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Studies on the Optimization and Characterization for the Biosynthesis of Inulinase under Solid state Fermentation

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Abstract: Inulinases constitute an important class of enzymes for producing fructose and fructooligosaccharides, which are extensively used in pharmaceutical and food industry. The production of inulinase has been reported from various fungal, yeast and bacterial strains. A local fungal isolate identified as *Aspergillus niger* was tested for the optimization of inulinase production in solid state fermentation. Various fermentation parameters such as pH, temperature, inoculum size, incubation time, carbon sources and nitrogen sources were analyzed. Four different substrates banana peel, garlic peel, wheat bran and Rice bran were used. Among the four substrates the use of banana peel (12 g) at 40 % moisture resulted in maximum production of inulinase. Furthermore, the optimal conditions such as pH, temperature, and suitable buffer to obtain maximum stability and activity of the enzyme were identified. Enzyme activity was found to be highest at pH 4.8 and at 45°C while present in the acetate buffer. The high residual activity of the enzyme at 45°C and also at 80°C indicated that the enzyme was more stable at these temperatures
Keywords: Inulinase production, Solid state fermentation, *Aspergillus niger*, inulin degradation.

INTRODUCTION

Inulinase is one among the industrial enzymes which has caught the attention of researchers.[1] Inulinases are used for production of high fructose syrups, fructooligosaccharides, ethanol and inulooligosaccharides which are extensively used in pharmaceutical and food industry.[2]

Inulinases which were first isolated from plants degrade the polymer inulin into fructose in a single step.[3] Inulin a carbohydrate reserve composed of fructans is widely prevalent in many plants, particularly in roots, and tubers such as dahlia and chicory. Hence such plants become good sources of high fructose calorie reduced sweeteners.[4]

Although these inulinases were first isolated from plants, it is difficult to extract them in sufficient quantities. The use of microorganisms to produce these enzymes will become a viable alternative to obtain this enzyme in large quantity and in turn would increase the potential for using inulinases in the production of fructose from inulin.[5]

Microorganisms which have been reported to produce high level of inulinase include various fungal, yeast and bacterial strains such are *Aspergillus aureus, Aspergillus oryzae Aspergillus awamori, Aspergillus ficcum, Aspergillus niger, Fusarium oxysporum, Penicillium purpurogenum, Rhizopus sp, Streptomyces sp, Acetobacter sp, Artrobacter sp, Bacillus subtilis, Escherichia coli, Staphylocoocus sp, Schizosacchromyces alluvius.*[6]

Inulinase production has been reported to be produced in both submerged and solid state fermentation. Despite the industrial enzymes

including inulinases are widely produced in submerged fermentation, solid state fermentation can still hold potential for economical production of enzymes in small scale units at relatively higher volumetric productivity.[6] The selection of microorganism is an important aspect in SSF for the production of enzymes and the organism is desired to grow at low water activity so as to include under GRAS (Generally recognized as safe) list. The present work is focused on to optimize the production of inulinases under Solid state fermentation using the fungus *Aspergillus niger*.[7]

MATERIALS AND METHODS

Isolation and Screening

The strain, *Aspergillus niger* used in the present study was isolated from the rhizosphere soil of different rhizosphere region and the potential inulinase producing strain was screened from those isolated cultures by using inulin containing medium. The strain which produced zone of clearance was taken for further studies and it was maintained in PDA slants. The medium used for screening composed of Inulin-10g, Yeast extract-10g, $NaNO_3$-10g, KH_2PO_4-5g, $MgSO_4.7H_2O$-1, Agar Agar-15, Distilled water -1litre, pH- 5. The screened *Aspergillus niger* strain was identified through microscopic studies.

Solid State Fermentation (SSF)

Fermentation was carried out in conical flasks containing 10 g of different hot air oven dried solid substrates like wheat bran, banana peel, garlic peel and rice bran, which was supplemented with stock nutrient solution. Moisture was adjusted to 50 %. Each flask was plugged with non-adsorbent cotton and autoclaved at 121^{o}c for 20 mins. The flasks were inoculated with 1ml of *Aspergillus niger* spores suspension and incubated for 72 hours at room temperature.

Preparation of nutrient solution

The nutrient solution was prepared using Yeast extract-10g, $NaNO_3$ -10g, KH_2PO_4 -5g, $MgSO_4.7H_2O$ - 1g, Distilled water -1 liter, pH- 5.

Optimization studies on inulinase production under SSF

Effect of inoculum size on inulinase production

10 gm of each substrate was transferred into 4 conical flasks. Inoculum size was varied between 1 – 4 ml.

Effect of incubation time on inulinase production

About 10 gm of each substrate was transferred discretely in 4 conical flasks. Inulinase enzyme activity was determined at regular 24 hours interval. Based on the production level, the incubation time was determined for further SSF studies.

Effect of moisture level on inulinase production

The level of moisture content of the each substrate was varied by adding different amounts of nutrient solution respectively at 30%, 40% 50% 60% and 70%. Then the fungal culture was inoculated and incubated at room temperature.

Effect of pH on inulinase production

About 10 gm of each substrate was transferred separately in 6 conical flasks. Each flask was labeled according to the different pH values 3, 4, 5, 6, 7 and 8. Then the pH was adjusted by changing the pH of nutrient solution. The flasks were sterilized and inoculated with 1ml of fungal spore suspension. The flasks were incubated in shaker at 27 $^\circ$C.

Effect of temperature on inulinase production

The SSF set up was made by four different substrates individually supplemented with nutrient solution separately in 4 conical flasks. The flask containing medium was sterilized, cooled and inoculated with fungal strain and incubated at different temperature at 27^{o}C, 37^{o}C, 47^{o}C and 57^{o}C.

Effect of carbon sources on inulinase production

About 10 gm of each substrate was transferred separately into 6 conical flasks. They were further supplemented with different carbon sources such as sucrose, glucose, lactose and maltose. Then the fungal spore suspension was inoculated and the flasks were incubated at 27 $^\circ$C.

Effect of nitrogen sources on inulinase production

About 10 gm of each substrate was transferred separately into 6 conical flasks. They were further supplemented with different nitrogen sources such as yeast extract, peptone, beef extract, ammonium nitrate, and potassium nitrate. Flasks were inoculated with 1ml of fungal spore suspension and then incubated.

Effect of increasing the concentration of suitable nitrogen source (yeast extract) on inulinase production

10 gm of banana peel was transferred to 5 conical flasks. The nutrient solution was prepared by varying the concentration of a nitrogen source yeast extract from 1% to 5% and was supplemented to those substrates to determine the optimum concentration of N_2 source.

Enhanced production of inulinase under SSF

10 gm of banana peel was supplemented with 40% of nutrient solution. The mixture was sterilized and inoculated with 0.1% w/v of culture suspension. After 2 days of incubation enzyme activity was assayed.

Characterization studies on inulinase enzyme

Optimization of pH for inulinase activity and stability

The effect of pH on inulinase activity was determined by incubating the crude sample at different pH varying from 4 to 5. The optimal pH for stability was tested by pre incubating the sample for 120 minutes in the buffer with pH varies from 4 - 5. The residual inulinase activity was measured using assay method.

Optimization of temperature for inulinase activity and stability

The optimal temperature on inulinase activity was determined by incubating the crude sample at different temperatures such as 45^{o}C, 55^{o}C, 65^{o}C, 75^{o}C and 80^{o}C. The optimal temperature for enzyme stability was tested by pre incubating another set of samples at different temperature from 45^{o}C to 80^{o}C. The residual inulinase activity was measured using assay method.

Optimization of suitable buffers for inulinase activity and stability

The suitable buffer on inulinase activity was determined by incubating the crude sample in 0.1 M of acetate and phosphate buffers. The suitable buffer for enzyme stability was tested by pre incubating another set of samples using same two buffers. The residual inulinase activity was measured using assay method.

Assay method for inulinase

The reaction mixture contained 0.2 ml of appropriately diluted culture and 0.8 ml of 0.5% inulin. It was dissolved in 0.1M of sodium acetate buffer (pH=5) and it was incubated at 50^{o}C. After the incubation for 20 min the increase in reducing sugars (fructose) was estimated by using 3, 5-dinitrosalicylic acid. Absorbance was measured at 550 nm. One unit of inulinase activity was defined as the amount of enzyme which produces 1 mol of reducing sugar (fructose) per min under the above conditions.

RESULTS AND DISCUSSIONS

Isolation of *Aspergillus niger* strains

A total of 7 fungal strains were isolated from collected soil samples of different rhizosphere region. These isolates were further subjected to screening process to identify inulin utilizing strain. Among the 7 *Aspergillus* strains active inulinase producing strain formed colonies which solubilized inulin particle in the agar plate and yielded a clear surrounding zone which was an indicator for inulin utilization.

Optimization studies on inulinase production under SSF

Effect of inoculum size on inulinase production

Among the four substrates (banana peel, garlic peel, wheat bran and rice bran) banana peel yielded maximum inulinase activity of (200 U/gds) followed by wheat bran and rice bran giving inulinase activities of (160 U/gds) and (137.2 U/gds) respectively at an inoculum volume of 1 ml. However, garlic peel yielded maximum inulinase activity of 155.8 U/gds only at an inoculum volume of 3 % (Fig. 1). It was observed that size of inoculum did not show significant influence on the production of inulinase. Usually the size of inoculum plays a significant role in the production of metabolites under SSF.[8] It is important to provide the SSF optimal size of inoculum, in order to obtain sufficient biomass which will inhibit the growth of undesirable microorganisms if at all present. On the other hand, high inoculum size would result in excess growth of biomass there by making the SSF deprived of sufficient substrate for product formation.[9]

Effect of incubation time on inulinase production

The maximal inulinase production 179.8 U/gds was obtained at 48 hrs culture incubation in banana peel while for other substrates such as for garlic peel (157.5 U/gds), wheat bran (152 U/gds) and rice bran (122 U/gds) at 72 hrs incubation for providing maximum inulinase activity (Fig. 2) .

Effect of moisture on inulinase production

The highest inulinase activity of 212.5 U/gds was obtained for banana peel at 40 % moisture and also banana peel produced maximal inulinase activity among other substrates at different moisture level. The Garlic peels showed maximum inulinase activity (148.2 U/gds) at 60% moisture (Fig 3).

Effect of pH on inulinase production

The maximum inulinase activity of 186.3 U/gds was obtained at pH 5 in banana peel followed by wheat bran (167.5 U/gds), and rice bran (132 U/gds) (Fig 4). However, garlic peels showed maximum inulinase activity (144 U/gds) at pH 4. On contrary to this, maximum production of inulinase from inulin by *X.campestris* was obtained

at pH 7, which was also shown to be the optimal pH for biomass growth.[10]

Effect of temperature on inulinase production

Aspergillus niger showed maximum inulinase production at 27 ^{0}C for all substrates in the order of banana peel, garlic peels, wheat bran and rice bran of (194.7 U/gds), (161.3 U/gds), (155.3 U/gds) and (150 U/gds) respectively (Fig. 5).

Effect of carbon sources on inulinase production

Inulinase production was studied for different substrates with 1 % carbon source in the nutrient solution. The maximum production was achieved in banana peel (201.9 U/gds) when supplemented with inulin as carbon source. A reasonable inulinase activity was also obtained when banana peel was supplemented with sucrose. The maximal inulinase activity of 158.6 U/gds was observed in garlic peel supplemented with inulin as carbon source (Fig 6). Inulin was followed by sucrose in giving maximum enzyme activity. No significant amount of inulinase was obtained in wheat bran and rice bran as compared with banana and garlic peels. The earlier studies revealed that *X.campestris* grown on sucrose showed considerable inulinase production 7.48 U/gds and comparatively lower inulinase production was observed when inulin was used as a carbon source [10] and the maximum inulinase activity from inulin by *Streptomyces sp.*[11]

Effect of nitrogen source on inulinase production

Various nitrogen sources (Yeast Extract, Beef Extract, Peptone, Ammonium nitrate and Potassium nitrate) of 1 % were added along with nutrient solution to the solid substrates. Yeast extract produced maximum inulinase activity in banana peel (199.1 U/gds), garlic peels (161 U/gds), wheat bran (159 U/gds), rice bran (108 U/gds). Only a little amount of inulinase activity was observed in peptone and potassium nitrate (Fig 7).

Effect of increasing nitrogen source (yeast extract) on inulinase production

The maximum inulinase activity was observed in 1g of yeast extract in banana peel yielded 199.1U/gds (Fig 8) and rest of the concentration of yeast extract not shown significant yield and also not arrested the growth of *Aspergillus* sp.

Characterized studies on inulinase enzyme

Optimization of pH for enzyme activity and enzyme stability

The effect of pH on the inulinase activity is presented in Fig 9(a). Inulinase at pH 4.8 and pH 4.6 resulted in activities of 194 U/ml and 186 U/ml respectively. Inulinase was found to be stable after 2 h of incubation at pH 4.8 with residual activity of 92 %. However, at other pH values enzyme was found to be only moderately stable. The results are shown in the Fig. 10(a). The inulinase enzyme is highly active at the optimum pH range of 4.5 to 5.0.[12] Similar results were also reported for inulinase production by *Aspergillus niger*.[13]

Optimization of temperature for enzyme activity and stability

The optimal temperature yielding maximum enzyme activity and enzyme stability was found to be 45°C. Also the enzyme was found to be more stable even at temperature as high as 80°C. The activities of inulinase at temperatures 45 ºC and 55 ºC were 192 U/ml and 189 U/ml. The residual activity of inulinase at 45 ºC after an incubating period of 120 min was 189 U/ml, confirming the enzyme is more stable at 45 ºC (Fig 9(b)).

Optimization of suitable buffers for enzyme activity and enzyme stability

The selection of suitable buffer is important for any enzyme based reactions. In the current study two buffers; acetate and phosphate buffers were tested for its suitability for inulinase enzyme. Acetate buffer was found to be suitable, giving a maximum activity of 197.5 U /ml and it was more stable after incubation of 120 min. It provided a residual activity of 195U/ml which indicated the 97 % of enzyme was stable. On the other hand, enzyme activity in phosphate buffer was found to be 190 U/ml and it provided its residual activity after incubation of 178 U /ml which it is 12 % less stable compared to acetate buffer. The results are shown in fig.9(c).

Graphical representation for optimization of inulinase under SSF

Fig.1 Effect of inoculum size on inulinase production under SSF

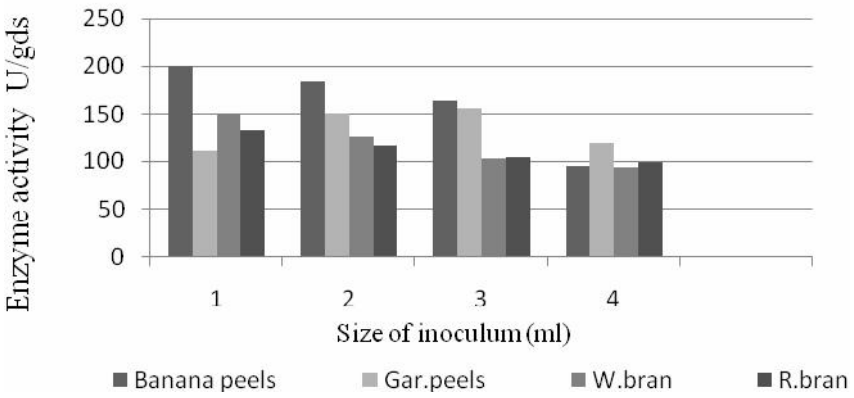


Fig.2 Effect of incubation time on inulinase production under SSF

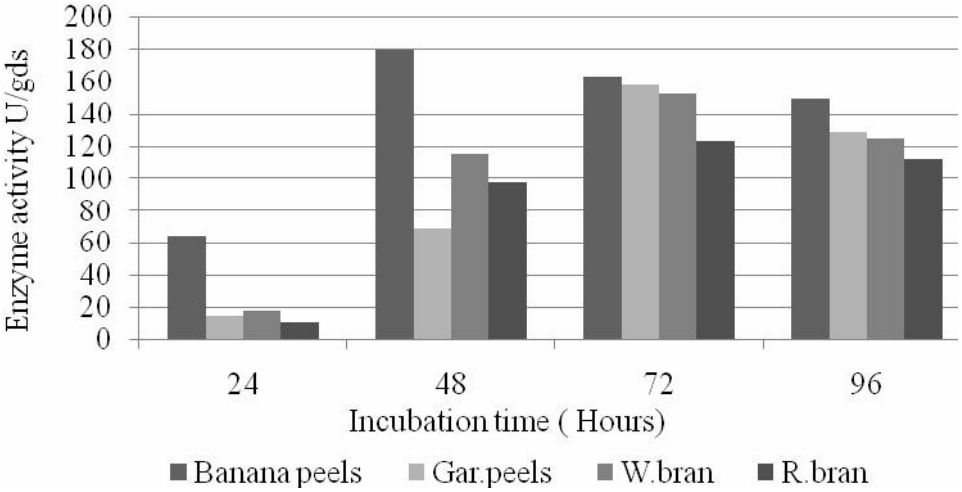


Fig.3 Effect of moisture level on inulinase production under SSF

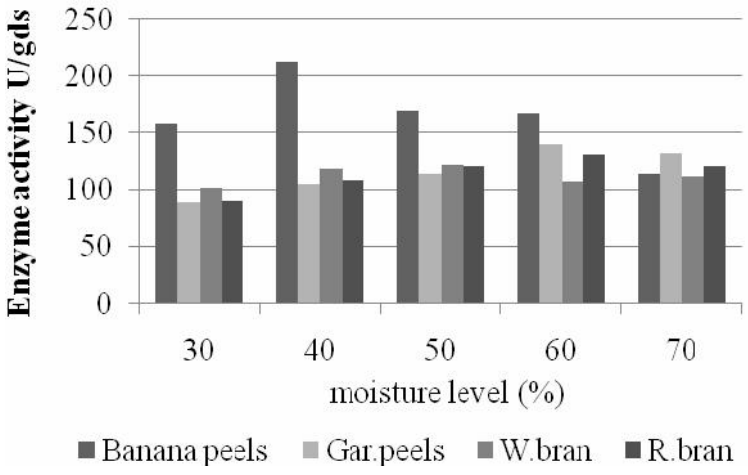


Fig.4 Effect of pH on inulinase production under SSF

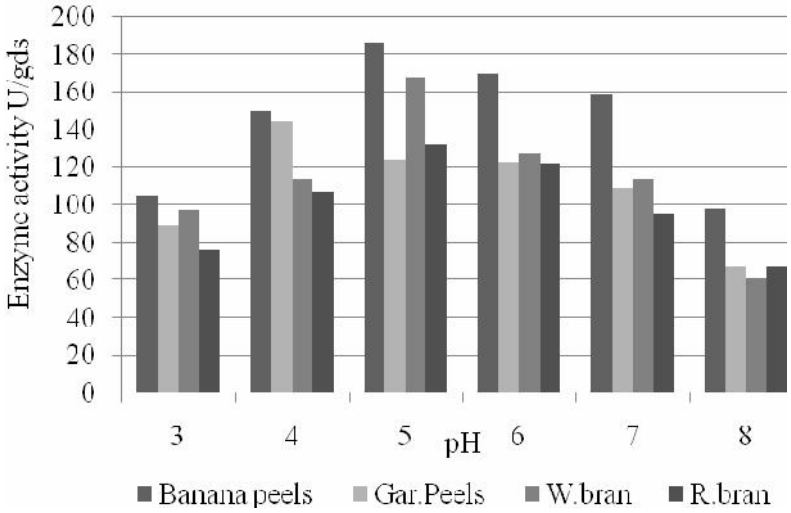


Fig. 5 Effect of Temperature on Inulinase Production under SSF

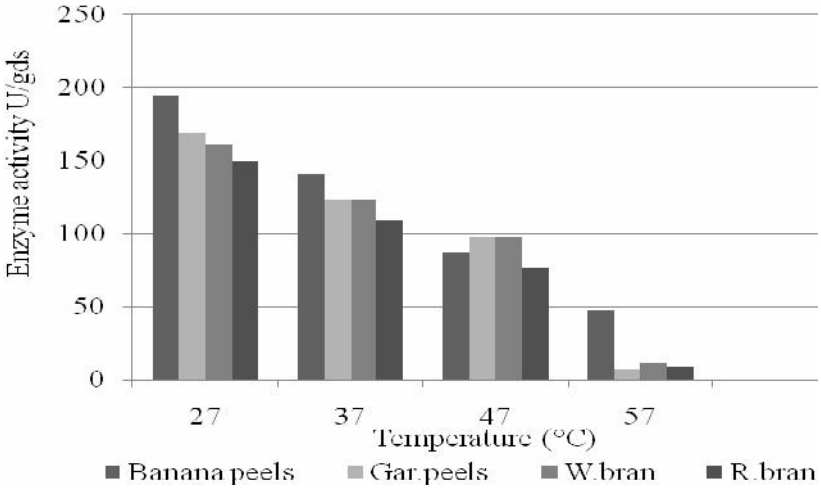


Fig.6 Effect of carbon sources on inulinase production under SSF

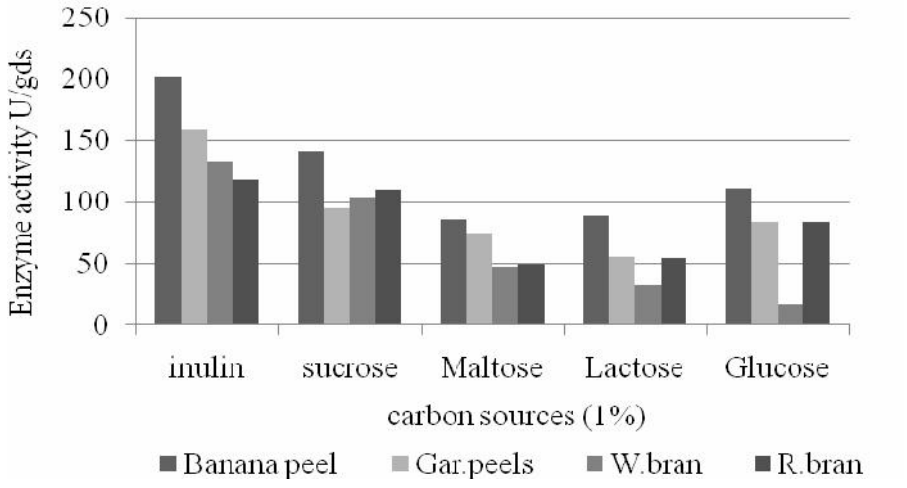


Fig 7 Effect of nitrogen sources on inulinase production under SSF

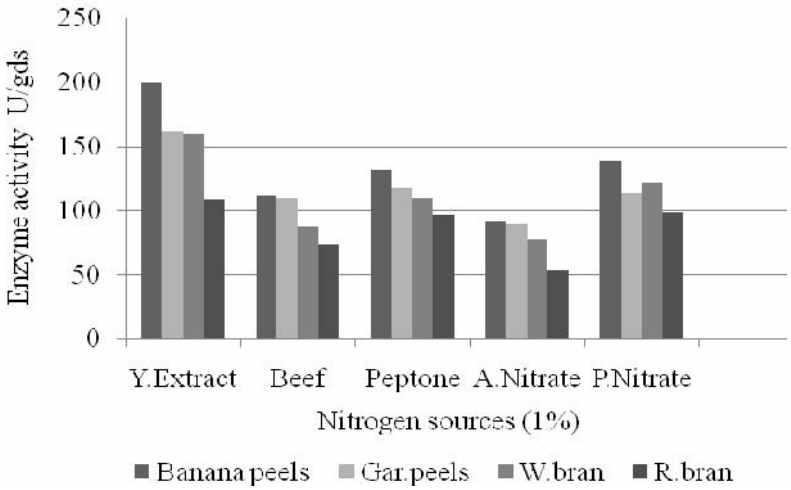


Fig. 8 Effect of increasing concentration yeast extract on inulinase production SSF

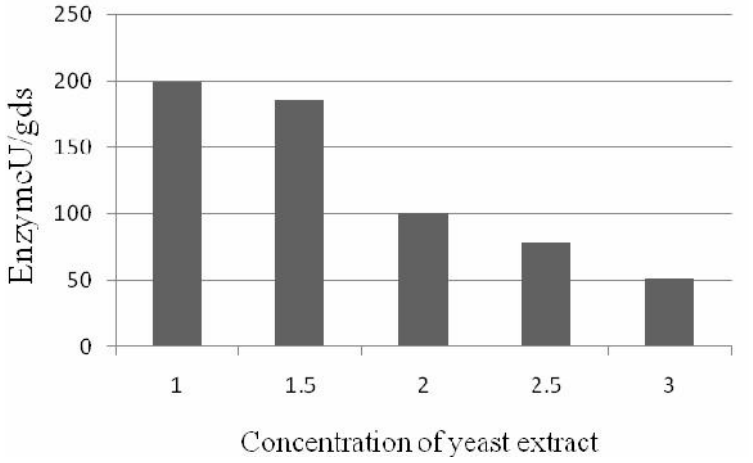


Fig 9(a).Optimization of pH for enzyme activity and enzyme stability

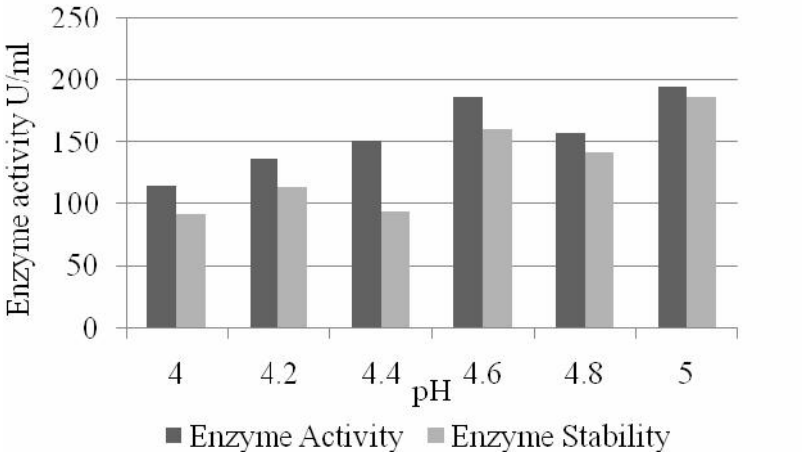


Fig 9(b)Optimization of Temperature for enzyme activity and enzyme stability

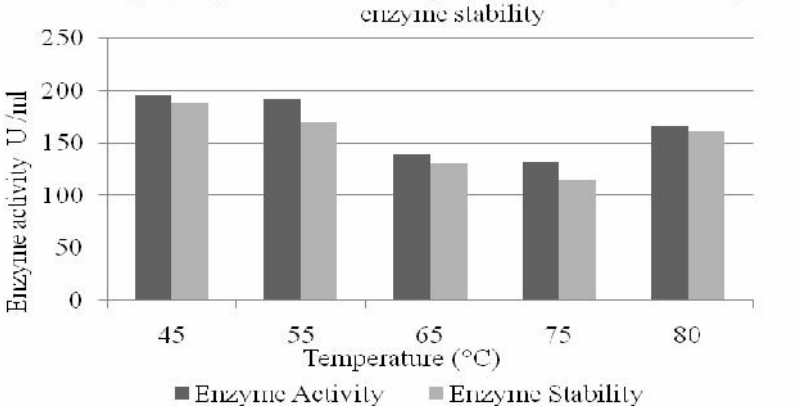
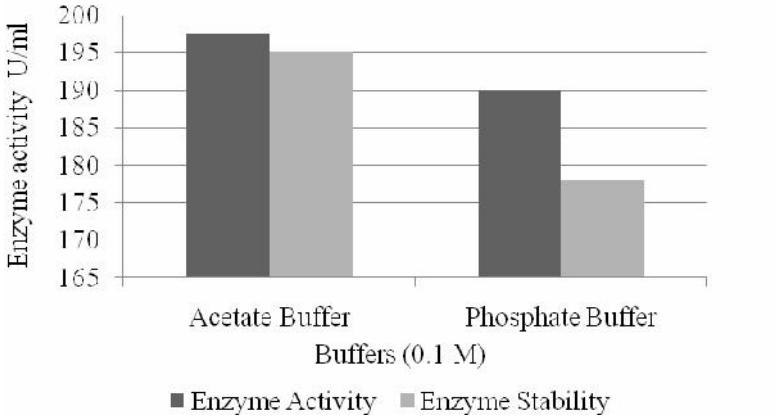


Fig 9(c) Optimization of suitable buffers on enzyme activity and enzyme stability



CONCLUSION

In this present study, we concluded that Solid State Fermentation is more efficient for the production of inulinase while using the substrate banana peel yielding 237 U/gds at 40% moisture with 1% of yeast extract at the incubation time of 48 hours. There is significance in this research work, as this is the first report on inulinase production from banana peel and garlic peel by *Aspergillus niger* using SSF. This study clearly indicates banana and garlic peels can be used as alternative feed stocks in the production of microbial inulinase using a more economical method. Though the pure inulin is costlier, these research pave the way to limiting the cost and for the commercialization is possible to scale up the inulinase production by *Aspergillus niger* through SSF which have diverse application for the industrial sector.

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