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PRODUCTION, OPTIMIZATION AND ENZYMATIC REMOVAL OF OLIGOSACCHARIDES FROM SOYMILK BY ALPHA GALACTOSIDASE ENZYME FROM ROCK SOIL *PSEUDOMONAS* SP. MCCMB3

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ABSTRACT

Alpha-galactosidase is a glycoside hydrolase enzyme that hydrolyses the terminal alpha-galactosyl moieties from glycolipids and glycoproteins. *Pseudomonas* sp. MCCMB3 an alpha galactosidase producer was isolated from the rock soil of Kulasekaram, Kanyakumari district, South India. Totally 10 strains were isolated, when compared with other strains, predominant enzyme activity has been observed in the strain of *Pseudomonas* sp. MCCMB3. The maximum amount of enzyme was produced using nutrient broth during optimization. Environmental and nutritional parameters were optimized for the enhanced enzyme production. The enzyme production was maximum at pH 8.0 and temperature 50°C. Glucose and ammonium carbonate induces enzyme production as carbon and nitrogen sources respectively. Based on the zymographic analysis, the molecular weight of the purified enzyme was about 80 KDa. The alpha galactosidase enzyme produced by *Pseudomonas* sp MCCMB3 degrades raffinose and stachyose present in soymilk which is used in other industrial applications.

Keywords: Pseudomonas, alpha galactosidase, nutrient broth, raffinose, stachyose.

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INTRODUCTION

Alpha galactosidase is a glycoside hydrolase enzyme that hydrolyses the terminal alpha – galactosyl moieties from glycolipids. It is encoded by the Galactosidase Alpha (GLA) gene [1]. It is not synthesized by humans and thus the presence of oligosaccharides could hinder digestion and cause flatulence, since they are utilized by the gas generating intestinal microorganisms. They can be used to clear these oligosaccharides and upgrade the nutrition of legume food [2]. Enzyme treatment with microbial alpha galactosidase would be promising for the elimination of these oligosaccharides [2]. Majority of bacterial alpha galactosidase belongs to family 36 of glycosyl hydrolases [3]. In the past few years, however alpha galactosidase have been considered effective food additives to remove anti- nutrient oligosaccharides, which occurred in soybean meal containing diets [4].

This enzyme is a homodimeric glycoprotein that hydrolyses the terminal alpha-galactosyl moieties from glycolipids and glycoprotein. It predominantly hydrolyses ceramide trihexoside and it can catalyses the hydrolysis of melibiose into galactose and glucose [5]. A variety of mutations in GLA gene affect the synthesis, processing and stability of this enzyme which cause Fabry's disease, a rare lysosomal storage disorder and sphingolipidosis that results from a failure to catabolise alpha- D- galactosyl glycolipids moieties. Two enzyme replacement therapies are available to functionally compensate for alpha galactosidase deficiency. Human alpha galactosidase A contain two recombinant forms includes Agalsidase alpha and Agalsidase beta. Both recombinant forms have the same amino acid sequence as the native enzyme. Agalsidase Alpha and Beta differ in the structure of their oligosaccharides side chains [6].

The enzyme can be used in various fields like production of sugars, processing of soymilk, conversion of blood type and treatment of Fabry's disease [7]. Fabry's disease of human is due to a deficiency of thermolabile lysosomal alpha galactosidase A, while consuming this enzyme will improves disease. Type B erythrocytes, which contain 3-O-α-D-galactopyranoside, can be transformed into type O erythrocytes by exposure to alpha galactosidase [8]. Alpha galactosidase may be used in the future for such medical purposes as enzymotherapy.

Soy milk is prepared from soybean seeds and is nutritionally comparable to cow's milk except the presence of flatulence causing raffinose family sugars and beany flavour. 40 % of the sugars present in soy are alpha galactosides (stachyose and raffinose) that cannot be digested by humans due to the lack of pancreatic alpha galactosidase. Therefore, alpha galactosides pass along the small intestine without being degraded or absorbed and are then taken up by gas-producing bacteria, in the large intestine, causing flatulence and intestinal disorders in symptomatic individuals [9].

This enzyme is widely distributed in bacteria, fungi, plants and animals[10]. Alpha galactosidase from bacterial strain was purified to homogeneity, but the purified enzyme was characterized and sequenced[11]. However, there is no other work has been performed in this aspect based on literatures. Hence, the present study, reported the findings of alpha galactosidase enzyme produced by *Pseudomonas* sp. MCCMB3 isolated from rock soil which degrades degrades raffinose and stachyose present in soymilk.

METHODS

Isolation of bacteria

Rock soil was collected from Kulasekaram, Kanyakumari district, South India using sterile polythene bags and brought to the laboratory of Department of Microbiology, Malankara Catholic College, Mariagiri. The collected samples were serially diluted using sterile distilled water. From the 10^{-1} suspension, 1ml

was transferred to 9 ml of sterile distilled water and subsequently diluted to 10^{-2} - 10^{-7} dilution. From the required dilutions, 0.1ml suspension was drawn and spread over the surface of nutrient agar medium (Himedia). The isolated bacteria were sub-cultured in nutrient agar slants and incubated at room temperature for 48 h to achieve a good growth and then preserved in refrigerator for further analysis[12].

Screening for alpha galactosidase producer

The colonies isolated from rock soil were streaked onto nutrient agar medium. Inoculated plates were incubated for over night at 37°C to obtain colonial growth. The blue colour around the colonies was visualized by adding a drop of X- gal (Genei) indicator over the colonies. The presence of blue colour formed by the hydrolysis of galactosyl moieties were noted as alpha galactosidase producers.

Characterization of α- galactosidase producer Cultural characterization

Microscopic, macroscopic, biochemical, and physiological characteristics of the potent alpha galactosidase producer (MCCMB3) were determined by using microbiological methods [12].

Molecular Characterization

The genomic DNA of *Pseudomonas sp*. MCCMB3 was isolated by CTAB/NaCl method[13]. The 16SrRNA eubacterial primer (5'- AAGCAACGCGAAGAACCTT- 3'; 5'- CACCGGCAGTCTCCTTAGAG-3'), was used for the amplification of DNA. The reaction mix contains Taq polymerase buffer (2 µl), dNTP mix (2 µl), Primer (F+R) (2 µl), DNA template (1 µl), Taq polymerase enzyme (0.2 µl), Double distilled water (12.8 µl). After initial denaturation at 95°C for 5 min, amplification was performed with 35 cycles of 1 min at 95°C, 1 min at 55°C, 1 min at 72°C followed by a final extension at 72°C for 10 min. The 16SrRNA gene sequence obtained from the isolate *Pseudomonas* sp. MCCMB3 was compared with other bacterial sequences by using NCBI BLASTn for their pair wise identities. Phylogenetic trees were constructed in MEGA 5.1Beta 3version (www.megasoftware.net) using unweighted pair group method with arithmetic mean (UPGMA) algorithms.

Enzyme assay and protein estimation

Alpha galactosidase activity was measured using p-nitro phenyl α- D- galactopyranoside (pNPG) as substrate[14]. The reaction mixture contained 0.1ml of approximately diluted enzyme solution, 0.8 ml of 0.1M acetate buffer (pH 5.0) and 0.1ml of 2 mM pNPG solution in distilled water. The mixture was incubated for 15 minutes at 55ºC and then the reaction was arrested by the addition of 3ml of 0.2 M sodium carbonate solution. The quantity of p-nitro phenol liberated was measured by the absorbance at 405 nm. One unit of enzyme activity was defined as the amount of enzyme that liberated 1 µmol of p-nitro phenol per min per ml of enzyme under assay conditions.

Production and optimization of alpha galactosidase production

The potent alpha galactosidase producer MCCMB3 was cultivated in nutrient broth (Himedia) under submerged culture condition. For the production of alpha galactosidase, the culture was inoculated in 1000 ml flask containing 250 ml nutrient broth. It was incubated at 37°C for 48 h in a rotary shaker at 250 rpm.

To increase the production of alpha galactosidase enzyme different parameters such as pH, temperature, carbon and nitrogen sources were optimized. The various carbon sources including sucrose, maltose, glucose, lactose and xylose were used in the optimization process at 1% (w/v). Both organic (peptone, yeast extract) and inorganic (ammonium sulphate, ammonium carbonate, urea) nitrogen sources were supplemented to the medium at 1% (w/v) to evaluate their effect on alpha galactosidase production. The effect of pH and temperature on the production of enzyme was also studied. The effect of temperature was evaluated using different incubation temperatures (20, 30, 40, 50 and 60°C) and the effect of pH was tested using a

range of pH (3-8). The culture was inoculated into the production medium with one parameter at a time and incubated at 37°£C for 48 h. The cell free supernatant (CFS) was collected by centrifugation at 8000 x g for 10 minutes. The CFSЧ was used for testing alpha galactosidase activity.

Purification of Enzyme
Purification by Ammonium sulphate Precipitation

The bacterial strain MCCMB3 was grown for 48 h at 37°C under optimized condition. The alpha galactosidase enzyme from the cell free supernatant was precipitated by ammonium sulphate at 50% saturation at 4°C[15]. The precipitate was collected by centrifugation at 8000 x g for 10 min at 4°C and dissolved in 1M PO_4 buffer of pH 7.0. The quantitative estimation of purified enzyme was also determined as previously described.

Molecular weight determination
SDS- PAGE Profile

The molecular weight of purified enzyme was determined using SDS- PAGE with 4% stacking gel and 10% separating gel. The PAGE gel was electrophoresed and stained with coomassie brilliant blue[15]. Molecular weight was determined by comparing the relative mobility of standard protein molecular weight markers (Bovine serum albumin).

Degradation of Raffinose and Stachyose in Soymilk

Soymilk used for the enzymatic degradation was prepared by the method of Mulimani and Ramalingam [16]. To 100 ml of soymilk, 10 ml of purified enzyme was added, stirred well and incubated for 2 h. Every 30 min time intervals, 10 ml of the reaction mixture were withdrawn, to which 10 ml of absolute ethanol was added to arrest the reaction by precipitating proteins. The contents were filtered through Whatmann filter paper no.1 and the filtrate was concentrated to 1 ml syrup in a thermostat water bath maintained at 75°C. The oligosaccharides in the concentrated syrup (both enzyme treated and untreated) were separated and estimated by the method as described by Tanaka et al[17].

RESULTS AND DISCUSSION

Isolation and Screening of Alpha galactosidase Producers

Totally 10 bacterial strains were isolated from the rock soil obtained from Kulasekaram, Kanyakumari District. Among the 10 bacteria, 3 bacterial strains showed blue colour on nutrient agar plates while adding X- gal over them. When compared with other strains predominant enzyme activity has been observed in the strain of *Pseudomonas* sp. MCCMB3. Therefore this strain was selected for further optimization and production of alpha galactosidase enzyme. Characterization of alpha galactosidase producer

Cultural Characterization

Considering the microscopic, macroscopic, biochemical and physiological tests performed, the strain was identified as *Pseudomonas* sp. MCCMB3. The biochemical characteristic of the strain was a Gram Negative bacterium that was found to be Catalase, Oxidase, Methyl red and Citrate positive. The strain grew well between temperature ranges from 30 - 40°C with an optimum growth at 40°C. The cultural characteristics of *Pseudomonas* sp. MCCMB3 on various growth media are tabulated in table1.

Table 1
Microscopic, macroscopic, biochemical and physiological tests for the strain Pseudomonas sp. MCCMB3

Test Performed	Results
Simple staining	Rod
Gram Staining	Gram negative
Growth on MacConkey agar	Non - lactose fermented colonies
Catalase	Positive
Oxidase	Positive
Motility	Motile
Indole	Negative
Methyl red	Positive
Voges Proskauer	Negative
Citrate	Positive
TSI	H_2S negative
Urease	Negative
Nitrate	Positive
Growth on Cetrimide agar	Bluish green colonies
Nutrient agar	White coloured medium rounded
Cetrimide agar	Bluish green colonies

Molecular Characterization

The DNA was isolated and subjected to 16SrRNA amplification which resulted in a PCR product of length 1466 bp. The 16SrRNA sequence of the isolate was blasted using megablast tool of GenBank (http://www.ncbi.nlm.nih.gov/). The evolutionary history was inferred using the UPGMA method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) is shown next to the branches. The evolutionary distances were computed using the Nei-Gojobori method and are in the units of the number of synonymous differences per synonymous site. Evolutionary analyses were conducted in MEGA5.1Beta 3version. Based on this data the isolate was confirmed as *Pseudomonas* sp. MCCMB3 (Fig 1). The sequence used in the analysis was deposited in GenBank, EMBL in Europe and the DNA Data Bank of Japan with an accession number JN575262

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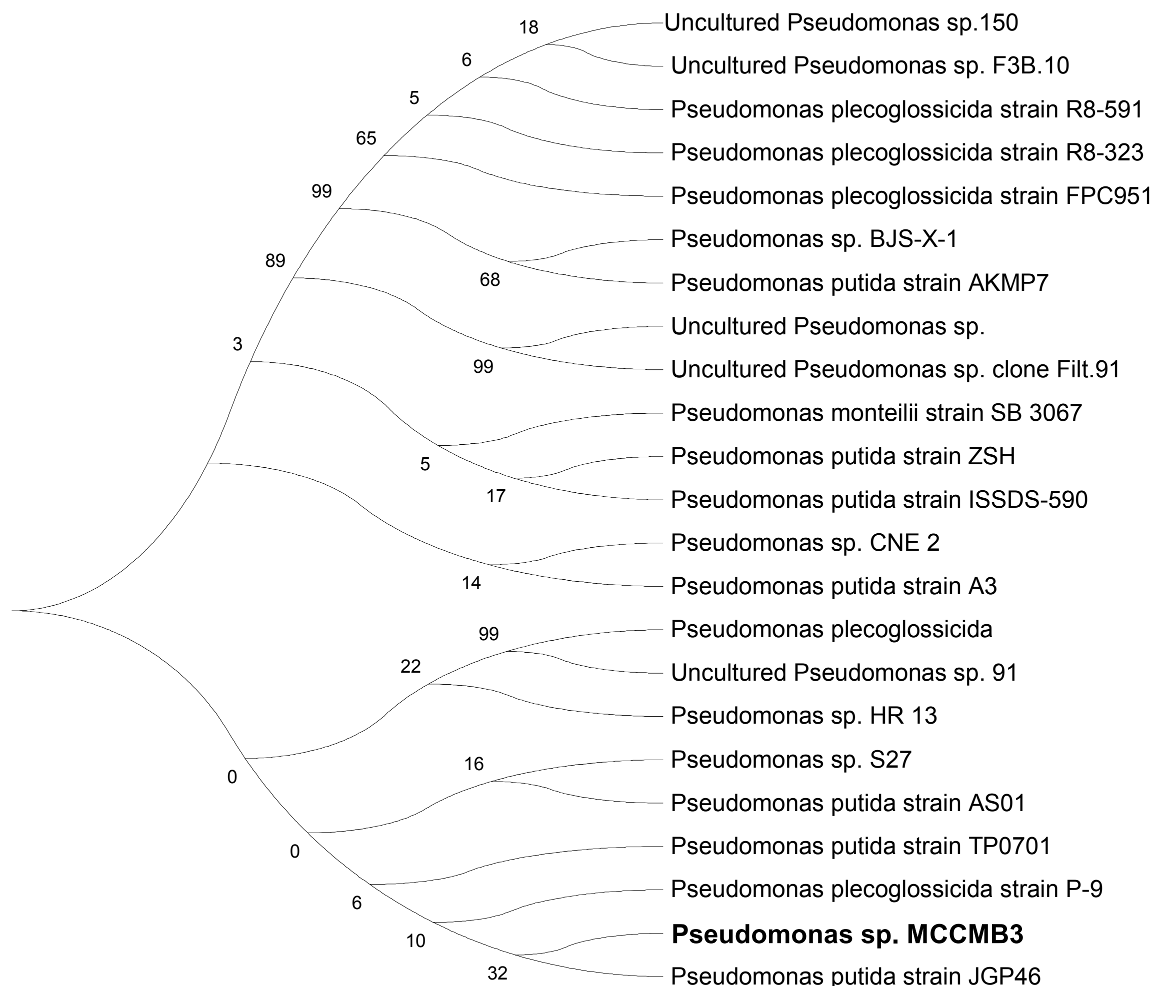

Figure 1
Phylogenetic analysis of Pseudomonas sp. MCCMB3 inferred using the UPGMA method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test. The evolutionary distances were computed using the Nei-Gojobori method.

Optimization of Alpha galactosidase production by Pseudomonas sp. MCCMB3

pH is one of the most important factor that determines the growth and morphology of microorganisms as they are sensitive to the concentration of hydrogen ions present in the medium. The results suggested that there is a stimulation of enzyme synthesis at pH 7.0 and optimum production was obtained at pH 8.0 (Fig 2). Anisha and Prema, [18] investigated that alpha galactosidase from bacterial sources were found active near neutral pH. Similarly maximum yield of alpha galactosidase by *Thermus* sp. was obtained in a medium with an initial pH of 7.0 [19]. Increasing the initial pH of the medium above 6 resulted in gradual increase of alpha galactosidase production. This showed that *Pseudomonas* sp. MCCMB3 required a pH of 8.0 for the production of alpha galactosidase enzyme.

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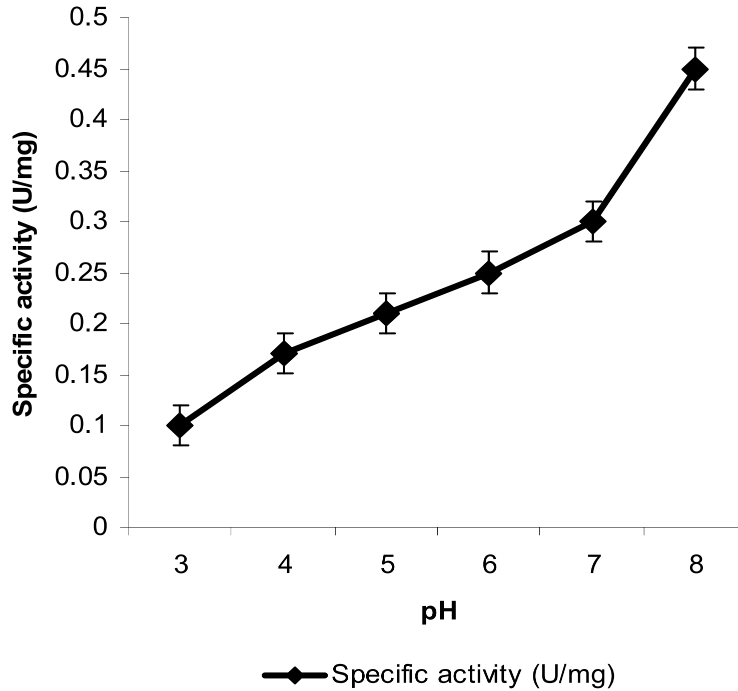


Figure 2
Effect of pH on alpha galactosidase production in Pseudomonas sp. MCCMB3

The effect of temperature on alpha galactosidase production was related to the growth of the organism. The effect of temperature on bacterial growth and alpha galactosidase production were studied. The production of enzyme and bacterial growth was determined at different temperatures ranging from 20 to 60°C also with an optimum enzyme production was observed at 50° C (Fig 3). In addition Al- Kandari and Sharifa [20] studied a *Lactobacillus helveticus* strain which produced alpha galactosidase at 37°C but with stability below 30°C. But few investigators reported that the purified alpha galactosidase enzyme proved to be quite thermo stable, retaining high levels of activity even after incubation at 70°C [21].

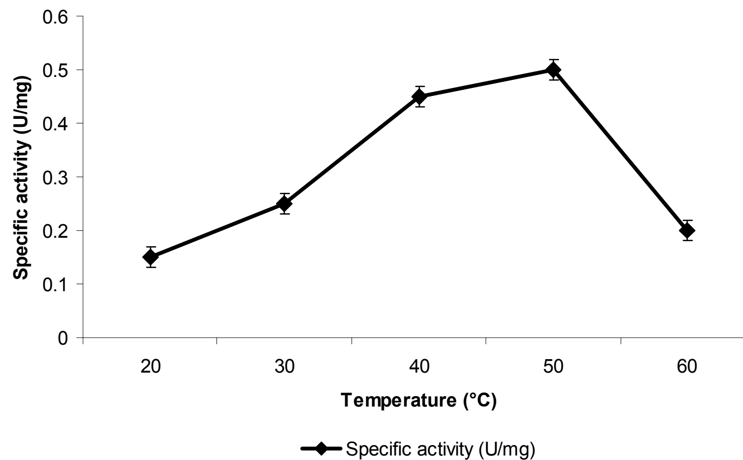


Figure 3
Effect of Temperature on alpha galactosidase production in Pseudomonas sp. MCCMB3

Previously Ramalingam et al [22] reported that different sugars induced alpha galactosidase to a varied extent from *Penicillium purpurogenum*. In the present study, among the sugars tested glucose showed maximum enzyme induction (Fig 4); whereas minimum alpha galactosidase induction was seen with maltose and sucrose as carbon source in the medium. This result also been agreed with several other published works such as Montelongo and Luis [23] investigated that growth on galactose, raffinose, melibiose and lactose produced the highest levels of enzyme activity in *Lactobacillus salivarius*. On the other hand, glucose, galactose, sucrose and high concentration of melibiose failed to induce the enzyme from *Lactobacillus helviticus* [24].

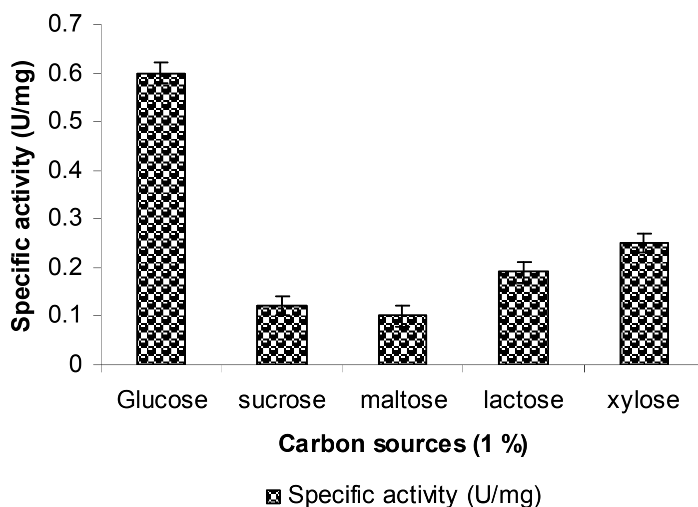


Figure 4
Effect of Carbon Sources on alpha galactosidase production in Pseudomonas sp. MCCMB3

In the investigation of the effects of various nitrogen sources on alpha galactosidase enzyme production ammonium carbonate was found to be the most promising source. The use of other nitrogen sources such as ammonium sulphate, peptone and urea was found to be slightly inhibitory on the production of alpha galactosidase by *Pseudomonas* sp. MCCMB3 (Fig 5). Yeast extract had moderate inhibition on enzyme production. The obtained result was contradictory to the previous literatures depicts that the production of alpha galactosidase by *Penicillium politans* was high at low concentration of yeast extract [25]. The supplementation of malt extract and urea resulted in significant alpha galactosidase production in *Penicillium janthinellum* [26].

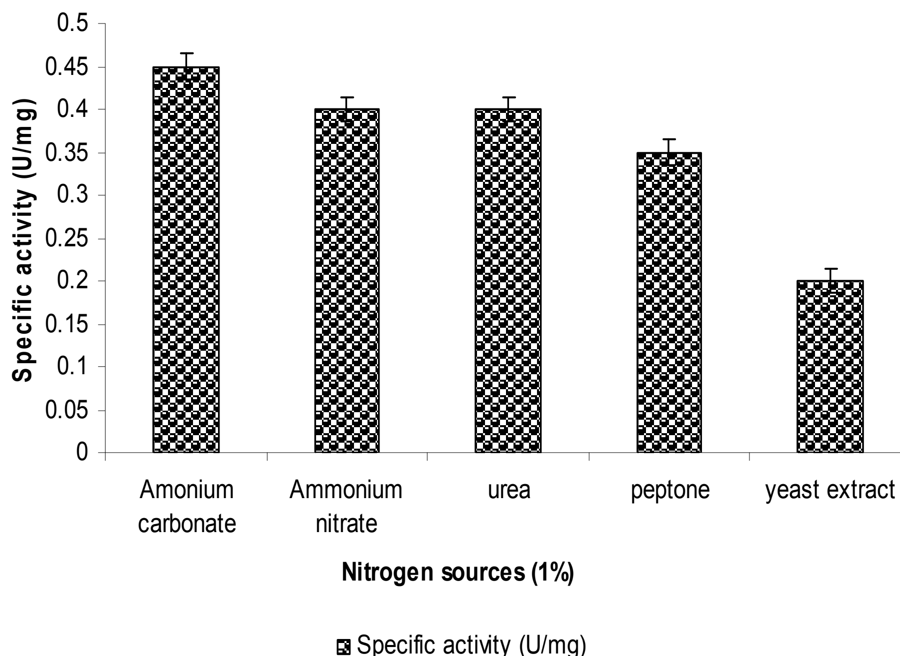
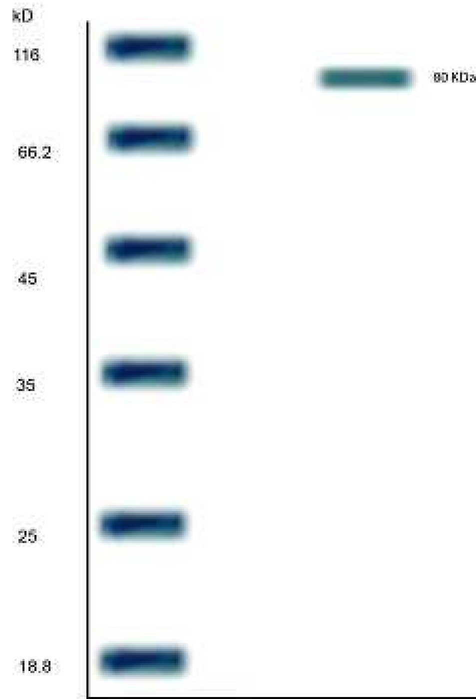


Figure 5
Effect of Nitrogen sources on alpha galactosidase production in *Pseudomonas* sp. MCCMB3

Purification of Alpha galactosidase enzyme

The CFS was used for the purification of alpha galactosidase enzyme from *Pseudomonas* sp MCCMB3. The enzyme was partially purified by ammonium sulphate precipitation. The total enzyme activity of the protein precipitate by ammonium sulphate was found to be in 50% saturation. In SDS-PAGE the aliquot from ammonium sulphate precipitation showed a single band. T1he molecular weight of the final protein on SDS-PAGE was found to be 80 KDa (Fig 6). The molecular mass of alpha galactosidase from the present study slightly differs with that of earlier purified alpha galactosidase from other bacteria, such as *Saccharopolyspora erythraea* (45 kDa), *Pseudoalteromonas* sp. KMM 701(195 +/- 5 kD) [27].



Lane 1Standard Marker; Lane 2 Alpha galactosidase enzyme

Figure 6
SDS-PAGE of Alpha galactosidase Protein in Pseudomonas sp. MCCMB3

Application of alpha galactosidase enzyme Degradation of Raffinose and Stachyose in Soymilk

The partially purified alpha galactosidase completely hydrolysed flatulence causing raffinose and stachyose of soymilk in 1.45 h by the disappearance of these oligosaccharides (Table 2). The percent degradation of flatulence causing oligosaccharides (raffinose and stachyose) decreases to an increase in the duration of enzymatic treatment. The results indicate that alpha galactosidase from *Pseudomonas sp.* MCCMB3 has a vital role in soymilk processing and other industrial applications. Alpha galactosidase from plant and microbes has earlier been reported for the hydrolysis of raffinose and stachyose of soy milk [25].

Table 2
Stachyose and Raffinose content (% dry wt) of soymilk treated with alpha galactosidase enzyme from Pseudomonas sp. MCCMB3.

Time(min)	0	30	60	90	120
Stachyose	**5.0**	**3.1**	**1.4**	**0.3**	**0**
Raffinose	**3.0**	**2.3**	**1.8**	**0.2**	**0**

CONCLUSION

Recently many publications have described the production of microbial alpha-galactosidase and its biotechnological application than other source such as plants, because the microbial enzyme has certain specificities: smaller substrate specificity, existence in one molecular form, higher stability etc. Hence, the present research article emphasizes the production of alpha-galactosidase from the rock soil bacteria *Pseudomonas* sp. MCCMB3. From the obtained results, it is clear that this strain plays an important role in the industrial production of this enzyme and degradation of the raffinose and stachyose present in soymilk. Thus the strain *Pseudomonas* sp. MCCMB3 is a boon to the scientific society.

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REFERENCES

1. Elshafei AM, Foda MS, Enein AA, Afify AS and Ali NH, Purification and enzymatic properties of alpha galactosidase from *Penicillium janthinellum*, Acta Biotechnologica, 13: 351- 359, (1993).
2. Garro M, Graciela F, De Valdez GF and Savoy GD, Alpha galactosidase assay in fermented soymilk products. Environ Microb Methods Protocols, 16:121-124, (2004).
3. Henrissat BA, A classification of glycosyl hydrolases based on amino acid sequence similarities. Biochem J, 280: 309-316, (1991).
4. Kidd MT, Morgon GW, Zumwalt JR, Price CD, Welch CJ, and Brinkhaus FL, Alpha galactosidase enzyme supplementation to corn and soybean meal broiler diets. J Appl Poult Res, 10: 186-193, (2001).
5. Naumoff DG, Phylogenetic analysis of alpha galactosidases of the GH27 family. Mol Boi, 38: 388-399, (2004).
6. Fervenza FC, Torra R and Warnock DG, “Safety and efficacy of enzyme replacement therapy in the nephropathy of Fabry disease”. Biologics, 2(4): 823-843, (2008).
7. Ganter C, Bock A, Buckel P and Mattes R, Production of thermostable recombinant alpha galactosidase suitable for raffinose elimination from sugar beet syrup. J Biotechnol, 8: 301-310, (1998).
8. Tzortzis G, Jay M, Baillon G. and Gibson R, Rastall synthesis of a galactooligosaccharides with alpha galactosidase from *Lactobacillus reuteri* of canine origin. Appl Microbial Biotechnol, 63: 286-292, (2003).
9. Suarez FL, Springfield J, Furne J., Lohrmann TT, Kerr PS. and Levitt MD, Gas production in human ingesting soy bean flour derived from beans naturally low in oligosaccharides. Amer J Clin Nutr, 69: 135-139, (1999).
10. Ramalingam, Saraswathy N, Sathasivam S, Subha K. and Poorani S. Purification and properties of alpha galactosidase from white rot fungus *Pleurotus florida*. Indian J Biochem Biophys, 57: 78-82, (2007).
11. Shibuya H, Kobayashi H, Park GG, Komatsu YM, and Sato T, Purification and some properties of alpha galactosidase from *Penicillium purpurogenum*. Biosci Biotechnol Biochem, 59: 2333- 2335, (1995).
12. Cappuccino JG and Sherman N, Microbiology-A Laboratory Manual. Benjamin Cummins. New York. pp 97-100, (1996).

13. Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. Basic local alignment search tool. J Mol Biol, 215(3):403-10, (1990).
14. Borooah J, Leaback DH, and Walker PG, Biochemical Journal 78, 106-110, (1961)
15. Palanivelu, p. (2001). Analytical Biochemistry and separation techniques. A laboratory manual. 2nd edition. Published by Tulsi book centre, Madurai, Tamil nadu pp 51.
16. Mulimani, V.H. and Ramalingam, Enzymic hydrolysis of raffinose and stachyose present in soymilk by crude α-galactosidase from *Gibberella fujikuroi*. BioChem. Mol. Biol. Int., 36, 897–905, (1995).
17. Tanaka M, Thananunkul D, Lee TCand Chichester CO, A simplified method for the quantitative determination of Sucrose, raffinose and stachyose in legume seeds. J Food Sci, 40: 1087-1088, (1975).
18. Anisha GS, Prema, P. Selection of optimal growth for the synthesis of alpha galactosidase from mangrove actinomycetes. Indian J Biotechnol, 5: 373-379, (2006).
19. Ladero M, Ruiz G, Pessela BCC, Vian A, Santos A and García-Ochoa F, Thermal and pH inactivation of an immobilized thermostable b-galactosidase from *Thermus sp.* strain T2: Comparison to the free enzyme. Biochem. Eng. J. (ISSN: 1369-703X), 31: 14-24, (2006).
20. Alkandari K, Sharifa, I Purification, Characterization and hydrolytic activity of alpha galactosidase from *Lactobacillus helviticus* ATCC 10797, Acta Microbial Pol. 16:50- 60, (2006).
21. Benevides CC, Pessela Lafuente RF, Torres R, Mateo C, Fuentes M, Filho M, Vian A, Jose L, Guisan MJ and Alfonso V, Production of a thermoresistant alpha galactosidase from *Thermus* sp. Strain T2 for food processing. Biochemistry and Microbiology, 91-103, (2007).
22. Ramalingam, Saraswathy N, Sathasivam S, Subha K. and Poorani S. Purification and properties of alpha galactosidase from white rot fungus *Pleurotus florida*, Indian J Biochem Biophys. 57: 78-82, (2007).
23. Montelongo N and Luis J, Alpha galactosidase from *Lactobacillus salivarius*; Isolation, Purification and biochemical characterization. Mol Boi, 37: 38-39, (1995).
24. Ali NH, Mohamed LA, Ali TH Purification and Characterization of alpha galactosidase from *Penicillium politants* NRC-510. Advances in Food Science, 31(3): 164- 169, (2009)
25. Ramalingam, Ruthra, Saraswathy N and Sathasivam S Degradation of flatulence causing oligosaccharides in soymilk by alpha galactosidase – A novel thermo tolerant from *Penicillium purpurogenum*. Indian Journal of Biotechnology, 9: 160-165, (2010).
26. Bakunina IY, Sova VV, Nedashkovskaya OI, Kuhlmann RA, Likhosherstov LM, Martynova MD, Mihailov VV, Elyakova LA. Alpha-galactosidase of the marine bacterium *Pseudoalteromonas* sp. KMM 701. , 63(10):1209-15, (1998).
27. Gote MM, Umalkar H, Khan MI and Khire JM, Thermostable alpha galactosidase from *Bacillus stearothermophilus* (NCIM5146) and its application in the removal of flatulence causing factors from soymilk. Process Biochemistry, 39:1723-1729, (2004).