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PRODUCTION OF INULINASE BY THERMO-ALKALI TOLERANT STENOTROPHOMONAS MALTOPHILIA PSSB7 ISOLATED FROM AGRO-WASTES

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ABSTRACT

Thermo-alkali tolerant strain isolated from banana fields produced high levels of inulinase with banana peel as carbon source and showed good growth at pH 6.5 to 9.5 and 40oC to 65oC. It was identified as Stenotrophomonas maltophilia PSSB7 by 16S rDNA analysis, and it is deposited in NCL, Pune with strain number-NCIM 5323, with the accession number- FJ707375. The optimal parameters for the production of inulinase was as follows: pH 8.0, temp 45°C, higher levels of inulinase (5551± 4.583 U/L) was achieved with 1% of banana peel. And was found to be the best carbon source and the effect of different substrates influenced the biosynthesis of inulinase indicating its role as a constitutive enzyme in our strain. Sodium nitrate a best nitrogen source and Mn+ was found to be the most suitable one. All these conditions make it a potential strain for the industrial production from agro based wastes.

KEYWORDS: *Banana peel, Exoinulinase, Stenotrophomonas maltophilia, Thermotolerant, Thin layer chromatography.*

1. INTRODUCTION

Inulin is a special type of dietary fiber that is naturally found in common foods such as leeks, artichokes, asparagus, onions, garlic, banana, wheat, rye, and chicory root. Inulin is a polymer mainly comprising of fructose units and typically have a terminal glucose and fructose units joined by a β (2→1) glycosidic bond.[1] Bio-resources containing inulin have recently received attention as a renewable resource for the production of fructose syrup, fructooligosaccharide and ethanol fermentation.[2] The importance of fructose in human

nutrition has increased significantly due to these favorable functions such as a sweetening agent in food and drinks, enhances flavor, colour and product stability in many foods and beverages instead of sucrose, because of its beneficial effects in diabetic patients, increases iron absorption in children.[3] The enzyme world market is around one billion dollars per year and this demand has to be met by many enzymes produced by microbial inulinases using submerged fermentation.

Enzymatic hydrolysis is usually achieved by either exo-inulinase or by the synergistic action of exo-inulinase and endo-inulinase.[2] Most of the inulinases are exo-enzymes that split fructose units from the fructose end of the inulin molecule. Few endo-enzymes have been reported to liberate oligofructosides as primary products of hydrolysis.[4] Inulinase activity has been investigated in yeasts;[5], fungi[6] and bacteria.[7] Though the enzyme has been well studied in the case of yeast and fungi, relatively limited investigations has been carried out on bacterial inulinases. So far, the enzyme has been characterized from Gram-positive bacteria such as Bacillus[8]; *Streptococcus*[9] and two Gram-negative bacteria, the *Xanthomonas*[10] and *Pseudomonas.*[11]

In the present study the inulinase was characterized from Gram-negative bacterium, the *Stenotrophomonas* sp. (PSSB7), isolated from banana fields. In addition, owing to the cost of pure inulin (synthetic), the use of agro wastes, inexpensive inulin containing substrates such as chicory, sugar cane bagasse, wheat bran, molasses, sawdust and banana peel have been evaluated for the production of inulinase using this bacterium.

MATERIALS AND METHODS

2.1 Isolation and screening of bacteria for inulinase production

Different soil samples were collected from the cultivated fields of banana from Gulbarga and Raichur districts of Karnataka, India. Approximately 1g of soil sample was suspended in 50 ml of mineral medium [(gL-1) K_2HPO_4 6.3, KH_2PO_4 1.8, NH_4NO_3 1.0, $MgSO_4$ 0.1, $CaCl_2$ 0.1, $FeSo_4$ 0.1, $MnSO_4$ 0.1, Na_2MoO_4 0.006, agar 20 and inulin 2], pH 7.0. Inulin (2%) was sterilized separately and added to the medium, inoculated with 200µl and incubated at 37^oC for 24 h. Colonies showing highest zone of hydrolysis was detected by spraying TTC reagent (0.1% triphenyl tetrazolium chloride in 0.5M NaOH. The extra cellular production of inulinase activity was confirmed by the appearance of red zone of hydrolysis around the colonies.

2.2 Identification of the bacterium

DNA was prepared from the strain PSSB7 using the MoBio microbial DNA isolation kit (MoBio Laboratories Inc.) and the small subunit rRNA gene was amplified using the two primers 16S1 (5'-GAGTTTGATCCTGGCTCA-3') and 16S2 (5'-ACGGCTACCTTGTTACGACTT-3'). The purified 16S rDNA of approximately 1.5 kb was sequenced using five forward and one reverse primer as described earlier.[12] The deduced 16S rRNA gene sequence was subjected to BLAST sequence similarity search for the closest match in the database. The 16S rRNA gene sequence obtained was deposited in GenBank with accession number (EMBL) is FJ707375 and the strain is also deposited in NCL, Pune with accession number - *Stenotrophomonas maltophilia* PSSB7 - NCIM 5323.

2.3 Optimization of physico-chemical variables on the growth and production of inulinase by *Stenotrophomonas maltophilia* PSSB7.

The effect of pH and temperature on the growth and inulinase production was studied by growing the strain *Stenotrophomonas* (PSSB7) in 50 ml of mineral broth medium containing 3.0% sucrose (as carbon source) and 2.5% tryptone (as nitrogen source). The effect of pH was carried out between 4 and 10 and the culture was grown at 45^{o}C for 28 h. The effect of temperature was studied by incubating the cultures at different temperatures (30 to 70^{o}C) for 28 h. The cell free extract was used for assaying the inulinase activity and the growth of the bacterium was monitored by determining the absorbance at 600 nm.

2.4 Effect of various nutritional parameters on the growth and production of inulinase by *Stenotrophomonas maltophilia* PSSB7.

The effect of carbon and nitrogen sources on the production of inulinase by *Stenotrophomonas* (PSSB7) was studied - 50 ml of mineral medium devoid of carbon sources was prepared by using various carbon sources at a concentration of 2%. Various carbon sources tested were inulin, galactose, starch, sucrose, maltose, dextrose, xylose, glucose, fructose, sorbitol, chicory, wheat bran, sugarcane bagasse, banana peel and sawdust and the nitrogen sources used were peptone, beef extract, tryptone, yeast extract, urea, sodium nitrate, soybean meal and potassium nitrate by taking all parameters one at a time. The effect of divalent metal ions on the growth and enzyme production was also studied by using different metal ions Hg^{+}, Ag^{+}, Mn^{+}, and Ca^{+} at 1mM concentration.

2.5 Purification of Inulinase from *Stenotrophomonas maltophilia* (PSSB7).

This strain was grown in 500 ml of mineral medium for 28 h at 45^{o}C. The culture was centrifuged at 10,000×g for 10 min and the cell free supernatant was used for the enzyme purification. To the cell free supernatant, different concentrations (40-80% w/v) of ammonium sulphate were added slowly under mild-stirring conditions at 4^{o}C and dialyzed against 50 mM sodium phosphate buffer (pH 8.0) for 24 h at 4 °C. The dialyzed fraction containing the enzyme was applied on Sephadex G-200 and the bound enzyme was eluted with a linear gradient of 50 mM sodium phosphate buffer (pH 8.0) containing NaCl solution in the range of 0.0 - 0.2 M for 1 h with a flow rate of 2.0 ml/min and all the positive fractions of inulinase showing the highest activity were pooled and assayed.

2.6 Assay for the activity of Inulinase

In vitro assay of inulinase activity was carried out according to.[13] 0.2 ml of enzyme solution was mixed with 0.5 ml of 0.2% inulin solution in 0.3ml of 50mM sodium phosphate buffer (pH-8) and was maintained at 45^{o}C for 30min. The reaction was stopped by adding 1ml of 3, 5 Dinitrosaliycilic reagent and reducing sugar was estimated spectrophotometrically. One unit of inulinase activity was defined as the amount of enzyme required to hydrolyze 1µmol of inulin per min, under the standard assay conditions.

In situ activity of Inulinase on polyacrylamide gel was done according to the method of Gabriel and Wang.[14] The gel was soaked in 2% inulin dissolved in 50 mM sodium phosphate buffer (pH 8.0) for 30 min at 55°C and the enzyme reaction was fixed by mixture of methanol, water, and acetic acid (5:5:2, v/v/v). The gel was treated with 0.1% 2,3,5-triphenyl tetrazolium chloride (TTC) in 1 N NaOH for 30 min and the band showing inulinase activity appeared pinkish red in colour.

2.7 Identification of end products

To determine the hydrolyzed end products of banana peel by inulinase, the mixture of substrate and the enzyme was incubated at 55^{o}C for 60 min. The hydrolyzed products were analyzed by TLC following the procedure as described by Kim *et al.*[11] The hydrolyzed sugar products were separated on silica gel or cellulose TLC plates with the solvent system iso-propanol, ethyl acetate and water (6: 2: 2, v/v). The separated sugars were visualized by spraying the plates with 10% sulphuric acid or α-napthol and heating at 120^{o}C for 5min.

RESULTS

3.1 Isolation and screening for the inulinase production.

Bacterial strains were isolated from soil samples collected from banana fields of Gulbarga and Raichur districts, Karnataka, India using the mineral medium.[12] However, the activity of the strain PSSB7 (Fig. 1) showed the highest activity ranged from 160 to 600 U/L and further the characterization was carried out to identify the strain up to species level.

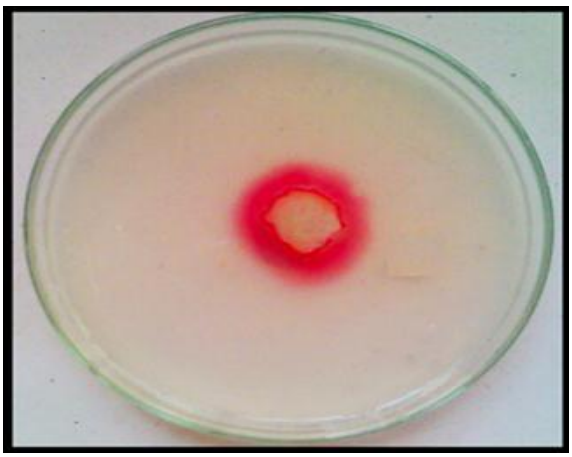

Figure 1: Hydrolysis of Inulin by *Stenotrophomonas maltophilia* PSSB7.

3.2 Identification of the bacterium:

16S rDNA sequence (1448 nucleotides) was carried out and subjected to BLAST sequence similarity, the search with the database revealed that the strain PSSB7 belonged to the genus *Stenotrophomonas* and closest relatives is *Stenotrophomonas maltophilia*. Strain DSM 50170; 16S rRNA accession number AB294553.[15]

3.3 Optimization of physico-chemical variables on the growth and production of inulinase by *Stenotrophomonas maltophilia* PSSB7.

The strain PSSB7 was grown for 72 h in the mineral medium containing 3.0% sucrose, 2.5% tryptone at 45^{o}C and assayed for the inulinase activity for every 4 h. The results indicated that the enzyme production followed the growth of the bacterium and the maximum growth of the bacterium and the production of the enzyme in synthetic media. The fermentation was carried out at various pH ranging from 4.0 to 10.0 and in the present study there was an ascending order of enzyme activity from pH 4.0 to 8.0 with a maximum peak of (583±10.53U/L) as shown in fig. 2.

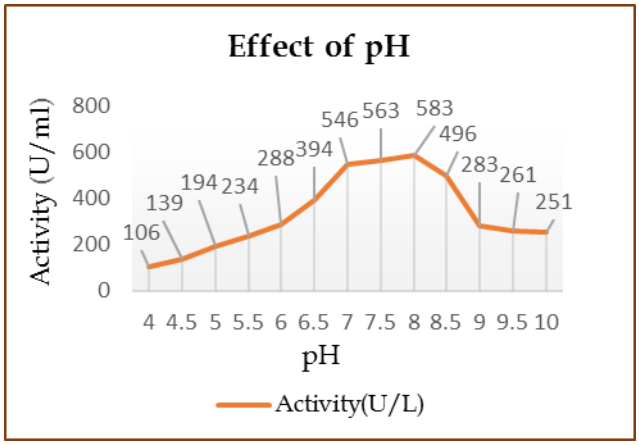

Figure 2: Effect of pH on inulinase production by PSSB7.

The effect of temperature on inulinase production showed the maximum inulinase activity at 45°C (655±5.29U/L) in synthetic media as shown in fig. 3 and the enzyme activity significantly reduced after 55°C (569± 4.35U/L).

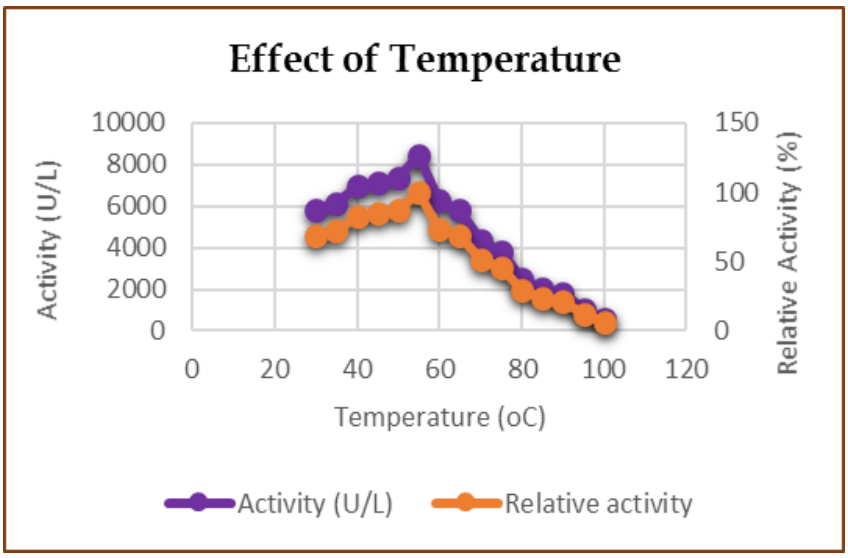

Figure 3: Effect of Temperature on inulinase production by PSSB7.

3.4 Effect of nutritional parameters on the growth and production of inulinase by *Stenotrophomonas maltophilia* PSSB7.

Effect of different substrates on production of inulinase was carried out by using various synthetic and natural carbon sources as listed in Table 1 at 1 % (w/v) concentration. Strain PSSB7 preferred inulin, galactose, starch and sucrose as favorable sources for inulinase secretion among the synthetic substrates. Among the natural substrates, the highest enzyme production was observed in banana peel followed by sugar cane bagasse and wheat bran, respectively. Among all the substrates tested, the highest enzyme production was observed

when 1% banana peel (5551± 4.583 U/L) was used as the substrate followed by sugar cane bagasse (4587±4.509U/L) which was 5 and 4-fold increase in enzyme activity respectively. Whereas, the other synthetic substrates such as maltose, dextrose, sucrose, xylose, glucose, fructose and sorbitol and the natural substrates as chicory, molasses and saw dust exhibited less activity in inulinase production.

The different organic and inorganic nitrogen sources were employed as substrates for the production of inulinase are listed in Table 2 at 0.5% (w/v) concentration. Among the various organic and inorganic nitrogen sources tested, sodium nitrate (0.5%) supported the maximum bacterial growth and inulinase production of (6478.6±2.517U/L), followed by yeast extract, ammonium sulphate, soyabean meal, ferrous ammonium sulphate, ammonium hydrogen phosphate and potassium nitrate.

3.5 Effect of metal ions

The effect of metal ions on inulinase was investigated by measuring residual activity after incubation with each metal ion at its optimum temperature and pH, for a fixed length of time using banana peel as the substrate. The metal ion Ba^{+2} had very little or no effect on the increased enzyme activity, but Mn^{+2} had significant effect on the enzyme activity wherein it enhanced the activity by almost two fold (11203±5.568U/L) as shown in Table 3 when compared to the control. The other metal ions used had a range of inhibitory effect on the inulinase activity.

3.6 Purification of inulinase from *Stenotrophomonas maltophilia* (PSSB7)

The inulinase produced was purified from the culture supernatant in early stationary phase by ammonium sulfate fractionation and gel filtration and the enzyme purified was 85.0 fold with a total yield of 11.0% (Fig. 4). The protein fraction subjected to gel filtration chromatography resolved into several peaks and the peak fractions from 15 to 29 showed the highest inulinase production. Upon size fractionation, the protein mixture yielded three major peaks and the peak 1 fractions from 10 to 18 exhibited the highest enzyme activities. A single band on SDS-PAGE was obtained and stained with coommasie blue and silver staining. The molecular weight of this inulinase was approximately 82.0 kDa as estimated by SDS-PAGE gel electrophoresis using protein markers of known molecular weight and was also confirmed by zymogram as shown in (Fig. 5).

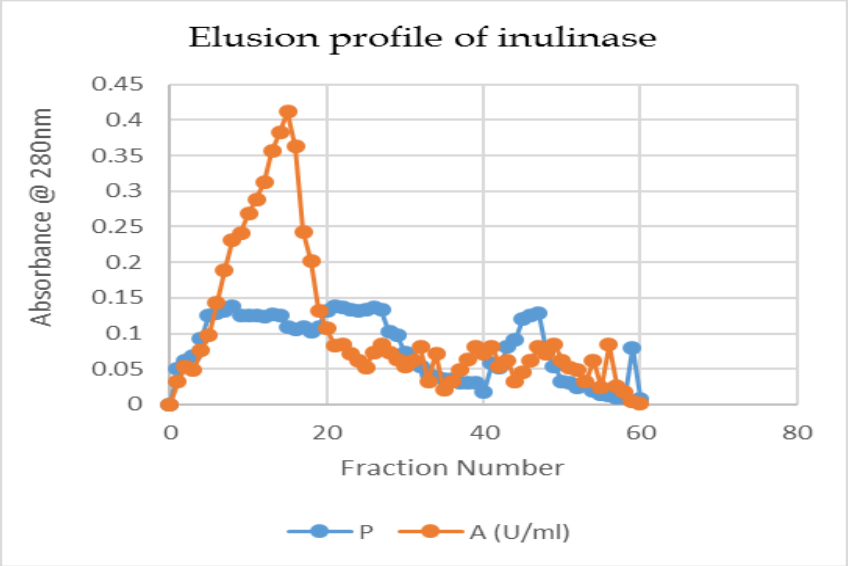

Figure 4: Elution profile of inulinase from Stenotrophomonas malatophilia PSSB7 on Sephadex G-200. P-Protein concentration, A-Activity (U/ml)

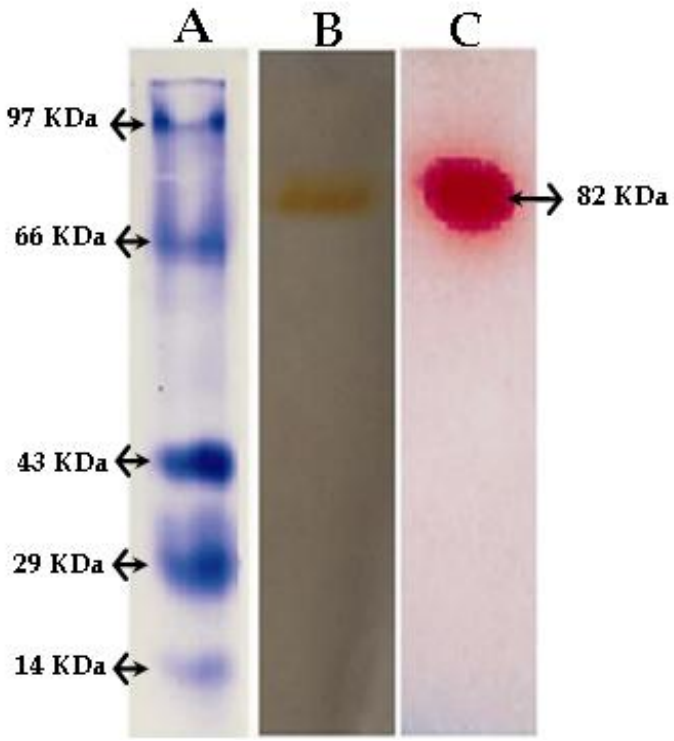

Figure 5: SDS PAGE and zymogram of purified inulinase from Stenotrophomonas malatophilia PSSB7 by silver nitrate staining.

Lane A: Standard Protein Marker

Lane B: Purified inulinase enzyme

Lane C: Activity staining of inulinase in native PAGE

3.7 Determination of Hydrolysis products:

Analysis of the hydrolyzed products of banana peel by inulinase was determined by TLC and indicated that the enzyme could completely hydrolyze the substrates to fructose at 55^{o}C in 30 min as shown in (Fig. 6). Further, the results suggested that the enzyme is an exo-inulinase as the end product is fructose.

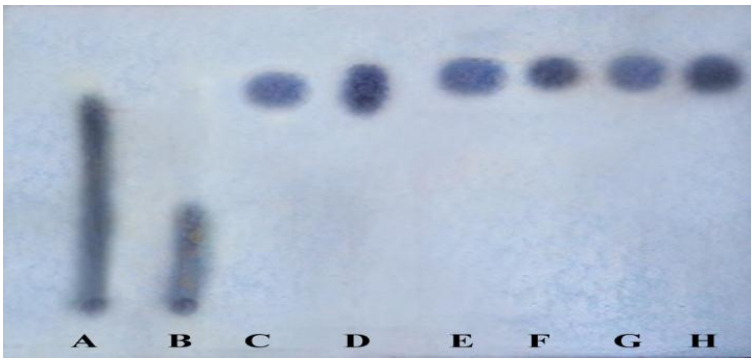

Figure 6: Thin layer chromatography of banana peel (1%w/v) hydrolysate by crude enzyme from *Stenotrophomonas maltophilia* PSSB7 (A-Banana peel, B-Glucose, C-fructose, D-H are test samples) at 30min reaction time.

DISCUSSION

4.1 Isolation and screening for the inulinase production

Inulin content ranges from 0.3 to 22.0 % (dry weight) in plants and are commonly used in human nutrition.[16] Thus, if the plants containing inulin are properly exploited and processed, they could be of a high quality and cheap source of carbohydrates and minerals for livestock. The active hydrolysis of (2-1) fructosyl fructose links present in inulin could be hydrolyzed by inulinase producing microbes.[5] So far, the enzyme inulinase was characterized from Gram-negative bacteria, *Xanthomonas* sp.[17] *P. mucidolens*[18], X. *oryzae*.[19] For the first time, inulinase was detected and reported from a Gram-negative bacterium PSSB7 identified as genus *Stenotrophomonas* based on 16S rRNA gene sequence isolated from banana fields by us using banana peel.

4.2 Identification of the bacterium

Stenotrophomonas sp. (PSSB7) produced maximum amount of the enzyme in 28 hr of its growth and the enzyme production declined after reaching the stationary phase indicating that the production of the enzyme is growth dependent.

Similar results were observed earlier in *Xanthomonas campestris*[17] and further our results are in accordance with Pandey *et al.*,[20] wherein the inulinase production of (0.4 U/ml) up to

24 h of fermentation by *A. niger* was reported. In contrast the enzyme production by *Aspergillus oryzae* took 48 h for the maximum growth and production of the enzyme.[21] Gupta *et al.*,[22] obtained 1.2 U/ml of inulinase produced by *A. oryzae* after nine days.

4.3 Optimization of physico-chemical variables on the growth and production of inulinase by *Stenotrophomonas maltophilia* PSSB7.

In contrast submerged cultures of *Streptomyces* sp. produced maximum inulinase (0.524 IU ml) from garlic powder.[23] Similar observations have been reported on inulinase induction by lignocelluloses such as chicory, onion peel, dandelion roots, and sugarcane bagasse.[17] Further, the activity depends on the cultivation conditions - cultivation of microorganism in solid state fermentation produces more enzyme than liquid culture.[5] Thus, these results indicated that the activity of the enzyme depends on the production rather than the catalytic activity.

The strain PSSB7 showed higher inulinase production over a pH range of 6.5 to 8.5 with a maximum at pH-8. Growth and enzyme production of PSSB7 strain shows an optimum activity at pH 8.0 and retained 20% of the activity at pH 3 and 18% at pH 11, thus indicating that it is most suitable enzyme for the industrial applications compared to the enzymes so far characterized and indicating our strain as a moderate alkali tolerant in nature.

Inulinase activity was observed at optimum pH 8.0 (611 U/L) after 28 h and subsequently, enzyme titers decreased with an increase in pH. The growth of *Stenotrophomonas maltophilia* (PSSB7) was found to be good from pH 6.5 to 10.0 indicating that the strain is moderately alkalophilic in nature. Similar results were reported by Zherebtsov *et al.*,[24] showing higher inulinase synthesis from a bacterial strain *Bacillus polymyxa 29* when the pH of fermentation medium was 6.0 to 8.0. In contrast *Bacillus smithii T7*, the best pH for highest inulinase activity was 7.0.[8]

The bacterium could grow from 35^{o}C to 60^{o}C with an optimum growth temperature of 45^{o}C, indicating that the strain is moderately thermophilic in nature showing higher inulinase production at 45^{o}C as shown in fig. 3 and at 50^{o}C the enzyme activity significantly reduced showing less activity up to 70^{o}C, by retaining 30% after 12 h of incubation, indicating that the enzyme is heat-stable.

Our observations are quite similar with[8] reported optimum temperature found was 50°C for higher inulinase production. In contrast to the reports in the Bacillus *polymyxa 29*, where optimum temperature is 35°C.[24] Higher temperature for optimization of inulinase is an extremely important factor for the application in the commercial production of the fructose or inulo oligosaccharides. Since higher temperature ensures proper solubility of inulin and also prevent microbial contamination.

4.4 Effect of nutritional parameters on the growth and production of inulinase by *Stenotrophomonas maltophilia* PSSB.

Our results found are similar with the synthetic substrate which was reported earlier in *Staphylococcus* sp.,[20] *B. smithii T7*.[8] The use of synthetic inulin as a substrate for the enhanced production of inulinase is uneconomical and therefore, the current impetus is on the use of Inulin - rich economic agricultural waste for enzyme production. In contrast submerged cultures of *Streptomyces* sp. produced maximum inulinase (0.524 IU ml) from garlic powder.[23] Similar observations have reported on inulinase induction by lignocelluloses such chicory, onion peel, dandelion roots, and sugarcane bagasse.[17]

Among all the nitrogen compounds sodium nitrate was found to be the good source. The higher concentration of sodium nitrate was inhibitory for inulinase synthesis and this indicates that appropriate concentration of inulinase nitrogen source in the medium was remarkable. Similar results were reported by[22] in *Fusarium oxysporum* with $NaNO_3$. Anuradha *et al.*,[25] found with *Aspergillus versicolor* strain by using 0.2% (w/v) ammonium dihydrogen phosphate and 0.15% (w/v) sodium nitrate after 15 days of growth. In contrast Wei Gao *et al.*,[8] found ammonium hydrogen phosphate as the best nitrogen source.

4.5 Effect of metalions

The metal ions Cu^{+2} and Fe^{+2} reduced the activity by 20 to 30%, Ca^{+2}, K^{+} inhibited the activity by 50% and EDTA repressed by 40%. Whereas total inhibition of banana peel hydrolytic activity was caused by Hg^{+2} and Ag^{+}. Mn^{+} ion increased the inulinase activity by one fold and total inhibition was observed in Hg^{+} and Ag^{+} suggesting that some –SH groups in the protein are essential for enzyme activity. In contrast Sharma *et al.*,[23] NaHCO3, $AgNO_3$ and $CoCl_2$ were not tolerated and did adversely affect the inulinase activity.

4.6 Purification of inulinase from *Stenotrophomonas maltophilia* (PSSB7)

In the present study, the enzyme produced by PSSB7 was purified up to 85% and the purified enzyme showed the specific activity of 34 U/mg and this activity was higher when compared with the bacterial inulinases purified so far.

The inulinase produced by yeast and fungi had a molecular weight of 50 to 87 Kda[26] except in case of *K. fragilis* which had a molecular weight of 250 KDa.[20] Whereas the bacterial inulinases, so far characterized, had a molecular weight of 50 to 75 KDa except in case of *Xanthomonas oryzae* No. 5 which has 139 Kda.[19] The present enzyme produced by PSSB7 has a molecular mass of 82 KDa. Our results, thus, clearly indicate that the enzyme is a unique one when compared to all other characterized inulinases from yeast, fungi and bacteria.

4.7 Determination of Hydrolysis products

The inulinase produced by PSSB7 has completely hydrolysed the banana peel to fructose indicating that the enzyme is an exo-inulinase. Though most of the inulinases characterized from fungi and bacteria belong to the exo - category, the enzyme characterized from Gram-negative bacteria had only endo-inulinase activity.[10] Whereas the extra-cellular enzyme characterized from *Stenotrophomonas* sp. (PSSB7) exhibited exoinulinase activity.

CONCLUSION

To the best of our knowledge it's noteworthy to mention here that the production of inulinase by PSSB7 isolated from banana agro-waste is a novel one reported by us. As Inulin is expensive, the current impetus is on the use of banana peel a rich economic agricultural waste for the enzyme production. It is thermo-alkali tolerant in nature. The results obtained ensure that, another promising organism is being added at the industrial level for the production of ultra-high fructose syrup. Further, the studies are in progress to enhance the enzyme activity and characterization of purified enzyme to be exploited in a commercial way.

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