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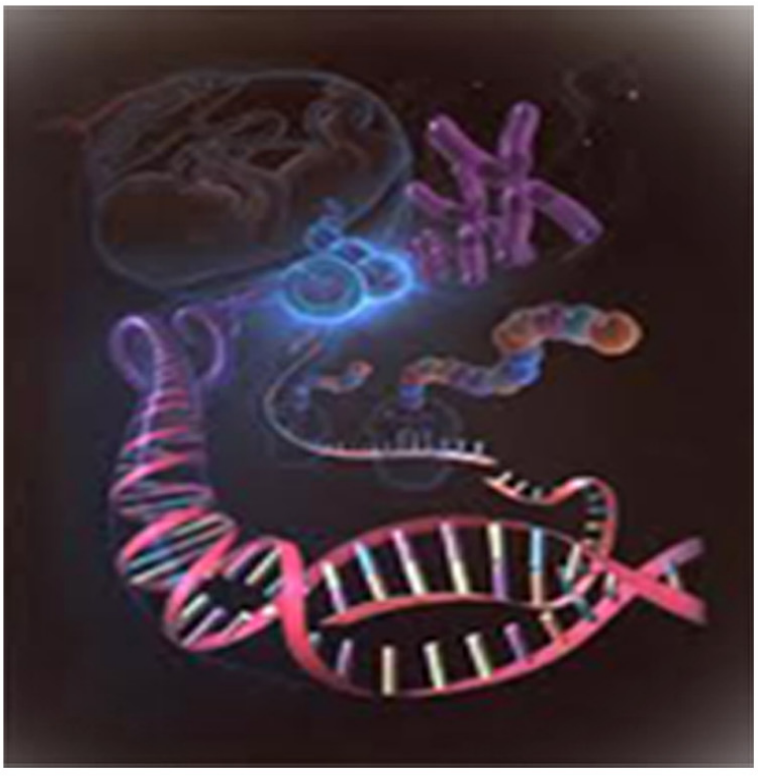

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Research Paper

ISOLATION, PURIFICATION AND EFFECT OF MODULATORS ON α-GALACTOSIDASE FROM DIFFERENT BACTERIAL ISOLATES

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α-D-Galactose-galactohydrolase (E.C.3.2.1.22), commonly referred to as α-galactosidase, catalyzes the hydrolysis of α-galactosidic linkages in oligo-saccharide such as raffinose, melibiose, stachyose and verbaschose. Legumes and its derivatives represent nutritionally high quality food products whose major drawback is their high content of α-galacto-oligosaccharides. These are not digested in the small intestine due to the natural absence of α-galactosidase in mammals. Legume seeds were moistened and exposed to air for bacterial contamination through bating technique. In this work after alpha galactosidase producing bacteria were screened and isolated. α-galactosidase from different bacterial isolates was purified by ammonium sulphate precipitation of 45% and 65% saturation and further purified by gel filteration chromatography using Saphadex G-100. Optimization of temperature and pH was determined as 40°C and 7.0 respectively. Effect of metallic ions on α-galactosidase was also investigated.

Keywords: α-galactosidase, Oligosaccharides, Enzyme modulators, Bacteria, Legumes

INTRODUCTION

α-Galactosidase (α-D-galactoside galactohydrolase, EC. 3.2.1.22) is an important enzyme in the processing of galactooligosaccharides (Ruben *et al*., 1973). This enzyme is also potentially important for hydrolysis of galactooligosaccharides especially raffinose and stachyose found in most of the legumes, because these are responsible for intestinal discomfort and flatulence (Cristafaro *et al*., 1974). Since humans and monogastric animals are deficient in pancreatic α-galactosidase, galactosides are not digested in the duodenum. Therefore, they pass into the large intestine where they are degraded by gas-producing intestinal bacteria, such as *Clostridium* spp. and *Bacteroides* spp., yielding considerable amounts of CH_4, CO_2, and H_2. The abnormal accumulation of flatulent rectal gas thus provokes gastrointestinal distress, such as abdominal pain, nausea, diarrhea, and increased peristalsis. (Rackis, 1981). Many attempts have been made to eliminate the oligosaccharides from legume based food. (Anisha *et al*., 2008). Enzyme treatment by microbial α-galactosidase offers a

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promising solution in the elimination of these oligosaccharides. In sugar industry this enzyme can also degrade the raffinose in molasses and thus increase the yield of crystallized sugars (Linden, 1982). Type B erythrocytes, which contain 3-O- α- D galactopyranoside can be transformed into type O erythrocytes by exposure to α- galactosidase (Ulezlo *et al.*, 1982). Fabry's disease of humans is due to a deficiency of thermolabile lysosomal α-galactosidase A (Ulezlo *et al.,* 1982). α-galactosidase may be used in future for such medical purposes as therapeutic enzyme. Some of the α-galactosidases are also known to catalyze transgalactosylation reactions especially at a high concentration of substrates. Interest in this enzyme stems from their potential technological and medicinal applications. The most important industrial application of α-galactosidase is presently in beet sugar industry, pulp and paper industry, soy food processing and in animal feed processing (Linden, 1982) (Prashanth *et al.*, 2005). Several microorganisms are known to produce α-galactosidase (Mukesh *et al.*, 2012). Isolated α-galactosidase by two Bacillus strains SPE10 and SPE15 for food processing. Usually inducible by carbon source in the culture medium. An important point to consider for microbial source is for industrial production, economical and efficiency. In this study, we have purified α-galactosidases from four different bacterial isolates and their pH and temperature optimization was determined with some enzyme modulators effect.

MATERIALS AND METHODS

Screening of α-Galactosidases Producing Bacteria

Legume seeds were moistened and exposed to air for bacterial contamination through bating technique (Ulfig *et al.*, 2003). (Vanbreuseghem, 1952) and soil flooded with legume flours were also collected from different places of Shimoga to screen α-D-galactosidase producing bacteria. Based on morphological features bacteria found on legumes seeds and soil sample were inoculated separately on the petriplates containing sterile screening medium contained 1% Tryptone, 0.5% Yeast extract, 1% NaCl, 2% Agar and 0.01% pNPG. All medium components except pNPG were sterilized by autoclaving at 121°C, 15 lbs pressure for 20 min. pNPG was sterilized by filter sterilization. Inoculated plates were incubated at 37°C for 24 h.

Bacteria producing α-galactosidase will act on pNPG and releases p-Nitrophenol as product which diffuses into the medium with yellow color. This is a positive indication for α-galactosidase producing bacteria.

The obtained isolated colonies were repeatedly sub-cultured to obtain pure cultures. The pure cultures were maintained on Nutrient-agar slants at 4°C and were sub-cultured every 15 days.

Enzyme Assay

The reaction mixture contains: 50 µL 10 mM pNPG, 10 µL McIlvaine buffer, pH 5.8, and 140 µL of the enzyme Final reaction volume was 200 µL. Reaction mixture was mixed gently and incubated at 37°C for 15 min. The reaction was stopped by adding 3 mL of 0.25 M sodium carbonate. Absorbance was measured at 400 nm against buffer. Concentration of released para nitrophenol (pNP) was calculated by means of a standard curve with pNP, and enzyme activity was determined using following equation.

$$\text{Activity (UE/mL)} = \frac{DA400 \times F\ (\text{mmol/mL}) \times Vf\ (\text{mL}) \times df}{15\ \text{min} \times V.enz\ (\text{mL})}$$

DA400:Absorbance at 400 nm.

F: calculated factor from the standard curve of pNP.

Vf: Final total volume (3.2 mL).

V.enz: enzyme volume in the reaction.

df: dilution factor (if necessary).

One unit of enzyme (UE) is defined as the amount of enzyme released by 1.0 mM of pNP from pNPG per mL per min under the assay conditions.

Extraction of α-Galactosidase from Bacterial Cell

Cells were harvested by centrifugation at 10,000 rpm for 5 min at 4°C then washed twice with McIlvaine buffer 0.05 M (Na_2HPO_4 – Citric acid pH 5.8) and suspended in same buffer. Cells were suspended in 0.05 M buffer and sonicated 20 KHz for 4 min with a pause of 2 min and kept in ice bath. Cellular debris was removed by centrifugation at 10,000 rpm for 10 min at 4°C and α-Galactosidase activity in supernatant was determined. It serves as source of crude enzyme.

Ammonium Sulfate Precipitation

The crude enzyme extracted was adjusted to 10% saturation with ammonium sulfate, with gentle stirring at 4°C overnight. The solution was centrifuged to remove precipitated material. The supernatant was then adjusted to 15% and supernatant collected as mentioned early, the same steps were followed till 100% saturation with ammonium sulfate, and the precipitate was suspended in 0.1 M McIlvaine (pH 5.8) and dialyzed against the same buffer for 48 h to remove the salt.

Gel Filtration Chromatography

Sephadex G-100 was used for molecular sieve chromatography. 5 mL of the enzyme solution from Ammonium sulfate precipitation was applied to a Sephadex G-100 column (2.6 by 70 cm), which had been equilibrated with 0.1 M McIlvaine buffer (pH 5.8). The column was eluted with the same buffer at a flow rate of 60 mL /h. Every 2-mL fraction was examined at 280 nm for protein content and assayed for enzyme activity under standard conditions.

Further purified enzyme is concentrated by taking Microcentrifuge of 1.5 mL volume as the minicolumn. The base tube is carefully pierced so that resulting hole is small enough to ensure that Sephadex powder is unable to leak from the bottom of the tube. A measured amount of dry Sephadex G-25 is added to the tube. The protein solution is added to the empty holed microcentrifuge tube followed by the dry Saphadex. After standing for few min the concentrated protein solution is collected in lower vial.

Effect of pH on Enzyme Activity

Influence of pH on enzyme activity was determined by assaying α-galactosidase activity over a pH range 4.0-8.0, using 50 mM citrate phosphate buffer (pH 4.0-6.0) and potassium phosphate buffer (pH 7.0 and 8.0) by standard assay method. The pH stability was also determined in a same pH range by pre-incubating enzyme samples in above buffers at room temperature for the period of 30 min, the residual activities were measured using standard enzyme assay method.

Effect of Temperature on Enzyme Activity

The optimum temperature of α-galactosidase was determined by performing assays at temperatures, 10-80^{o}C. The temperature stability was also determined by incubating enzyme samples over a same temperature range for the period of 20 min. Aliquots (100 µl) were withdrawn after suitable time interval and residual enzyme activity was determined by standard assay method.

Effect of Enzyme Modulators

The impact of various metal ions such as Mg^{2+}, Fe^{2+}, Cu^{2+}, Ag^{2+}, Hg^{+}, Zn^{2+} and organic compounds like EDTA, on enzyme activity were studied. The metal ion at a final concentration of 0.1 mM was incubated for 20 min at room temperature, and enzyme activity was then assayed under standard conditions.

RESULTS AND DISCUSSION

Screening of α-galactosidases Producing Bacteria

Bacteria producing α-galactosidase would act on pNPG and releases p-Nitrophenol as product which diffuses into the medium with yellow color. Four colonies from different samples were selected and inoculated into screening medium, pH 7.0. After 24 h of incubation, the cells were harvested and disrupted by sonication. The supernatant obtained on centrifugation at 10,000 rpm, 10 min, 4°C was assayed for intracellular α-galactosidase enzyme activity. The extracted crude enzymes from different isolates were screened for α-galactosidase activity using pNPG substrate.

The isolated four colonies were repeatedly sub-cultured to obtain pure cultures. The pure cultures were maintained on nutrient-agar slants at 4^{o}C and were sub-cultured every 15 days. α-galactosidase activity was not detected in broth supernatant of any of the isolates, which indicates no extracellular activity. The isolates showing intracellular α-galactosidase activity were selected for further studies and labelled as A1, A2, A3 and A4. Identification of isolates were confirmed as *Enterobacter* sp., *Bacillus amyloliquefaciens* strain H102, *Bacillus* sp. and *Bacillus thuringiensis* strain 2PR56 respectively (Identification results not shown). Bacterial intracellular α-galactosidase were also reported in alkophilic strain *Micrococcus* (Akiba *et al.*, 1976), *Bifidobacterium adolescentis* DSM 20083 (Susanne *et al*., 1999) and *Bifidobacterium breve* (Xiao *et al.*, 2000).

Ammonium Sulfate Precipitation

The maximum α-galactosidase activity in cell extract of *Enterobacter* sp. and *Bacillus amyloliquefaciens* strain H102 isolate was found to be at 65% ammonium sulphate saturation. In case of *Bacillus* sp. and *Bacillus thuringiensis* strain 2PR56 isolates the maximum α-galactosidase activity was found to be at 45% ammonium sulphate saturation. Thus obtained precipitate was suspended in 0.1 M McIlvaine (pH 5.8) and dialyzed against the same buffer for 48 h to remove the salt.

Gel Filtration Chromatography

Around 600 µl of concentrated protein solution was collected after treatment with dry saphadex G-25 separately from four bacterial isolates. 900 µl of volume was retained in the column. The percentage of yield and fold of purification was tabulated in the Table 1. Similar method was followed by (Allan *et al*., 1984) to concentrate ^{125}I

Table 1: Purification Results of α-galactosidase from of Four Bacterial Isolates

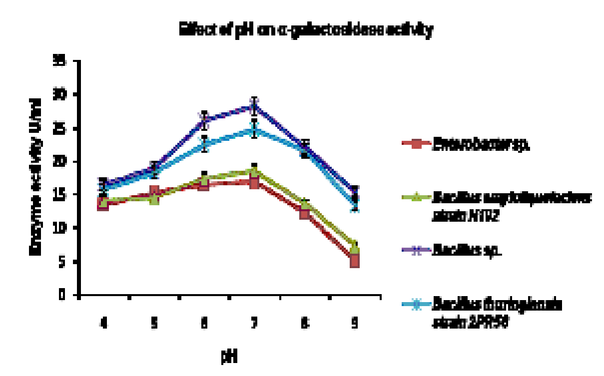
Purification Step	Total Protein (mg)				Activity(U/ml)				Specific Activity (U/mg)				Yield (%)				Purification Fold			
	Enterobacter sp.	*Bacillus amyloliquifaciens* strain H102	*Bacillus sp.*	*Bacillus thuringiensis* Strain 2PR56	*Enterobacter sp.*	*Bacillus amyloliquifaciens* strain H102	*Bacillus sp.*	*Bacillus thuringiensis* Strain 2PR56	*Enterobacter sp.*	*Bacillus amyloliquifaciens* strain H102	*Bacillus sp.*	*Bacillus thuringiensis* Strain 2PR56	*Enterobacter sp.*	*Bacillus amyloliquifaciens* strain H102	*Bacillus sp.*	*Bacillus thuringiensis* Strain 2PR56	*Enterobacter sp.*	*Bacillus amyloliquifaciens* strain H102	*Bacillus sp.*	*Bacillus thuringiensis* Strain 2PR56
$(NH_4)_2SO_4$ Precipitation	73	85	213	200	150	175	440	410	0.06	0.08	0.25	0.24	100	100	100	100	1	1	1	1
Gel filteration	1.2	1.7	0.35	0.31	13.9	18.8	43.4	40.1	0.62	0.91	2.81	2.98	9.2	10.7	9.8	9.7	10.3	11.3	11.2	12.4
Concentrated	6.1	8.3	15.2	13.7	28	36.4	85.6	78.5	0.58	0.81	2.7	2.2	18.6	20.7	19.4	19.1	9.6	10.1	10.8	9.1

labelled goat antibody.

Effect of pH on α-galactosidase Activity

α-D-galactosidase isolates of *Bacillus sp.,*and *Bacillus thuringiensis* Strain 2PR56 showed highest enzyme activity at pH 7.0 and poor/lesser activity at pH 4.0 and 9.0 While enzyme isolates of *Enterobacter* sp. and *Bacillus amyloliquifaciens* strain H102 showed very less activity at the same pH conditions used (Pederson and Goodman, 1980), who reported a neutral pH of 7.0 for the α-galactosidase isoenzymes isolated from *Bacillus stearothermophilus* (Figure 1). A broad pH range of 3.0 to 9.0 was reported in *Geobacillus stearothermophilus* NCIM-5146 (Gote *et al*., 2006) and pH range of 5.0 to 7.0 in *Bifidobacterium adolescentis* DSM 20083 (Susanne *et al.,* 1999) and in *Streptomyces griseoloalbus* (Anisha *et al.*, 2008).

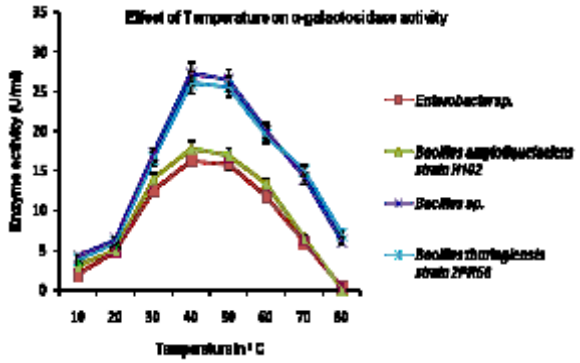
Figure 1: Effect of pH on α-galactosidase Activity



Effect of Temperature on α-galactosidase activity

The results shown in Figure 2 of α-D-galactosidase isolate of *Bacillus* sp., strain was found to be stable at temperature range of 40°C to 60°C, and *Bacillus thuringiensis* Strain 2PR56 showed gradual decrease in the activity beyond 40°C. Whereas enzyme isolates of *Enterobacter* sp. and *Bacillus amyloliquifaciens* strain H102 were found to be very unstable beyond 40°C. α-galactosidases from *Bifidobacterium breve* (Xiao *et al.*, 2000) and *Lactobacillus fermentum* (Carrera *et al.*, 2006). demonstrated an optimum temperature of 50°C similar to that optimum

Figure 2: Effect of Temperature on α-galactosidase Activity

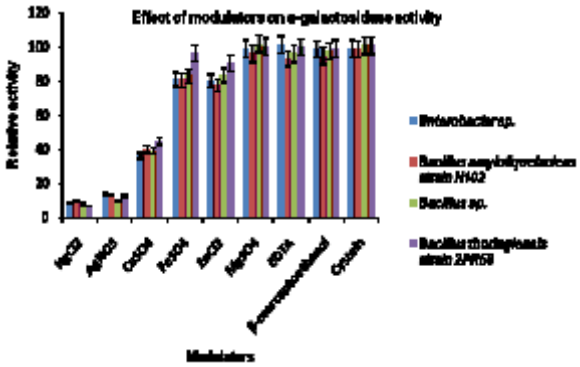


temperature range of 50-65°C was reported in *Streptomyces olivaceous* (Oishi and Aida, 1975).

Effect of Enzyme Modulators on α-galactosidase

The results shown in Figure 3, indicated that, of all the modulators tested, $HgCl_2$ shown to be a potent inhibitor for α-D-galactosidase of all isolates, whereas $MgSO_4$ has been found to be an activator, presence of which has increased the enzyme activity. Similarly EDTA as a positive modulator increased the activity of the enzyme isolate from *Enterobacter* sp. and *Bacillus thuringiensis* strain 2PR56. Cystein increased the activity of the enzyme isolate from *Bacillus* sp., and *Bacillus thuringiensis* Strain2PR56. This has shown that, the activities of the same enzyme isolates from different bacterial species respond to positive modulators like EDTA and cystein differentially.

Figure 3: Effect of Enzyme Modulators on α-galactosidase Activity



α-galactosidases isolated from *Bacillus stearothermophilus* NUB 3621 (Fridjonsson *et al.*, 1999). *Bifidobacterium breve* (Zhao *et al*., 2008) and *Bacillus* sp. (Akiba and Horikoshi, 1976) were reported to be strongly inhibited by Ag^{2+} and Hg^{+} ions). Researcher suggested that the inhibition may be due to reaction with amino, carboxyl and imidazolium group of histidine present at the active site. Unlike α-galactosidase of *Escherichia coli* (Burstein and Kepes, 1971).

CONCLUSION

Present investigation highlights on screening of α-galactosidase from bacterial isolates, further it will be useful to screen or modify the industrially important microorganisms for commercial exploitation. Optimization of temperature and pH reveals the effective medium required for enzyme maximum activity which can be implicated in fermentation studies with appropriate medium constituents considering the effect of modulators for its enhancement as well as inhibition. Further studies are required for formulating α-galactosidase to therapeutic and industrial use.

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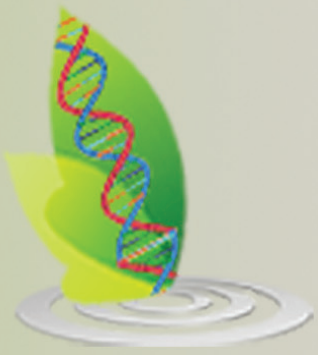

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