Isolation and Production of Inulinase From Banana Peel by Using Aspergillus Niger Under Submerged Fermentation

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Abstract- Inulinase consist of an important class of enzymes for the production of fructose and fructo-oligosaccharides, which are extensively used in pharmaceutical, food industry and beverages. Inulinase have also found their application for inulin substrates hydrolysis for single-cell oil and single-cell protein production. The isolated fungus was identified as *Aspergillus niger.* It has been isolated from numerous habitats. This enzyme is found in many plants species and is widely distributed in fungi, yeast and bacterial strains. In the present study; rhizosphere soil samples were collected and inoculated for the isolation of fungal species present in soil. A local fungal isolate identified as *Aspergillus niger* was tested for the optimization of inulinase production in solid state fermentation and submerged fermentation for various parameters such as pH, temperature, incubation time, carbon and nitrogen sources. Five different substrates wheat bran, banana peel, orange peel, sugarcane bagasse, garlic peel were used. Among five banana peel substrate resulted in maximum production of inulinase for incubation time in days. The inulinase production using banana peel as substrate was higher in submerged fermentation (61 U/ml) than solid state fermentation (52 U/ml). The optimum pH and temperature was found to be 5.4 and 37°C respectively. In submerged fermentation inulinase showed higher activity when inulin (72 U/ml) with respect to sucrose (67 U/ml) was used as carbon sources. Yeast extract showed higher activity in submerged fermentation (66 U/ml) was used as nitrogen sources.

Index Terms- Inulinase, Submerged fermentation, *Aspergillus niger*, inulin, Banana peel.

I. INTRODUCTION

The agro-industrial residues such as coffee husk, wheat straw, pineapple waste, mixed fruit, maosmi waste, sugarcane bagasse, banana peel, rice bran, wheat bran, and kiwi fruit peel have been investigated for their potential to be used as substrates. Inulinase is one of the most important enzymes in food processing industries mainly for extraction and clarification of high fructose syrups, fructo-oligosaccharides, ethanol and inulo-oligosaccharides.[1]

Banana is a fast growing and high biomass yielding plant. India is the largest producer of banana next to mango. Banana has a protective outer layer (a peel or skin) with a fleshy, edible inner portion. Banana peels waste are readily available as agricultural waste, yet they seem to be under utilized as a potential growth medium for local yeast, fungi strains, despite their rich carbohydrate content and other basic nutrients that can support fungi growth. It contains 50% cellulose, 25% hemicelluloses and 25% lignin which can be easily utilized by the fungus for metabolism. The indigenous yeast and fungi with good fermentation attributes, which may enhance ethanol yield and other enzyme and minimize cost of production, could be obtained from ripe banana peels.[7]

Aspergillus which includes a set of fungi that are generally considered asexual, although perfect forms (forms that reproduce sexually) have been found. *Aspergillus niger which* is distinguished by its black spores, so that it usually called Black Mold. They are geographically widely distributed, and have been observed in a broad range of habitats because they can colonize a wide variety of substrates. *Aspergillus niger* is commonly found as a saprophyte growing on dead leaves, stored grain, compost piles, and other decaying vegetation.

The spores are widespread, and are often associated with organic materials and soil. The primary uses of *Aspergillus niger* are for the production of enzymes and organic acids by fermentation. While the food, for which some of the enzymes can be used in preparation. The fungi are heat tolerant filamentous fungus. *Aspergillus niger* was used for the optimization of inulinase production parameters in solid state fermentations and also to clarify the specific fungal strain with the best enzyme (Inulinase) production activity.[9]

Sources of Inulinase:

Inulinase can be produced from a wide array of organisms including plants, bacteria, molds and yeast.[14] Inulin is predominantly found in plants belonging to either *Liliaceae*, (e.g., leek, onion, garlic and asparagus). Dandelion, Chicory and Jerusalem artichoke have been proven to contain inulinase that can be extracted and purified, but according[6] these plant sources are not as productive as the microbial ones, which seem to be the only source capable of producing enough enzymes for industrial applications. For this reason, in the last three decades, significantly efforts have been made to find the best microbial source for the extraction of inulinase.[12] A number of fungal, yeast, and bacterial strains have been used for the production of inulinase. Among them, fungal strains belonging to *Aspergillus sp., and Penicillium sp.* and yeast strains belonging to *Kluyveromyces sp.* (diploid yeast) are apparently the most common.[11]

Classification of Inulinase

Inulin is hydrolyzed by enzymes known as Inulinase. Endo-inulinases and Exo-inulinases are glycosidase that perform the endo-hydrolysis and exo-hydrolysis of 2, 1-β-D-fructosidic linkages (β-2, 1-fructanlinks) in inulin. It is clear from the literature that two different actions can be exerted by inulinase on inulin molecules: an external action and an internal action, the corresponding two kinds of inulinase are called exo-inulinase and endo-inulinase.[3 & 5]

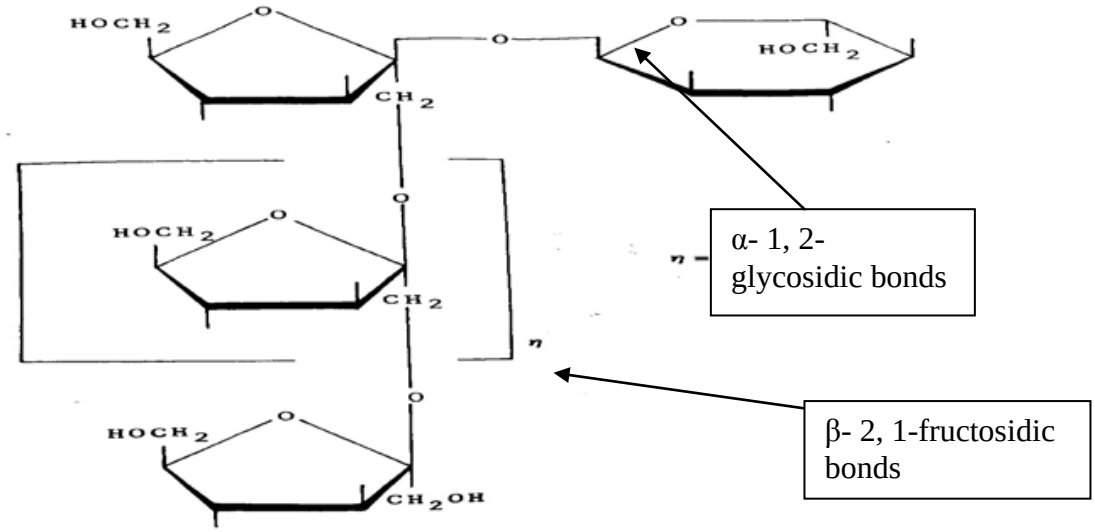

Figure 1: Schematic Structure of inulin structure.[13]

Exo-inulinase splits of the terminal fructose units from inulin, exo-inulinase action begins with the separation of the first D-fructose molecule and goes on until the last linkage within the molecule of inulin is broken and a molecule of D-glucose is released. Exo-inulinases (β-D-fructo hydrolase), split terminal fructose units in sucrose, raffinose and inulin to liberate fructose.[10]

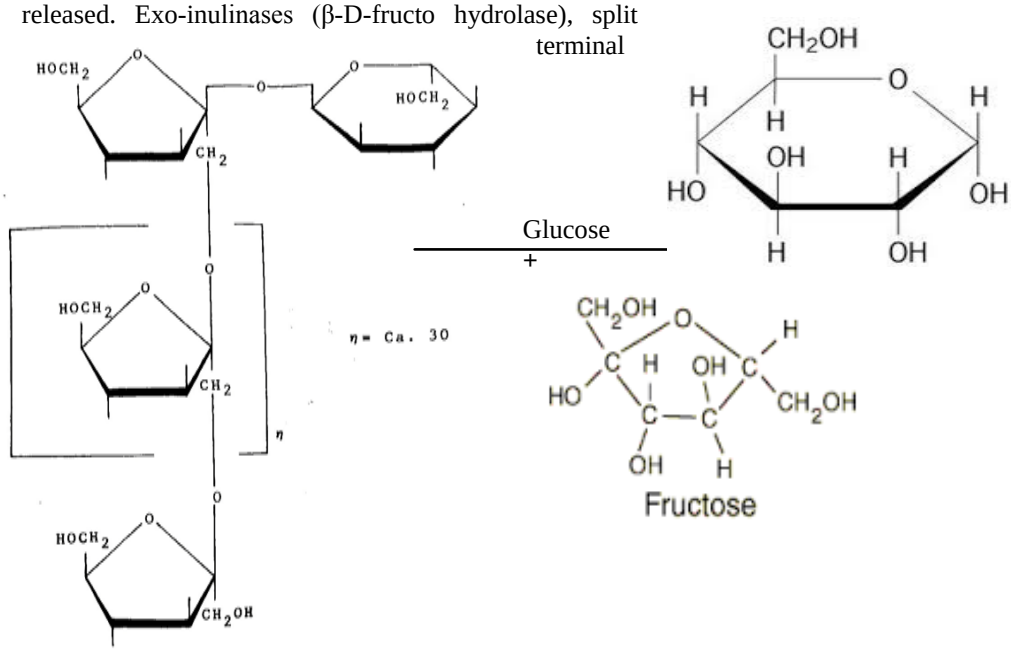

Figure 2: Action of Exo-inulinase

Endo-inulinase breaks down inulin into inulo-oligosaccharides (IOSs), it acts on the internal linkages and yields a set of inulo-oligosaccharides[5]. Endo-inulinases (2, 1-β-D-fructan fructo hydrolase) are specific for inulin and hydrolase it by breaking bonds between fructose units that are located away from the ends of the polymer network, to produce oligosaccharides. The property of having an exo- or an endo-action depends on the microbial origin of the enzyme.[2]

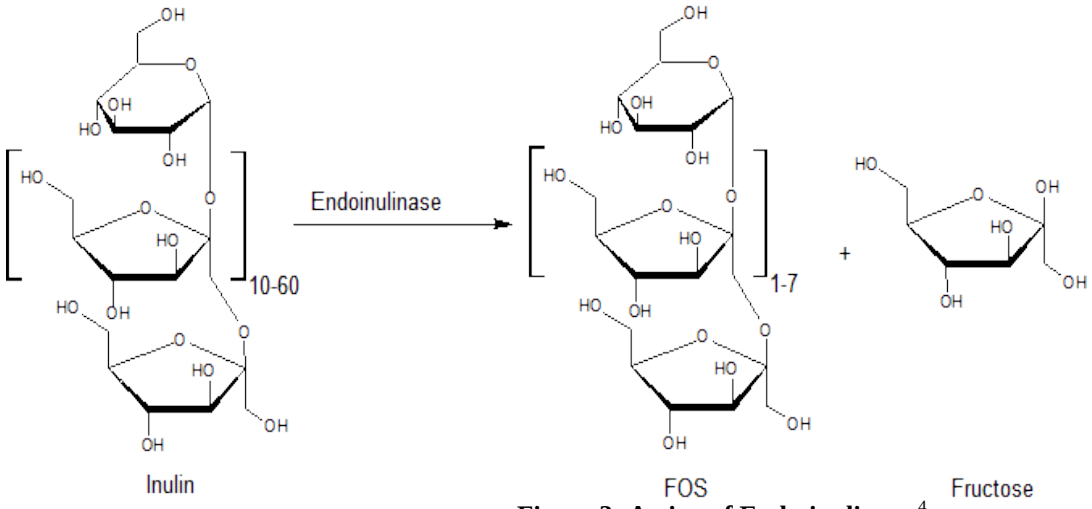

Figure 3: Action of Endo-inulinase [4]

In the present study, *Aspergillus niger* was isolated from soil sample and screened for production of Inulinase from food waste. Inulinase production had been reported to be produced in submerged fermentation. The optimization of inulinase production by incubation time, pH, temperature, carbon sources, nitrogen sources, while using the food waste as substrate also had been studied.

II. MATERIALS AND METHODS

Isolation and screening of inulinase producing fungi

Fungus was obtained from rhizosphere soil for the study which was collected in sterilized petriplates of different rhizosphere region (from village). Isolated fungus was maintained on PDA media slants at 27°C and subculture every month and stored at 4°C**.** The inulinase producing strain was screened from those isolated cultures by using inulin containing medium. The strain which produced zone of clearance was taken. The medium which was used for screening composed of- Inulin-10gm, Yeast extract-10g, $NaNO_3$-10g, KH_2PO_4-5g, $MgSO_4.7H_2O$–1g, Agar - 15g, Distilled water -1 liter, pH- 5. [9]

Submerged Fermentation

Submerged fermentation of food waste (banana peel) was carried out on a rotator shaker at 150 rpm at 37°C in 250ml of Erlenmeyer flask that filled nutrient solution. It was autoclaved at 121°C for 20 minutes. That flask was inoculated by *Aspergillus niger* spores. The samples were collected after 3, 6, 9 and 12 days. The samples were centrifuge at 5000 rpm for 15 minutes. The supernatant was used as enzyme extract and used for enzyme assay.

III. OPTIMIZATION OF INULINASE ENZYME PRODUCTION

Effects of pH on Inulinase activity

To determine the optimal pH, inulinase activity was measured over a pH range of 4 to 5.6 using inulin as substrate. Sodium acetate buffer was used to obtain the pH gradient. Sodium acetate buffer with different pH ranges (4, 4.4, 5, 5.4 and 5.6) was prepared, and then 1 gm of substrate (inulin) was dissolved in 100 ml of each previous buffer with pH range (4 to 5.6) to substrate concentration1%. 0.1 ml of crude enzyme (enzyme extract) was added to 0.9 ml of inulin solution in test tubes, and the mixture was incubated for 15 min. in water bath at 50°C. The reaction was stopped by adding 1ml of 3, 5 dintro salicylic acid solution. All tubes were boiled in boiling water bath for 5 min., then directly cooled in cold water bath. 10 ml of distilled water was added to each tube, and mixed well. Absorbance was read at 540nm.

Effects of temperature on Inulinase activity

The effect of temperature on inulinase activity was examined over a temperature range .The substrate solution(0.9ml) was added to 0.1ml of enzyme solution and incubated for 15 minute at different temperatures (0,20,27,37 and 50)°C**,** then the activity was estimated. The reaction was stopped by adding 1ml of 3, 5 Dintro salicylic acid solutions. All tubes were boiled in boiling water bath for 5 min., then directly cooled in cold water bath. 10 ml of distilled water was added to each tube, and mixed well. Absorbance was read at 540nm

Effects of carbon sources on Inulinase production

To study the effect of different carbon sources on Inulinase production, about 10 gm of each substrate was transfer separately into four 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 pH-5.4 and 37°C for 3 days and enzyme assay was done.

Effects of nitrogen sources on Inulinase production

To study the impact of different nitrogen sources on inulinase production, about 10 gm of each substrate was transferred separately into four conical flasks. They were further supplemented with different nitrogen sources such as yeast extract, peptone, ammonium nitrate, and potassium nitrate. Flasks were inoculated with 1ml of fungal spore suspension and then incubated at pH-5.4 and 37°C for 3 days and enzyme assay was done.

Enzyme Assay Method

Inulinase activity was determined by the method.[8] A blank was also prepared that does not contain enzyme. 0.1 ml of crude enzyme (enzyme extract) was added to 0.9 ml of inulin solution in test tubes, and the mixture was incubated for 15 min. in water bath at 50°C . The reaction was stopped by adding 1ml of 3, 5 dinitro salicylic acid solution. All tubes were boiled in boiling water bath for 5 min., then directly cooled in cold water bath. 10 ml of distilled water was added to each tube, and mixed well. Absorbance was measured in spectrophotometer at 540 nm, and then enzymatic activity is determined by depending on fructose standard curve. One unit of Inulinase activities defined as amount of enzyme that is needed to produce 1 mol of fructose (reducing sugar) per minute.

IV. RESULTS, OBSERVATIONS AND DISSCUSSION

Isolation of the fungi

The fungus was successfully isolated from the rhizosphere soil on PDA. The fungi culture was identified as *Aspergillus niger* on the basis of its morphological characteristics. *Aspergillus niger* which was distinguished by its black spores, so that it usually called Black Mold and They were geographically widely distributed, and was observed in a broad range of habitats because they can colonize a wide variety of substrates. The isolated fungus identified as *Aspergillus niger* species was screened for inulinase production on PDA containing inulin. The petriplates and slants containing fungal culture showed the formation of Black spores zones around the fungal colonies.

Production of inulinase under Submerged fermentation for different food wastes

In submerged fermentation *Aspergillus niger* was cultivated on different food wastes (banana peel, sugarcane bagasse, orange bagasse, garlic peel and wheat bran). The enzyme activity was measured from the third day of incubation and further reading was taken after an equal interval of three days. The enzyme activity in submerged fermentation of different inulin waste by *Aspergillus niger*. *Aspergillus niger* growing on banana peel showed the highest enzyme activity of 62 U/ml on 9th day as compared to different substrates such as wheat bran (61 U/ml), orange peel (36 U/ml), sugarcane bagasse (41 U/ml), and garlic peel (36 U/ml) at 9th day of incubation. (Figure- 4).

Production of inulinase under submerged condition

The isolated fungus was cultivated on nutrient medium and enzyme activity observed from third day of incubation and mean after three days interval. The activity was higher at 9th day of incubation (Figure-5). Highest activity measured was 61 U/ml.

Effect of temperature on inulinase activity

The effect of temperature on inulinase activity was examined over a temperature range. The substrate solution (0.9ml) was added to 0.1ml of enzyme solution and incubated for 15 minute at different temperatures (0, 20, 27, 37 and 50) °C**,** and then the enzyme activity was measured. The reaction was stopped by adding 1ml of 3, 5 dinitro salicylic acid solution. All tubes were boiled in boiling water bath for 5 min., then directly cooled in cold water bath. 10 ml of distilled water is added to each tube, and mixed well. Absorbance was read at 540nm. Inulinase enzyme showed maximum activity at 37°C was noted as optimum temperature. The inulinase produced by *Aspergillus niger* showed the highest activity at 37°C (Figure-6).

Effect of pH on inulinase activity

Inulinase activity was measured over a pH range of 4 to 5.6 using inulin as substrate. The pH gradient was obtained by sodium acetate buffer. Sodium acetate buffer with different pH ranges (4, 4.4, 5, 5.4 and 5.6) was prepared. 1 gm of substrate (inulin) was dissolved in 100 ml of each previous buffer with pH range (4 to 5.6) to substrate concentration 1%. The optimal pH was found to be 5.4 for inulinase. The inulinase production from *Aspergillus niger* was active in pH range 5 to 5.6, and exhibiting the highest activity at 5.4 pH (Figure-7).

Effects of carbon sources on inulinase production

Four carbon sources (glucose, sucrose, lactose and maltose) were tested against inulin for inulinase production . The flask was maintained as control inulin was found to be carbon source in comparison to other sources. Inulinase production was studied for substrate with carbon sources in the nutrient solution. In submerged fermentation inulinase showed higher activity when inulin (72 U/ml) with respect to sucrose (67 U/ml) was used as carbon sources showed in (Figure-8).

Effects of nitrogen sources on inulinase production

Various nitrogen sources (peptone, ammonium nitrate, yeast extract and potassium nitrate) were added along with nutrient solution to the solid and submerged substrates. The presence of Yeast extract effectively produced maximum inulinase activity by *Aspergillus niger* with Yeast extract produced maximum inulinase activity in banana peel under submerged fermentation (55 U/ml) in (Figure-9). But potassium nitrate and ammonium nitrate had an inhibitory effect on inulinase production compared to the others

Graphical representation of optimization of Submerged Fermentation

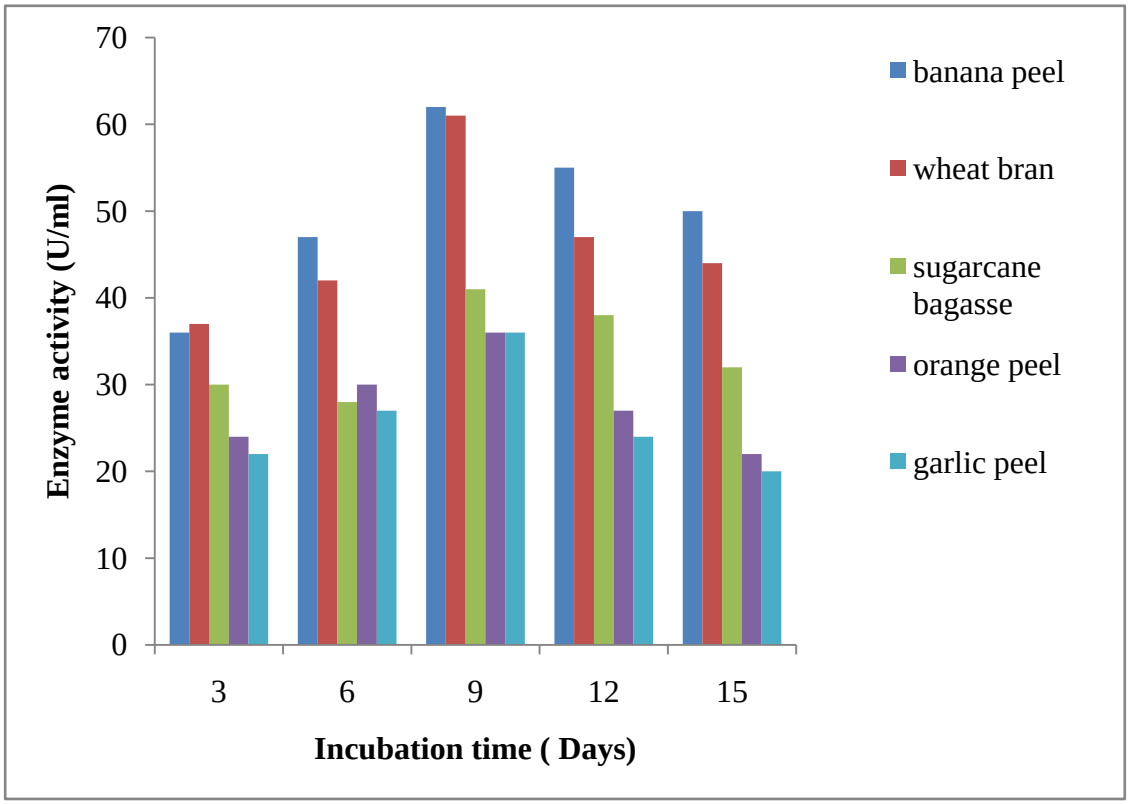

Figure 4: Inulinase activity of *Aspergillus niger* in submerged fermentation of different food wastes [enzyme activity (U/ml) v/s incubation time (days)].

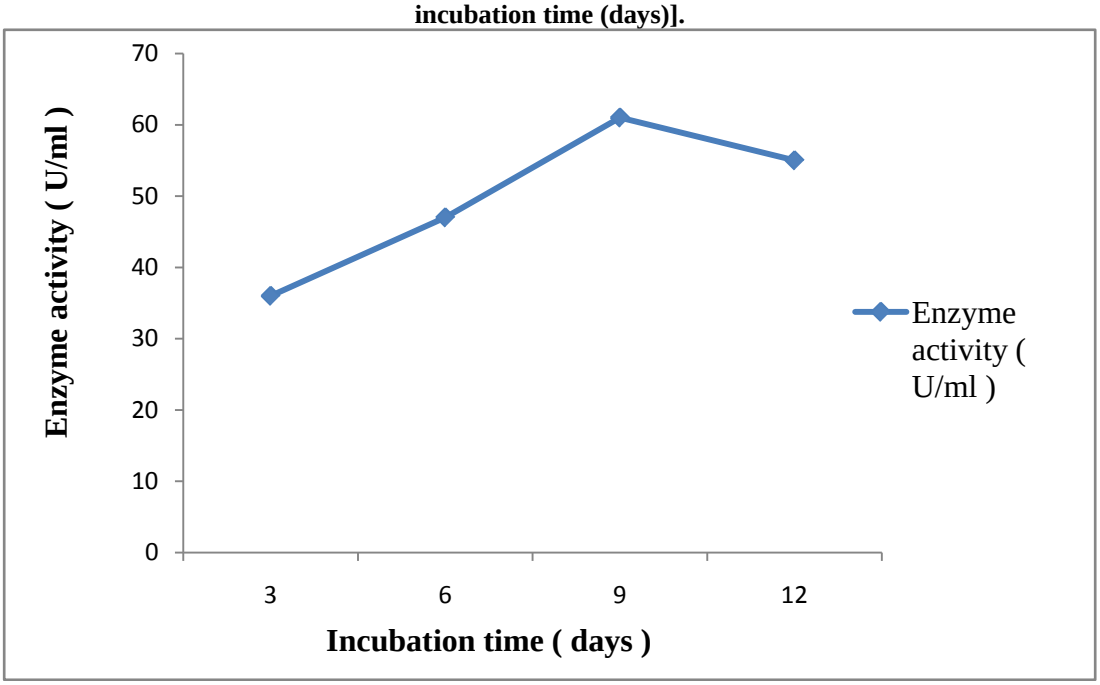

Figure-5: The activity of inulinase produced by *Aspergillus niger* after particular days of incubation under submerged condition [enzyme activity (U/ml) v/s incubation time (days)].

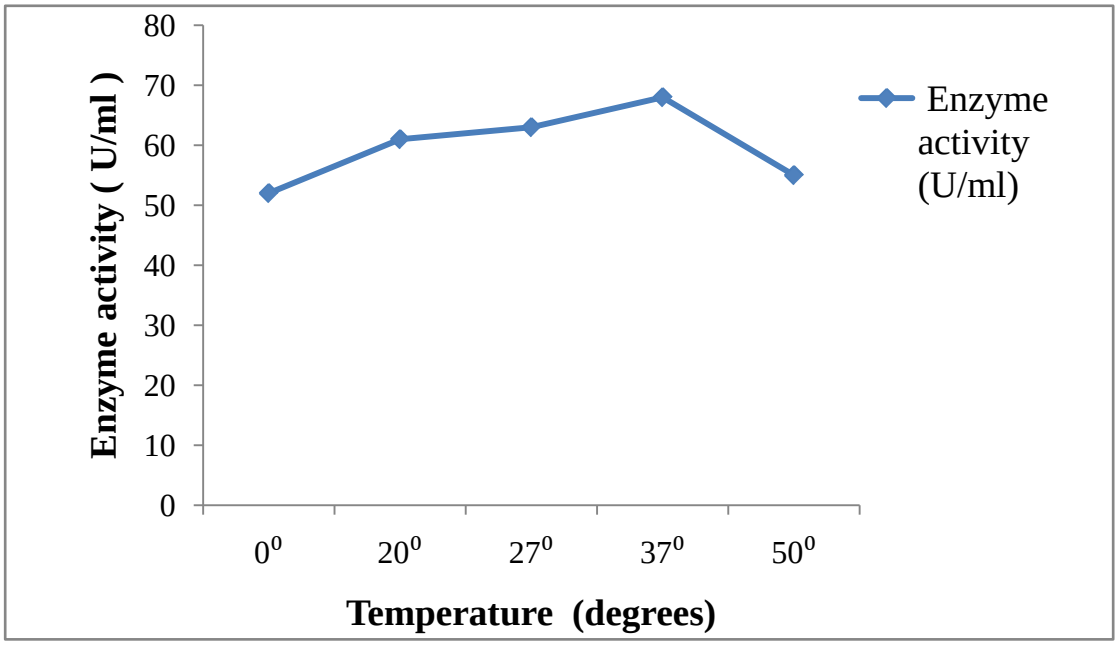

Figure 6: Effect of temperature on activity of inulinase from *Aspergillus niger* [activity (U/ml) v/s temperature (degree)]

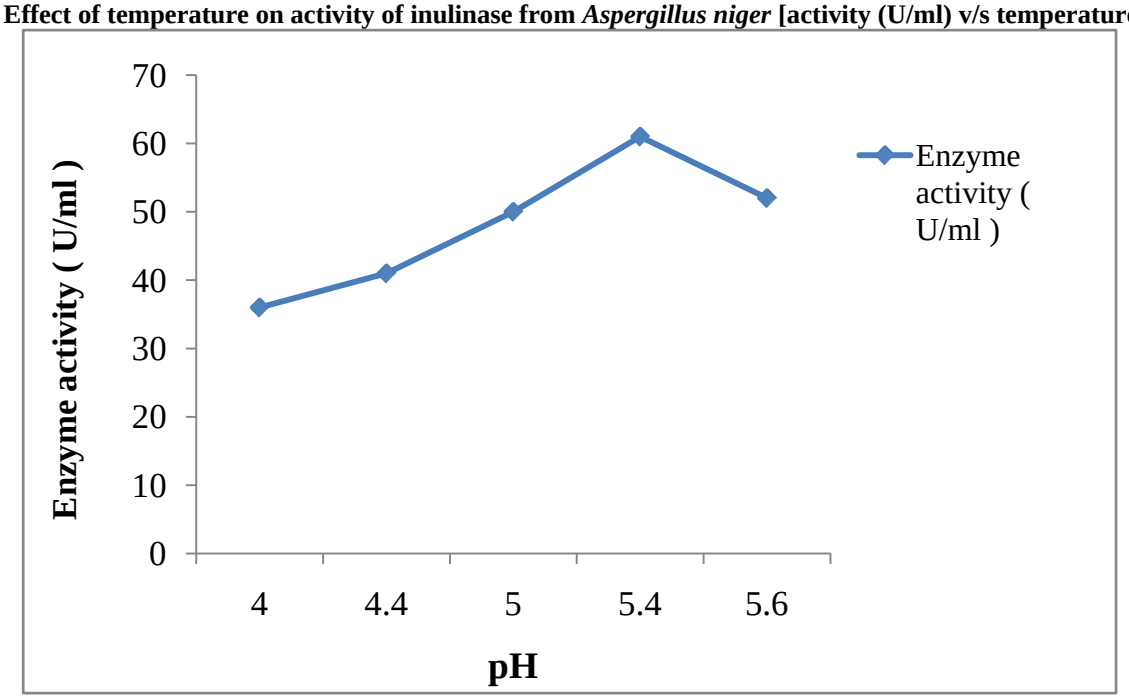

Figure 7: Effect of pH on activity of inulinase from *Aspergillus niger* [enzyme activity (U/ml) v/s pH].

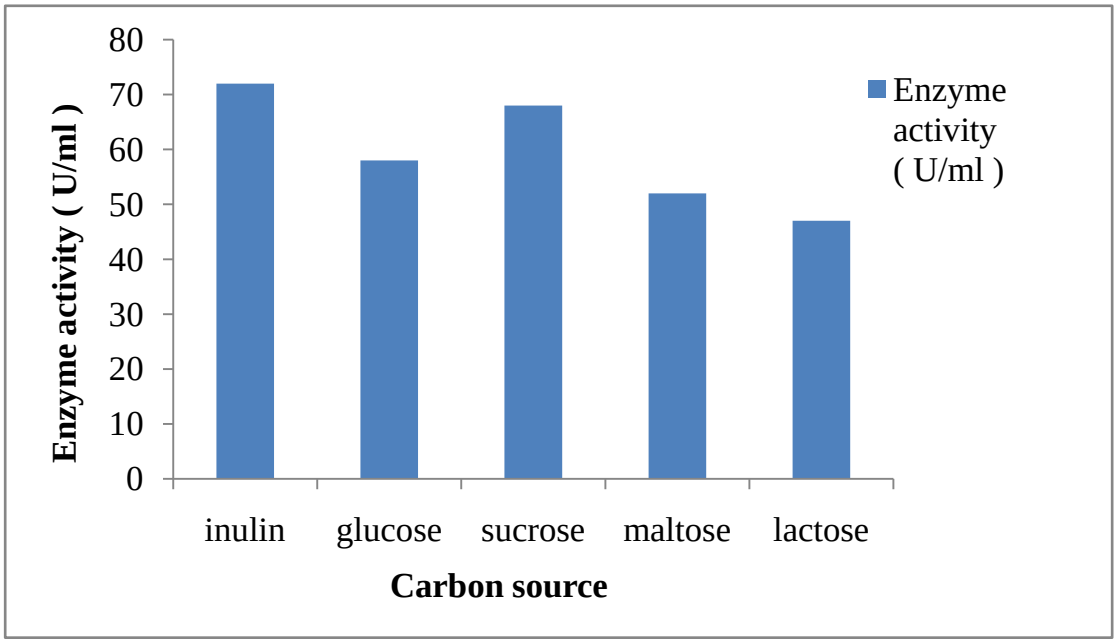

Figure 8: The enzyme activity reached the maximum in particular carbon source under submerged condition [enzyme activity (U/ml) v/s carbon sources].

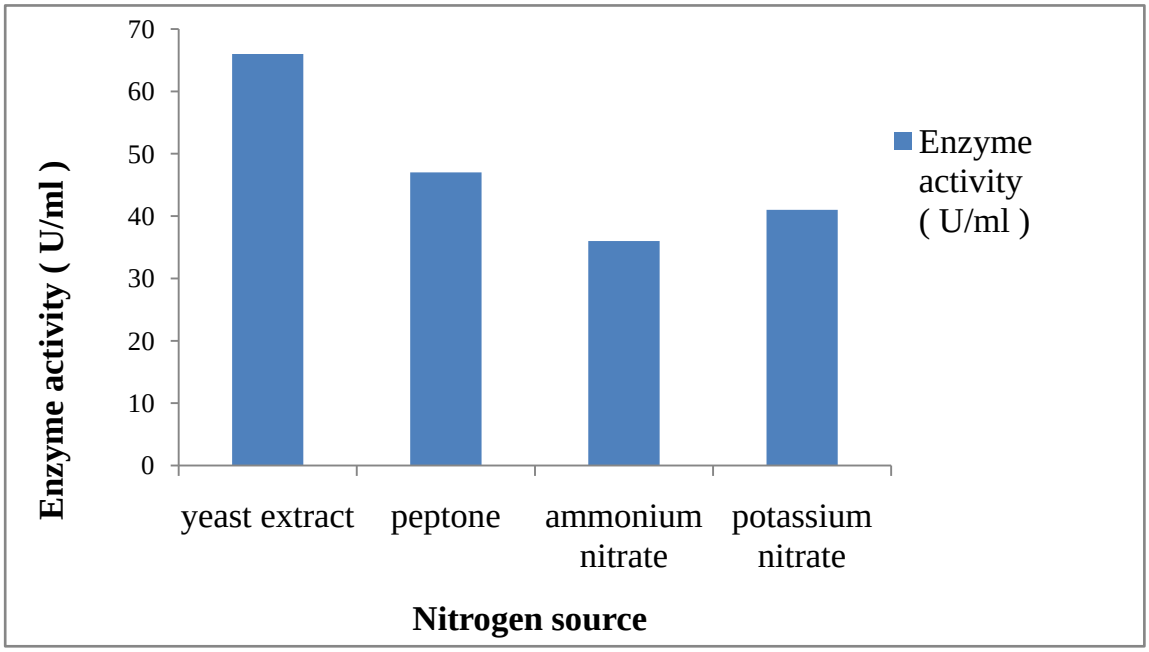

Figure 9: The enzyme activity reached the maximum in particular nitrogen source and under submerged condition [enzyme activity (U/ml) v/s nitrogen sources].

V. CONCLUSION

In this study, the Inulinase production using banana peel as substrate was higher in submerged fermentation (61 U/ml) than solid state fermentation (52 U/ml). The optimum pH and temperature was found to be 5.4 and 37°C respectively. In submerged fermentation inulinase showed higher activity when inulin (72 U/ml) with respect to sucrose (67 U/ml) was used as carbon sources. In solid state fermentation inulinase showed higher activity when inulin (55 U/ml) with respect to sucrose (50 U/ml) was used as carbon source. Yeast extract showed higher activity in both solid state (55 U/ml) and submerged fermentation (66 U/ml) when it was used as nitrogen source. This study clearly indicates that using pure inulin is costlier so research is the way to limiting the cost and for the commercialization is possible to scale up the inulinase production by *Aspergillus niger* through submerged fermentation. Banana peels can be used as alternative

feed stocks in the production of microbial inulinase using a more economical method.

REFERENCES

[1] Bender J. P., Mazutti M. A., Oliveira D., Luccio M., and Treichel H.,, "Optimization of inulinase production by solid state fermentation using sugarcane bagasse as substrate". Journal of Biotechnology. 2003; 12: 1123-1131.

[2] Cairns, A. J., "Fructan biosynthesis in transgenic plants". Journal of Exp. Bot.. 2003; 54: 549-567.

[3] Ettalibi M. and Baratti C. "Purification, Properties and comparison invertase, Exoinulinase and Endoinulinase of *Aspergilus ficcum"*. Journal Applied Microbiology and Biotechnology. 1980; 26: 13-20.

[4] Gill P. K, Sharma A. D., Harchand R. K., and Singh P., "Effect of Media Supplements and Culture Conditions on Inulinase Production by an Actinomycete Strain,". Journal of microbiology. 2006; 87 (3): 359-362.

[5] Kango N., "Production of Inulinase using tap roots of Dandelion (Taraxacum officinale) by *Aspergillus niger*," Journal of Food Engineering. 2008; 85 (3): 765-770

[6] Kochhar A., Gupta A. K., and Kaur N.,"Inulinase from *Aspergillus versicolor*: A potent enzyme for production fructose from inulin." Journal of Science Industrial. 1997; 56: 721-726.

[7] Kumar, G. P. ; Kunamneni, A. ; Prabhakar, T. and Ellaiah, P. "Optimization of process Parameters for the production inulinase from a newly isolated *Aspergillus niger*AUP1". World J Microbiol and Biotechnol. 2005; 21(8): 1359-1361.

[8] Miller G. L., "Use of Dinitrosalicylic Acid Reagent for Determination of Reducing Sugar. Analytical Chemistry. 1959; 31: 426-428,

[9] Narayanan M., Srinivasan B., Gayathiri A, Ayyadurai A. and Arunkumar Mani. "Studies on the Optimization and Characterization for the Biosynthesis of Inulinase under Solid state Fermentation". Journal of biotechnology. 2013; 5(1): 376-384.

[10] Onodera S. and Shiomi N. "Purification and substrate specificity of endo-type inulinases from *Penicillium purpurogenum"*. Agriculture Biological Chemistry. 1988; 52: 2569-2576.

[11] Pandey A., and Selva kumar P., "Solid State Fermentation for the Synthesis of Inulinase from *Staphylococcus* sp. and *Kluyveromyces marxianus*," Journal of Bioresource technology. 1999; 69: 123-`129.

[12] Ricca E., Calabro V., Curcio S., and Iorio G., "The production of fructose by Chicory inulin enzymatic hydrolysis: a kinetic study and reaction mechanism". Journal of Process Biochemistry. 2009; 44: 446-470

[13] Stolzenburg, K., "Jerusalem artichokes raw material for inulin and fructose production". Zuckerindustrie. 2005; 130: 193-200.

[14] Vandamme E. J. and Derycke D. G, "Microbial Inulinases: Fermentation Process, Properties, and Applications," Advances in Applied Microbiology. 1983; 29 (1): 139-176.

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