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Full Length Research Paper

Optimization studies on mannanase production by *Trichosporonoides oedocephalis* in submerged state fermentation

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The study evaluated various agricultural wastes and process parameters for mannanase production by *Trichosporonoides oedocephalis* in submerged state fermentation. Different agricultural wastes were screened as substitutes to commercial substrate for mannanase production using substitution method. Process parameters affecting enzyme production were optimized using one-factor-at-a-time approach. Mannanase production was conducted in mineral salt medium and enzyme activity determined by dinitrosalicylic acid method. Among tested carbon sources, cassava peels proved to be the best for mannanase production (11.767 U/ml), while the lowest (0.100 U/ml) was observed with pineapple peels. Maximum mannanase activity and productivity (37.963 U/ml, 4.745 U/ml/hr) were observed after 96 hrs of incubation. Initial pH of the culture medium was also optimized and it was observed that enzyme activity increased as the pH of fermentation medium increased. The best nitrogen sources and inoculum concentrations (spores/ml) were ammonium nitrate and 1×10^6, respectively. The optimal culture conditions obtained from this study helped to standardize the requirements for optimum production of mannanase using agrowastes, thereby contributing to better fish feed formulation incorporating plant ingredients, especially in the larval stages of fish fingerlings when the enzyme system is not efficient.

Key words: *Trichosporonoides oedocephalis*, mannanase, agro-wastes, submerged fermentation, optimization

INTRODUCTION

Hemicelluloses are complete polysaccharides consisting of linear and branched chain in the cell walls of higher plants which are closely associated to the cellulose and lignin forming lignocelluloses biomass (Moreira and Filho, 2008). Mannan is the most abundant of polysaccharide present in softwood hemicelluloses. Manno-oligosaccharides are generated when mannan is hydrolysed by the combination of β-mannanase (EC.3.2.78), β-mannosidase (EC. 3.2.1.25) and β-glucosidase (EC.3.2.21) with debranching enzymes such as galactosidase (EC.3.2.1.22) and acetyl esterase (EC.3.1.1.6) (Gubitz et al., 1996; Singh et al., 2003; Petkowicz et al., 2007).

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The growing interest in mannanase production for industrial applications is due to its importance in the bioconversion of agro-industrial residues. Various mannanases from fungi, yeasts and bacteria as well as from germinating seeds of terrestrial plants have been produced (Ferreira and Filho, 2004; Heck et al., 2005; Juhasz et al., 2005; Jiang et al., 2006; Lin et al., 2007). Production of β-mannanase by microorganisms is more promising due to its low cost, high production rate and readily controlled conditions (Meenakshi et al., 2010). Mannanases could be used in prebiotic preparation which is expected to improve the growth performance of animal. Manno-oligosaccharides, product of gaur gum galactomannan by β-mannanase was found to be a substrate which could prevent the colonization of *Escherichia coli* and *Salmonella* sp, leading to an improvement of animal growth performance (Ishihara et

al., 2000).

Mannanase enzyme is important in paper industry including bioleaching pulp (Jahwarhar et al., 2012) waste bioconversion of biomass to fermentable sugars (Chandrakant and Bisaria, 1998), increasing the quality of feed quality (Marini et al., 2006) and reduces viscosity of coffee extracts (Hagglund et al., 2003). Endo-β-1,4-mannanases (EC.3.2.1.78) randomly hydrolyze the main chain of hetero mannans, the major softwood hemicellulose (Mabrouk and El Ahwany, 2008). Mannanases have been tested in several industrial processes, such as extraction of vegetable oils from leguminous seeds, viscosity reduction of extracts during the manufacture of instant coffee and manufacture of oligosaccharide (Phothichitto et al., 2006; Sanchez, 2009; Meenakshi et al., 2010) as well as applications in the textile industry (Mabrouk and El Ahwany, 2008). In paper industry, mannanases have synergistic action in the biobleaching of the wood pulp, significantly reducing the amount of chemicals used (Ninawe and Kuhad, 2006).

In our earlier study, *Trichosporonoides oedocephalis* was screened using plate assay technique, and it was observed to give considerable mannanase activity. Based on this information, this fungal strain was selected for this present study. The objectives of the study are therefore to screen selected agro-wastes as substrates for mannanase production as well as evaluate the effect of variation of certain process parameters on mannanase production under submerged fermentation.

MATERIALS AND METHODS

Fungi isolate

Trichosporonoides oedocephalis isolated from agro-wastes previously confirmed positive for mannanase activity by plate assay in our previous work was used in this study. The fungal isolates were identified in the Microbiology Research Laboratory, Federal University of Technology, Akure, Ondo State, Nigeria according to the method designed by Pitt and Hocking (1997) on the bases of cultural characters (colour, shape of colony, surface and reverse pigmentation and texture of the colony) as well as microscopic structure (septate or nonseptate hyphae, structure of hyphae and conidia). The fungal isolate was maintained on locust bean gum containing agar plates and sub-cultured at regular intervals. They were incubated at 30±2° C until the entire plates were covered by active mycelium and stored at 4°C in refrigerator on agar slants.

Chemicals and agro-wastes

The selected agro-wastes (yam peel, potato peel, pineapple peel, rice bran, wheat bran, orange peel, copra meal and cassava peel) utilized as carbon sources were procured from farm field, local market and domestic sources. The substrates were washed, sun dried and oven-dried at 70^{o}C with Model DHG Heating Drying Oven for a period of 2 hrs, sieved to 40 mm mesh size and stored in air tight transparent plastic containers to keep it moisture free (Gubitz et al., 1996). Locust bean gum was purchased from Sigma Chemicals (St. Louis, MO). All other chemicals were of analytical grade.

Media preparation and enzyme production

For the production of mannanase in submerged state fermentation, the isolate was grown at 30°C in 250 ml Erlenmeyer flask in Mandels and Weber's medium modified by (Iqbal et al., 2010). This medium contained the following ingredients (g/L): Peptone 2, yeast extract 2, $NaNO_3$ 2, K_2HPO_4 1, $MgSO_4.7H_2O$ 0.5, KCl 0.5 and $FeSO_4.7H_2O$ traces. Final pH was adjusted to 6.0 and then sterilized at 121°C for 15 minutes. After sterilization, each flask was inoculated with two discs of 8mm diameter of the organism from mannanase containing agar medium using sterile cup borer (El-Naggar et al., 2006). The flasks were incubated at 28°C for 5 days at static condition. Sterile medium supplemented with copra meal without organism served as the control.

Enzyme extraction

The submerged state cultures were prepared by adding 10-fold (v/w) 0.1 M phosphate buffer (pH 6.8) and shaking (180 rpm) at 30°C for 60 min. The solid materials and fungal biomass were separated by centrifugation at 6000 rpm for 15 min at 4°C using refrigerated centrifuge (Centurion Scientific Limited). The clear supernatant was used for enzyme assays and soluble protein determination. Each treatment was carried out in triplicates and the results obtained throughout the work were the arithmetic mean of at least 3 experiments.

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% Locust Bean Gum (LBG) with 0.5 ml of supernatant at 45^{0}C for 60 min (modified method of (El-Naggar et al., 2006). 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 incubation period on mannanase activity

Fermentation period is an important parameter for enzyme production by *T. eodocephalis*. In this study, the fermentation experiment was carried out up to 192 hours and production rate was measured at 24 hours intervals. Mannanase assay was carried out according to standard assay procedures (El-Naggar et al., 2006).

Effect of agrowaste (carbon sources) on mannanase activity

Effect of various carbon compounds namely: orange peels, yam

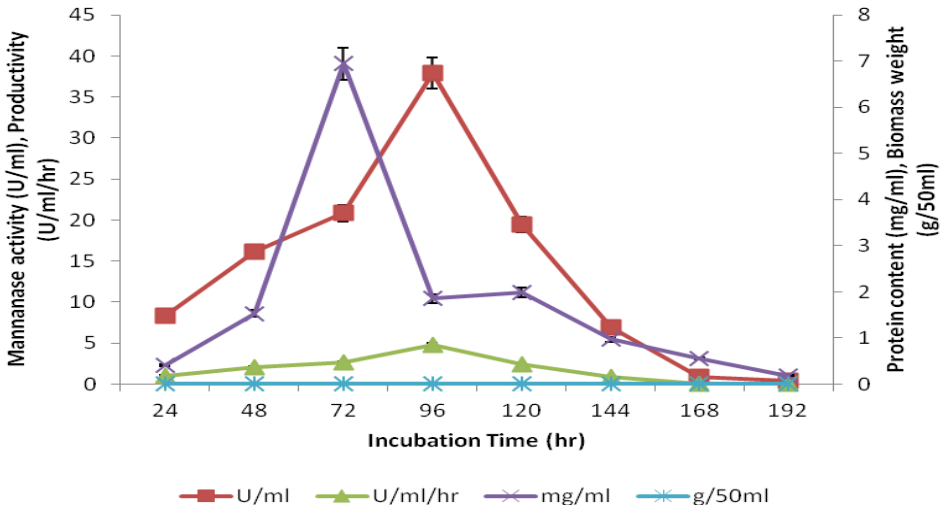

Figure 1. Time course profile of mannanase activity, productivity and protein production of *T. oedocephalis.*

peels, wheat bran, rice bran, cassava peels, pineapple peels, potato peels, groundnut and palm kernel cake were evaluated with Locust Bean Gum (LBG) serving as control. The broth was distributed into 250mL flasks containing 50ml modified Mandels and Weber's medium and 0.5% of each carbon sources were then added before inoculation of the strain and after culture inoculation, the flasks were incubated for 5days at 30^{o}C (El-Naggar et al., 2006).

Effect of pH on mannanase activity

The medium used was of the same composition as that used in the previous experiment. The medium was initially adjusted using NaOH or HCl to cover a pH range from 4.0 to 9.0 (All adjustments were made before sterilization). After inoculation, the flasks were incubated under static condition for 5 days at 30°C. After 5 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 (El-Naggar et al., 2006).

Effect of different nitrogen sources on mannanase activity

The appropriate nitrogen source for mannanase production by the *T. eodocephalis* was determined by supplementing the fermentation medium with different nitrogen sources (soya beans, locust beans, yeast extract, whey, peptone, urea and ammonium chloride) at 0.2% level, replacing the prescribed $NaNO_3$ of the fermentation medium (Javed et al., 2006).

Effect of inoculum concentrations (spores/ml) on mannanase activity

The best inoculum concentration for mannanase production by the *T. eodocephalis* was determined by inoculating the fermentation medium with different spore concentrations ranging from 1×10^3 to 1×10^7 (Abd-Aziz et al., 2008).

Statistical analysis

Data presented on the average of three replicates (±SE) are obtained from there independent experiments.

RESULTS AND DISCUSSION

The effect of incubation period on mannanase activity

Data presented in Figure 1 showed the effect of different incubation periods on mannanase production by *T. eodocephalis.* From the results, it was found that mannanase revealed its best production at 96 hrs of incubation (37.963 U/ml). At longer incubation periods beyond 96 hrs, the activity decreased sharply and at 192 hrs the mannanase activity was 99.03% lower than the activity obtained at 96 hrs of incubation. On the other hand, the productivity reached the maximum of 4.745 U/ml/h at 96 hrs incubation period showing that there was relationship between mannanase activity obtained per hour and production of mannanase. The decrease in the production of mannanase by *T. eodocephalis* after 96 hrs of incubation period might be due to the depletion of nutrients and accumulation of other by-products like proteases in the fermentation medium initiating autolysis of cells (Meenakshi et al., 2010; Malik et al., 2010). The result obtained was in contrast with the findings of other works that recorded maximum mannanase production at 72 hrs for *Penicillium ocittanis* (Blibech et al., 2010). The contrast in our findings against other workers concerning optimum incubation time might be due to differences in

Table 1. Effect of different carbon sources on mannanase production by *Trichosporonoides oedocephalis.*

Carbon sources	Final pH (U/ml)	Mannanase activity	Protein (mg/ml)	Yield (U/g)
WB	4.23^{e}±0.07	0.283^{b}±0.01	0.139^{a}±0.00	0.567^{b}±0.01
GNS	4.47^{f} ±0.04	0.283^{b}±0.01	0.139^{a}±0.00	0.567^{b}±0.01
CP	4.85^{g}±0.03	11.767^{g}±0.01	0.139^{a}±0.00	23.533^{g}±0.02
PAP	4.94^{h}±0.08	7.033^{f}±0.00	3.611^{b}±0.01	14.067^{f}±0.06
RB	3.91^{d}±0.03	0.467^{c}±0.01	0.139^{a}±0.00	0.933^{c}±0.02
PPP	3.74^{c}±0.03	0.100^{a}±0.01	0.139^{a}±0.00	0.200^{a}±0.01
ORP	3.40^{b}±0.01	1.950^{d}±0.03	0.139^{a}±0.00	3.900^{d}±0.03
LBG (control)	3.33^{a}±0.03	3.517^{e}±0.00	0.139^{a}±0.00	7.033^{e}±0.10

Values are presented as Mean±S.E (n=3). Means with the same superscript letter(s) along the same column are not significantly different (P>0.05). **RB=**Rice bran, **WB**= Wheat bran, **PPP=**Potato peels, **GNS**= Groundnut shell, **ORP=**Orange peels, **LBG**= Locust bean gum, **PAP**= Pineapple peels, **CP**= Cassava peels.

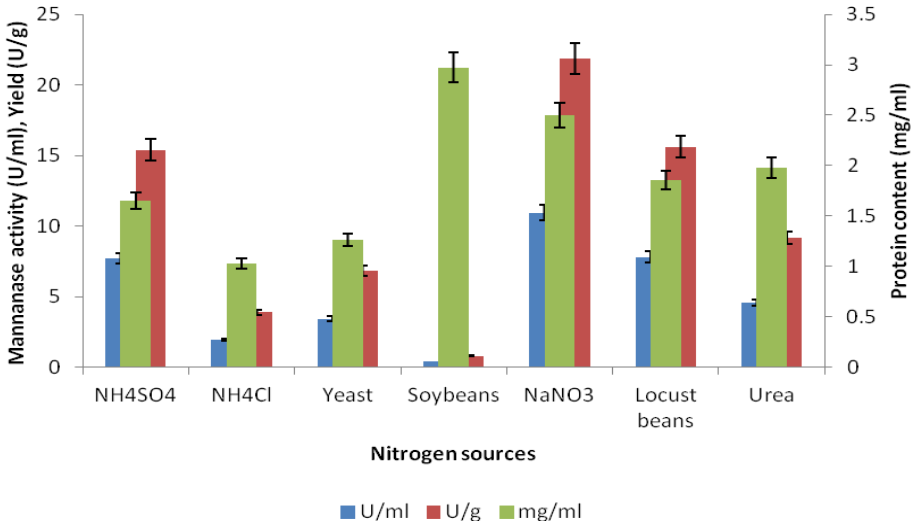

Figure 2. Effect of different nitrogen sources on mannanase activity of *T. oedocephalis.*

growth rate and physiology.

Effect of carbon source 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-industrial by-products were evaluated as substrates for mannanase production by *T. oedocephalis* in comparison to galactomannan (control). Mannanase synthesis of *T. eodocephalis* was highest when cassava peels was utilized as a carbon source compared to other agro-wastes. Enzyme activity (11.767 U/ml) of cassava peels was 3.4 fold increase compared to locust bean gum. *T. oedocephalis* grew well on various raw materials of commercial potential with significant differences in the rate of enzyme production. The large variation in mannanase yield may be due to the nature of cellulose or hemicellulose, presence of some components (activators or inhibitors) in these materials and variations in the substrate accessibility (Mabrouk and El Ahwany, 2008).

Effect of different nitrogen sources on mannanase activity

In this present work, different nitrogen sources such as ammonium sulphate, yeast extract, locust beans, ammonium chloride and soy beans were added separately to the fermentation medium at 0.2% replacing ammonium nitrate (control) in the mineral salt medium (Figure 2). Among the tested nitrogen sources; ammonium nitrate gave maximum enzyme activity and yield. The result obtained could be attributed to the fact

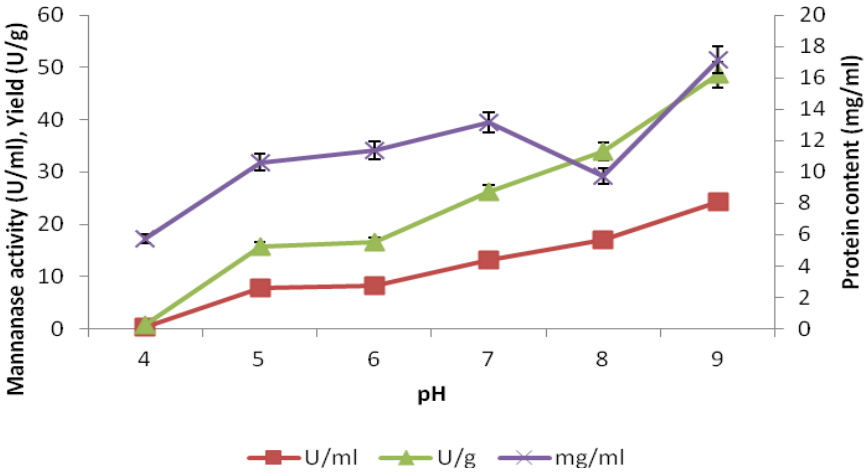

Figure 3. Effect of different pH values on mannanase activity of *T. oedocephalis.*

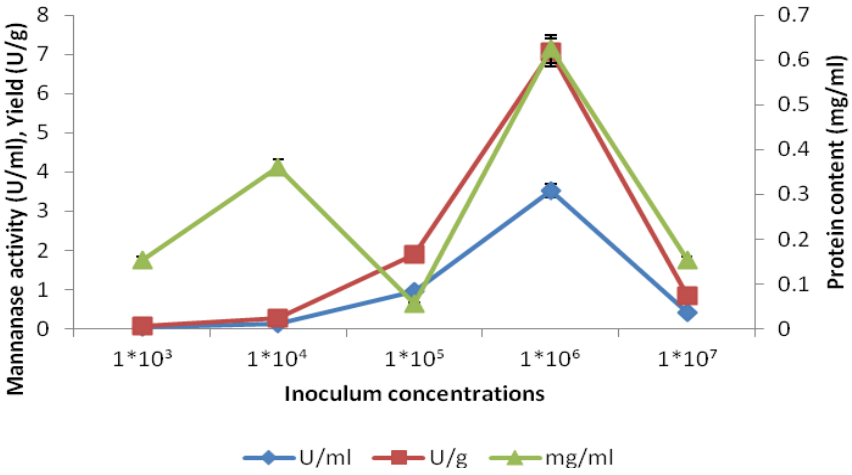

Figure 4. Effect of different inoculum concentrations on mannanase activity of *Trichosporonoides oedocephalis.*

that the ammonium nitrate provided both the ammonium as well as sulphate ions for conidial cell growth and enzyme production (Mekala et al., 2008).

The effect of initial pH on mannanase activity

The medium used was of the same composition as that used in the previous experiment. The medium was initially adjusted using NaOH or HCl to cover a pH range from 4.0 to 9.0 (All adjustments were made before sterilization). After inoculation, the flasks were incubated for 6 days at 28°C. Mannanase activity and yield increased throughout the experiment Figure 3. The increase in mannanase activity at high pH might be due to the fact that acidic proteases that are active in degrading mannase protein are in active.

The effect of inoculum concentrations (spores/ml) on mannanase activity

Inoculum concentration has profound effect on the production of mannanase and protein synthesis. The best inoculum size on mannanase production by *T. oedocephalis* was recorded at 1×10^6 spores/ml, and as the inoculum size increased the production decreased as indicated in Figure 4. At an increased inoculum size, the fungus might over grow and led to the phenomena where the fungus lacked nutrient for the production of enzyme (Abd-Aziz et al., 2008). It has also been reported that the inoculums size influenced the mycelia growth and exo-

biopolymer production (Park et al., 2001; Lee et al., 2004). It was observed that small inoculum size controls and shortens the initial lag phase whereas larger inoculums size increased the moisture content to significant extent. The free excess liquid present an additional diffusion barrier together with that imposed by solid nature of the substrate and leads to a decreased in growth and enzyme production (Jatinder et al., 2006a, 2006b).

CONCLUSION AND RECOMMENDATION

The results highlight that *T. eodocephalis* has potential to be an indigenous source of mannanase production cultured on cheap agrowastes as sole carbon source in submerged state fermentation. The optimal process parameters for the production of mannanase were proposed at incubation period of 96 hrs, cassava peels, ammonium nitrate and 1×10^6 (spore/ml). The values obtained were greater than what was reported in the findings of El-Naggar et al. (2006) when *Aspergillus niger* was used as a mannanase producer. The mannanase activity of *T. eodocephalis* was twelve folds greater than what was reported for *A. niger*. Cassava peels are agro-industrial and domestic byproducts that could have good biotechnological potential. Nevertheless such waste was not tested extensively in previous studies. Process parameters optimization can be exploited at bioreactor level for industrial production of mannanase, a biotechnologically important hydrolytic enzyme.

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