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RESEARCH ARTICLE

SCREENING AND OPTIMIZATION OF NUTRITIONAL CONDITIONS FOR ENHANCED MANNANASE PRODUCTION BY *STREPTOMYCES SP. AZA12*.

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Abstract

Different actinomycete isolates were screened for the production of mannanase under submerged state fermentation. *Streptomyces sp. AZA12* was selected as the most potent isolate with highest enzyme activity and was therefore selected for further studies. The nutritional requirements of *Streptomyces sp. AZA12* for the production of mannanase in submerged fermentation were investigated. Different agricultural wastes were screened as substitutes to commercial substrate for mannanase production. Soyastraw was found to be the most effective carbon source with highest mannanase activity of 48.33 U/ml. Asparagine was obtained to be the best nitrogen source with highest activity of 93.38 U/ml out of all the nitrogen sources screened in this study. Process parameters like pH, temperature, time of incubation, affecting enzyme production were optimized using one-factor-at-a-time approach. Mannanase production was conducted in mineral salt medium and enzyme activity was determined by dinitrosalicylic acid method. The optimal culture conditions obtained from this study helped to standardize the requirements for optimum production of mannanase using agrowastes.

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Introduction:-

β -mannanase is the enzyme that catalyses the endolytic hydrolysis of β -1,4 mannosidic linkages in mannopolysaccharides such as β -1,4-mannan, glucomannan and galactomannan. Mannan and heteromannans are a part of the hemicellulose fraction in plant cell walls. (Dhawan, *et al.* 2008). Mannan endo-1,4- β -mannosidase or 1,4- β -D-mannanmannohydrolase (EC 3.2.1.78) commonly known as mannanase, randomly hydrolyzes β -1,4-glycosidic linkage of mannan, glucomannan, galactomannan and galactoglucomannan, to yield manno oligosaccharides (Zhengqiang Jiang, *et al.* 2006). Endo- β -mannanase is an endohydrolase, which is a member of glycosyl hydrolase family 5 and 26. As an endohydrolase it hydrolyses the internal bonds of a polysaccharide. Various mannanases from fungi, yeasts and bacteria as well as from germinating seeds of terrestrial plants has been produced. A vast variety of bacteria, actinomycetes, yeasts and fungi are known to be mannan degraders (Talbot and Sygusch, 1990; Puchart *et al.*, 2004). Production of β -mannanase by micro-organisms is more promising due to its low cost, high production rate and readily controlled conditions.

In our earlier study *Streptomyces sp. AZA12* was screened using plate assay technique, and it was observed to give considerable mannanase activity. Based on this information, the isolate was selected for this present study. The objectives of the study are therefore to screen selected agroresidues as substrates for mannanase production as well

as evaluate the effect of variation of certain process parameters on mannanase production under submerged state fermentation.

Material and method:-

Actinomycetes isolate:

Streptomyces sp. AZA12 isolated from soil sample previously confirmed positive for mannanase activity by plate assay method in our previous work was used in this study. The actinomycete isolate was identified in the Genomebio technology Pvt. Ltd laboratory, Pune, Maharashtra. The actinomycete isolate was maintained on guar gum containing agar slants and sub-cultured at regular intervals. They were incubated at $40 \pm 2^\circ \text{C}$ for 4 days and stored at 4°C in refrigerator.

Chemicals and agroresidues:

The selected agroresidues were procured from farm fields, local market, and domestic sources. The substrates were washed, sundried, oven dried at 50°C and crushed into fine particles and sieved through 1mm mesh size. The powdered substrates were stored in plastic container to keep it moisture free for further use. Guar gum was purchased from HiMedia chemicals and all other chemicals used were of analytical grade.

Media preparation and enzyme production:

For the production of mannanase in submerged state fermentation, the isolate was grown at 40°C in 250 ml Erlenmeyer flask. This medium contained the following ingredients (g/L): guar gum 0.5%, NaNO_3 2, K_2HPO_4 1, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.5, KCl 0.5 and $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ traces (Oladipo O. Olaniyi *et al.* 2013). Final pH was adjusted to 7.0 and then sterilized at 121°C for 15 minutes. After sterilization, each flask was inoculated with 5% spore suspension containing $1220 \times 10^7 \text{cfu/ml}$. The flasks were incubated at 40°C for 4 days at 150rpm. Sterile medium supplemented with guar gum without organism served as the control. After incubation the production medium was filtered to remove the biomass and the clear filtrate was used as crude enzyme for further study.

Enzyme assays:

Mannanase activity was assayed in the reaction mixture composing of 0.5 ml of 50mM potassium phosphate buffer pH 7.0 and 1% Guar Gum (GG) with 0.5 ml of supernatant at 45°C for 60 min. Amount of reducing sugar released was determined by the dinitrosalicylic acid reagent (DNS) (Miller, 1959). One unit of mannanase activity was defined as amount of enzyme producing 1 micromole of mannose per minute under the experimental conditions.

Optimization of process parameters:-

Effect of agroresidues (carbon source) on mannanase production:

Different agrowastes were used as carbon source and evaluated with Guar Gum (GG) serving as control. The broth was distributed into 250mL flasks containing 50ml minimal medium and 1% of each carbon sources were inoculated with spore suspension of potent strain, the flasks were incubated for 4days at 40°C at 150 rpm.

Effect of nitrogen sources on mannanase production:

The appropriate nitrogen source for mannanase production was determined by supplementing the fermentation medium with different nitrogen sources (organic and inorganic) at 0.2% level, replacing the prescribed NaNO_3 of the fermentation medium. (Oladipo O. Olaniyi *et al.* 2013).

Effect of NaCl on mannanase production:

The effect of NaCl on mannanase production was studied by supplementing the medium with different NaCl concentrations ranging from 1% to 8%. After incubation mannanase assay was carried out according to standard assay procedures (KhamphengPhothichitto, *et al.* 2006).

Effect of incubation period on mannanase production:

Fermentation period is an important parameter for enzyme production. In this study, the fermentation experiment was carried out up to 168 hours and production rate was measured at 24 hours intervals. Mannanase assay was carried out according to standard assay procedures (KhamphengPhothichitto, *et al.* 2006).

Effect of pH on mannanase production:

The fermentation medium used for mannanase production was initially adjusted using NaOH or HCl to cover a pH range from 3.0 to 9.0 (All adjustments were made before sterilization). After inoculation, the flasks were incubated

under shaking condition for 4 days at 40°C. After 4 days of incubation, the culture was centrifuged to obtain supernatants which were used for mannanase assay and protein content determination according to standard assay procedures.

Effect of temperature on mannanase production:

The effect of different temperatures was studied on mannanase production. After inoculation, the flasks were incubated at different temperatures ranging from 20°C to 50°C. After incubation mannanase assay was carried out according to standard assay procedures (KhamphengPhothichitto, *et al.* 2006).

Effect of sugars on mannanase production:

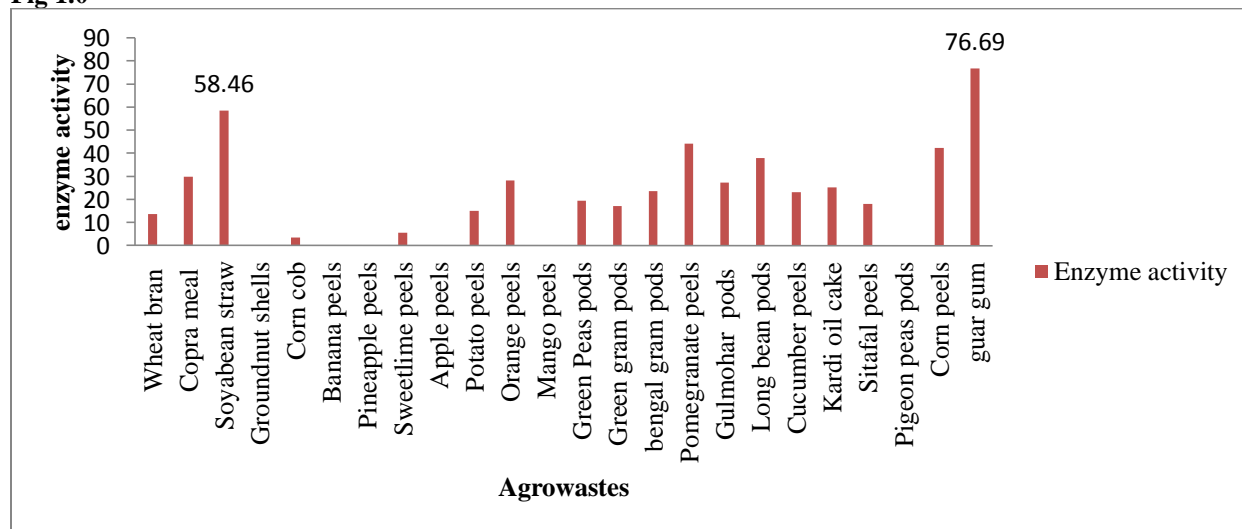
Different sugars were supplemented to evaluate the induction or repression on mannanase production. (Akinyele, *et al.* 2013). After incubation mannanase assay was carried out according to standard assay procedures (KhamphengPhothichitto, *et al.* 2006).

Results and discussion:-

Effect of carbon source (agroresidues) on mannanase activity:

Agro-industrial by-products are available in large amounts and they have been used for the production of several enzymes (Mabrouk and El Ahwany, 2008; Howard *et al.*, 2003). Table 1 shows that several types of agro-wastes were evaluated as substrates for mannanase production by *Streptomyces sp.AZA12* in comparison to guar gum (control). Mannanase synthesis by *Streptomyces sp.AZA12* was highest when soystraw was utilized as a carbon source compared to other agro-wastes (56.46U/ml). However, the value obtained for soystraw was significantly lower than that of guar gum (control) (76.69U/ml). It might be due to the fact that soyastraw provided adequate amount of nutrients like proteins, carbohydrates, fats, fibers, ash, calcium, magnesium, phosphorous, potassium, sulphur, amino acids etc. (Akinyele, *et al.* 2013).

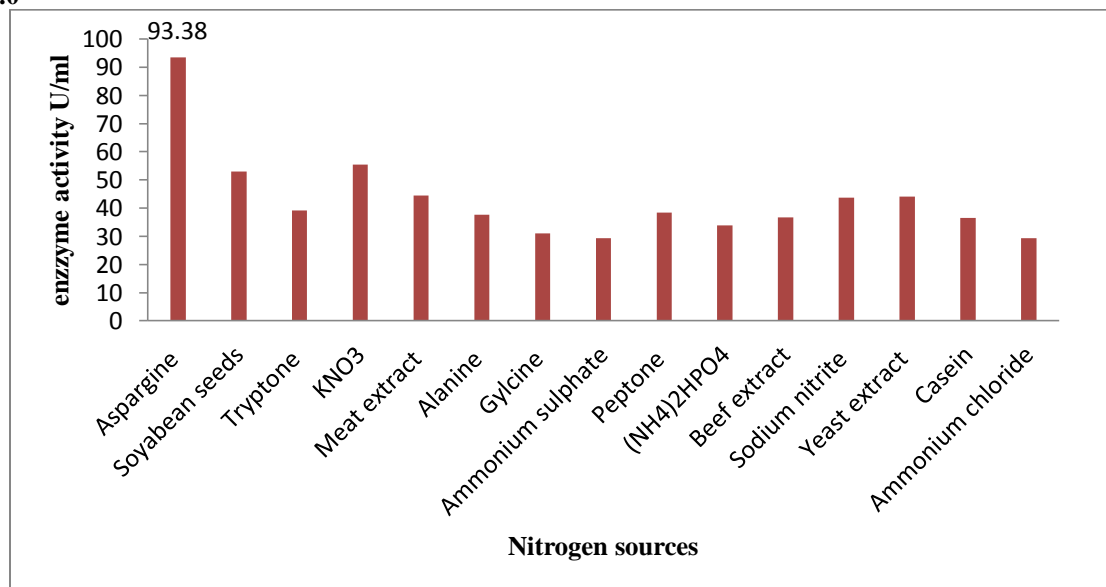
Fig 1.0



Effect of different nitrogen sources on mannanase activity:-

In this present work, different nitrogen sources were added separately to the fermentation medium at 0.2% replacing NaNO₃ (control) in the mineral salt medium. Among the tested nitrogen sources; asparagine gave maximum enzyme activity 93.38U/ml. Okeke and Obi (1993) reported the more enzyme production using inorganic nitrogen sources as compared to the organic nitrogen sources from *Arthrographis* sp. They mentioned that the use of the organic nitrogen sources could also be advantageous than the inorganic ones, as the organic sources are cheaper in cost and also not involved in the competition with active site of the enzyme.

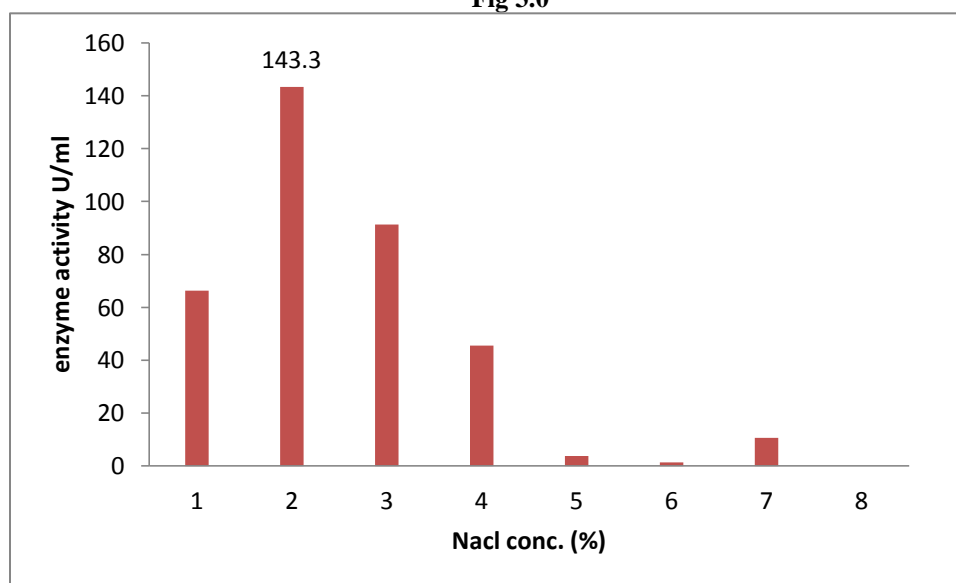
Fig:2.0



Effect of NaCl concentration:

The *Streptomyces* sp. AZA12 was able to grow at 8% of NaCl concentration but the mannanase activity decreased as the concentration of NaCl increased. After 3 % of NaCl concentration there was drastic decrease in mannanase activity and there was no enzyme activity at 8% NaCl.

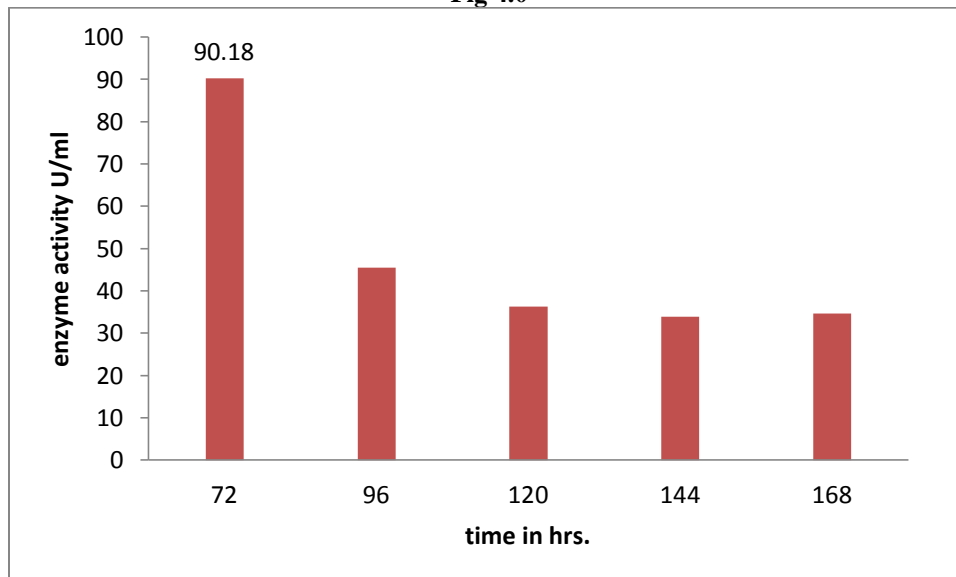
Fig 3.0



Effect of incubation period:-

Data presented in Figure 4 showed the effect of different incubation periods on mannanase production by *Streptomyces* sp. AZA12. From the results, it was found that mannanase revealed its best production at 72 hrs of incubation (90.18 U/ml). At longer incubation periods beyond 72hrs, the activity decreased. The decrease in the production of mannanase by *Streptomyces* sp. AZA12 after 72 hrs of incubation period might be due to the depletion of nutrients and accumulation of other by-products in the fermentation medium initiating autolysis of cells (Meenakshiet al., 2010; Malik et al., 2010). The result obtained was same as the findings of other works that recorded maximum mannanase production at 72 hrs for *Penicilliumocittanis*(Blibechet al., 2010).

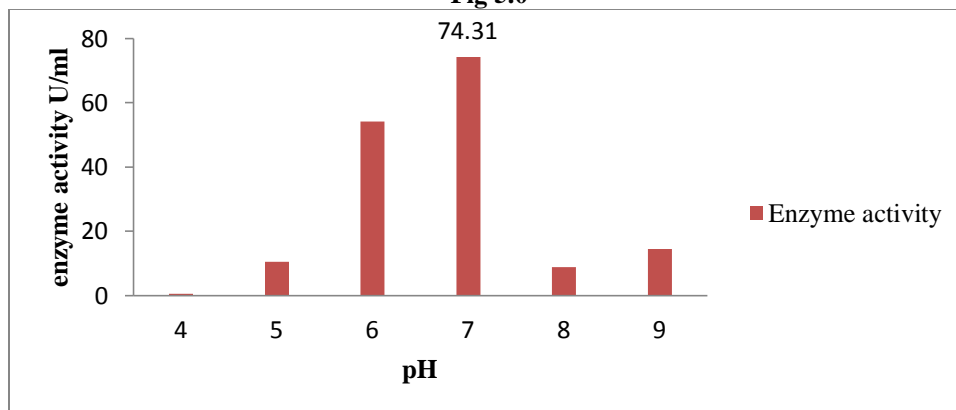
Fig 4.0



Effect of pH on mannanase production:

The effect of pH on mannanase production is shown in fig 5. The best mannanase production was seen at pH 7 with mannanase activity of 74.31 U/ml. At pH 8 there was drastic decrease in mannanase activity. The increase in mannanase activity at pH 7 might be due to the fact that acidic proteases that are active in degrading mannanase protein are inactive. Corresponding to the pH optimum of both bacterial and fungal mannanase, they were in the range of 3.0-6.0 (Sachtlehner A., *et al.* 1999). However, some of mannanases from *Bacillus subtilis* KU-1, *Bacillus* sp. KK01, *Bacillus* sp. strain JAMB-750, *Thermotoga neapolitana* 5068 and *Streptomyces ipomoea* showed higher optimum pH in the range of 7.0–10.0 (Takeda N., *et al.* 2004).

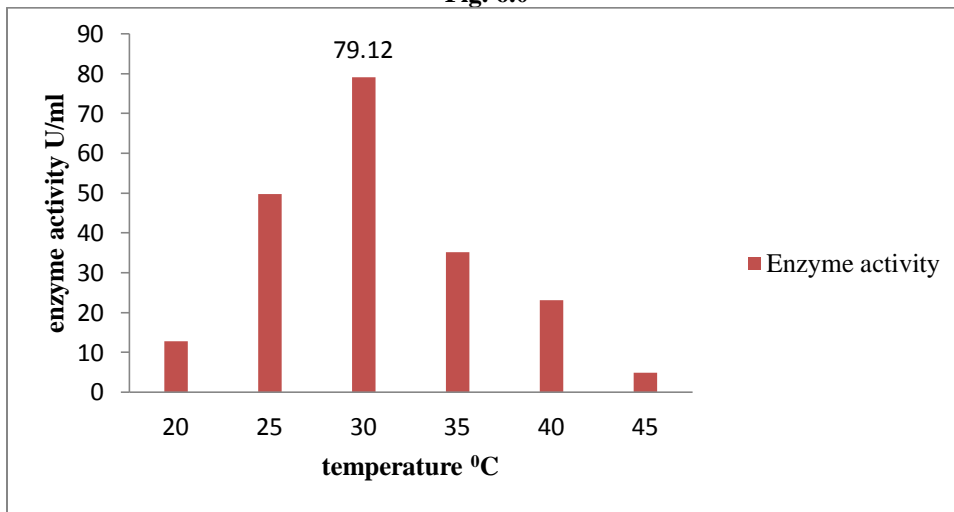
Fig 5.0



Effect of temperature on mannanase production:

The optimum temperature for the production of mannanase from *Streptomyces AZA12* was investigated from 20°C to 45°C. The medium was inoculated and incubated at different temperatures viz. 20°C, 25°C, 30°C, 35°C, 40°C and 45°C. Since the organism is a mesophile, very less growth as well as low amount of mannanase production was observed at higher temperature of 45°C. Maximum enzyme production was observed at 30°C (Fig. 6).

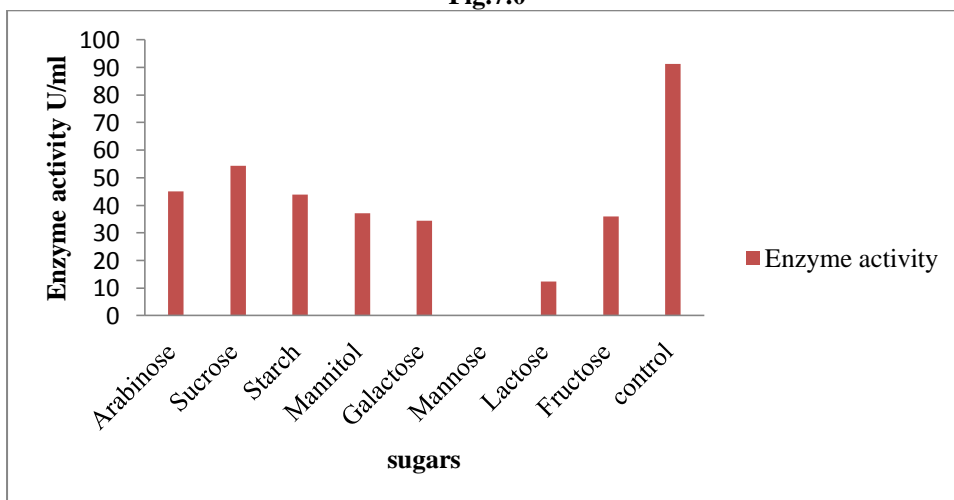
Fig. 6.0



Effect of sugars on mannanase production:

The effect of various sugars (0.2 g/l) (Akineyele, et al. 2013) supplemented to the enzyme production medium containing guar gum in order to evaluate its induction or repression effect on mannanase production were tested (fig 7). The highest activity was exhibited by guar gum (control). Addition of different sugars with guar gum was accompanied by severe inhibitory effects on enzyme production. Such results may be due to the catabolic repression processes when easily assimilated carbon sources were added (Moussa and Thawat, 2007; Mabrouk and El-Ahwany, 2008).

Fig.7.0



Conclusion:-

According to results *Streptomyces AZA12* has potential to be an indigenous source of mannanase production using cheap agrowaste soystraw as sole source of carbon in submerged fermentation. The optimal process parameters for the production of mannanase were screened of which asparagine as nitrogen source, 72hrs of incubation period, pH 7, 30°C temperature, was found to be the best for mannanase production. There was no induction in mannanase production after sugar supplementation to the production medium. Soystraw are agrowastes that could have good biotechnological potential.

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