better β-galactosidase synthesis occurs in the presence of nitrogen sources [32].On the other hand, these results were different from the report of [30,33] who indicated that the highest activity of this enzyme was obtained from *Aspergillus niger* and *Bifidobacterium longum* CCRC 15708, respectively, while using yeast extract.

In this result $MgSO_4.7H_2O$ showed maximum activity 0.3 U/ml and minimum activity 0.044 U/ml was observed with medium containing $(CuSO_4)$. $5H_2O$ in *Bacillus subtilis*. Thus, among all the metal ions, $MgSO_4.7H_2O$ emerged as the best metal ions for the β-galactosidase production. El-Hofi, *et al.*, [34] stated that the purified enzyme was assayed with different metal ions. Fe^{3+}, Zn^{2+} and Cu^{2+} strongly inhibited the enzyme. However, Mg^{2+}, Ca^{2+} and Mn^{2+} inhibited negligibly.

In the present study the crude extracts showed 80 µg/ml protein and 0.280 U/ml of β-galactosidase activity. The ammonium sulphate precipitation was showed 19.8 µg/ml protein and 0.203 U/ml activities which clearly indicated that there is 2.8 fold in the protein purification with 72.5% of recovery of protein by ammonium sulphate precipitation method. In dialysis, the total protein obtained was 16 µg/ml with the β-galactosidase activity of 0.186 U/ml the enzymes purified by 3.3 folds with a recovery of 66.4%.This result stated was almost similar to [30] who stated that the enzyme was extracted by conventional ammonium sulphate precipitation. The 40-60% fraction revealed maximum enzymatic activity for *Bacillus MTCC-864*. While [4], stated that the best ammonium sulphate concentrations between 30-70% for *Bacillus coagulans* RCS3 [35] reported that the perfect concentration was 80% for *Lactobacillus*. Also[34] found that the best concentration of ammonium sulphate was 50-60% to get on maximum specific enzymatic activity from *Durio zibethinus*. The purified β-galactosidase activity was subjected to SDS-PAGE was found to have a molecular weight of 60 Kda. Our results were almost similar with [36, 37] reported β-galactosidase with the molecular weight of respectively 65, 75 and 79.9 Kda.

CONCLUSION

In the present study β-galactosidase producing bacteria was isolated from milk and curd. Bacteria were tested for their ability to hydrolyze 5-bromo-4-chloro-3-indolyl-β-D-galactosidase (X-Gal) and O-nitro phenyl-β-D-galactopyranoside (ONPG). Further, identified by conventional methods,16SrRNA gene sequencing as *Bacillus subtilis* and the effect of various physical and chemical parameters in the production of β-galactosidase enzyme was checked physical character like incubation time, temperatures and pH, combination of different parameter using standard production media with variations chemical parameters in the carbon, nitrogen and metal ions, the process of optimization was carried out to maximize the production.The protein was further purified ammonium sulphate precipitation by dialysis and the protein bands on the polyacrylamide gel indicate the presence of enzyme β-galactosidase.However, further purification, separation and characterization of the putative proteins have to be carried out. β-galactosidase from *Bacillus subtilis*is the potential enzyme which will have the promising biotechnological approaches in industrial applications to serve the mankind.

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