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Full Length Research Paper

Screening and optimization of nutritional conditions for mannanase production by *Penicillium italicum* LAD-A5 in solid state cultivation

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Different fungal isolates were screened for the production of mannanase under solid state cultivation at static condition. *Penicillium italicum* LAD-A5 was selected as the most potent in producing enzyme of high activity. It was therefore selected for further studies. The nutritional requirements of *Penicillium italicum* LAD-A5 for the production of mannanase in solid state fermentation were investigated. The process parameters optimized include; carbon and nitrogen sources, inoculum concentrations, percentage moisture content and sugar supplementation. Utilization of different agrowastes on mannanase production was evaluated. Orange peels were found to be the most effective carbon source with highest mannanase activity of 129.130 U/ml. Yeast extract meal was obtained to be the best nitrogen source with highest activity of 128.380 U/ml out of all the nitrogen sources screened in this study. The best percentage moisture content for maximum enzyme activity was obtained at 50%. Inoculum concentration of 1×10^6 yielded highest mannanase activity of 37.562 U/ml. Addition of simple carbon sources to medium containing locust bean gum caused a slight catabolic repression of mannanase synthesis.

Key words: Screening, optimization, solid state cultivation, mannanase activity, *Penicillium italicum* LAD-A5.

INTRODUCTION

"Mannan exists in nature in two forms, galactomannan and acetylated galacto-mannan. Galactomannan is present in the seeds of leguminous plants and composed of a homogenous backbone of β-1-4 linked mannose residues. Acetylated galactomannan, a principal component of hemicellulose, has a heterogenous backbone of β-1-4 linked mannose and glucose units. Mannanases (EC 3.2.1.78; 1,4-β- D- mannan mannano-hydrolases) occur widely in microorganisms in fungi, yeasts, and bacteria as well as from germinating seeds of terrestrial plants (Ferreira and Filho, 2004; Heck *et al*., 2005; Jiang *et al*., 2006; Lin *et al*., 2007; Meenakshi *et al*., 2010) ".

"In recent years, mannanases have gained increasing attention because of their various biotechnological applications in the food, feed, coffee extraction, oil drilling, detergent, as well as pulp and paper industries (Lin and Chen, 2004; Dhawan and Kaur, 2007; Titapoka *et al*., 2008; Naganagouda *et al*., 2009). They can also be used in the production of mannooligosaccharides, which were reported to be excellent prebiotics stimulating growth of beneficial intestinal microorganisms (Gibson *et al*., 2000)". Mannanases are useful in many fields including biobleaching of pulp and detergent industry (Gubitz *et al.,* 1997), bioconversion of biomass wastes to fermentable sugars (Chandrakant and Bisaria, 1998), and upgrading of animal feed stuff (Ray *et al.,* 1982). It can be used to reduce the viscosity of coffee extracts (Hashimoto and Fukumoto, 1969). The coffee preparation using β-mannanases showed better volatile aroma, taste properties and visual appearance of the final drink (Nunes and Coimbra*,* 1998).

Mannanases could be used as valuable food sweetener or additives (Tomotari, 1990) and also have

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potential application for mannooligosaccharide preparation to be used as prebiotic, which is expected to improve the growth performance of animal. Manno-oligosaccharides are generated when mannan is hydrolyzed by endo-1,4-β–D-mannanase (EC 3.2.1.78) which catalyzes the random cleavage of β-D-1, 4-mannopyranosyl linkages within the main chain of galactomannan, glucomannan, galactoglucomannan and mannan (McCeary and Matheson, 1974). It has been reported that mannooligosaccharides are special nutrient or growth promoter for probiotics, such as *Bifidobacterium* sp. and *Lactobacillus* sp. In this study, the nutritional parameters for solid state fermentation were optimized to enhance mannanase production by *Penicillium italicum* LAD-A5.

MATERIALS AND METHODS

Microorganisms

Eleven fungal strains from agro-wastes previously confirmed positive for mannanase activity by plate assay were used in this study. The fungal isolates were identified in the Microbiology Research Laboratory, Federal University of Technology, Akure, Ondo State, Nigeria. The isolates were: *Penicillium italicum* LAD-A5*, Aspergillus flavus* LAD-2A*, A. flavus* LAD-10A, *A. glaucus* LAD-9A11*, Rhizopus japonicus* PAP-1C*, R. japonicus* YP-5B, *R. japonicus* LBW-8A, *R. japonicus* BN-7A, *R. japonicus* LBW-7B, *R. japonicus* LAD-9A12 and *Trichosporonoides oedocephalis* FCN-12C.

Screening for mannanase production

For the production of mannanase in solid state fermentation, the isolates were grown at 28^0C in 250 ml Erlenmeyer flasks containing 10 grams of the coarsely ground copra meal. Mandels and Weber's medium modified by El-Naggar *et al*. (2006) was used to adjust the moisture content from 50% to 80%.

Optimization of nutritional conditions

To ascertain the effect of nutritional conditions on mannanase production by *Penicillium italicum* LAD-A5, the present study was carried out using different carbon sources (yam peels, wheat bran, groundnut shell, palm kernel cake, cassava peels, pineapple peels, potato peels, rice bran, orange peels and locust bean gum (control)), inorganic and organic nitrogen sources (NH_4Cl, $NaNO_3$, NH_4NO_3, yeast extract, whey, peptone, soybeans, urea and locust beans), inoculum concentrations (1×10^3, 1×10^4, 1×10^5, 1×10^6, 1×10^7) and percentage moisture contents (20%, 40%, 50%, 60% and 80%). Different sugars were supplemented to evaluate its induction or repression effect on mannanase production.

Enzyme extraction

The solid state cultures were prepared by adding 10-fold (v/w) 0.1 M phosphate buffer (pH 6.8) and shaking (180 rpm) at 30^0C for 60 min. The solid materials and fungal biomass were separated by centrifugation (6000 rpm, 15 min at 4^0C). The clear supernatant was used for enzyme assays and soluble protein determination.

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 45^0C 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.

Statistical analysis

Experiment data was subjected to ANOVA of SPSS programming. Duncan's multiple range tests was used to identify significant differences between means of treatments.

RESULTS AND DISCUSSION

Screening of mannan-degrading fungal strains

All the tested fungal strains were able to produce extracellular mannanase in solid state fermentation although with differences in the rate of enzyme production. The highest mannanase activity was reached by *P. italicum* LAD-A5 followed by *Trichosporonoides oedocephalis* FCN-12C and *R. japonicus* YP-5B, while the lowest was recorded in *R. japonicus* LBW-8A (Table 1). Therefore, *P. italicum* LAD-A5 was selected for further studies. The highest yield and protein content was recorded for *P. italicum* LAD-A5 which shows that there was direct relationship between the yield, protein content of the tested cultures and the production of mannanase. It was also observed that the final pH of all the tested cultures was in acidic range (4.49-6.59). Mannanase activity had been reported in a variety of molds under solid state cultivation (Ademark *et al*., 1998; Regalado *et al*., 2000; El-Naggar *et al*., 2006; Sae-Lee, 2007; Dhawan and Kaur, 2007; Hashem *et al*., 2009) but few data are available on mannanase activity of *P. italicum*.

The effect of carbon sources

Substrate selection for enzyme production in a solid state fermentation (SSF) process depends upon several factors, mainly relating to substrate cost and availability and thus may involve screening several agro-industrial residues (Ray *et al*., 2007). Table 2 shows that several types of agro-industrial by-products were evaluated as

Table 1. Mannanase production by different fungal isolates in solid state cultivation.

Isolates	Final pH	Mannanase activity (U/ml)	Protein (mg/ml)	Yield (U/g)
1C	5.46^{g}±0.03	11.667^{d}±0.31	1.111^{g}±0.00	1.667^{d}±0.02
2A	5.93^{h}±0.03	16.482^{g}±0.06	0.694^{c}±0.00	2.355^{g}±0.01
5A	5.30^{f}±0.02	18.056^{j}±0.02	1.667^{j}±0.01	2.579^{j}±0.06
5B	4.49^{a}±0.06	17.500^{i}±0.15	1.250^{h}±0.01	2.500^{i}±0.01
7A	5.48^{g}±0.03	15.370^{f}±0.00	0.139^{a}±0.00	2.186^{f}±0.01
7B	6.38^{i}±0.03	6.204^{b}±0.21	0.972^{f}±0.01	0.886^{b}±0.01
8A	6.59^{j}±0.00	5.000^{a}±0.04	0.556^{b}±0.01	0.714^{a}±0.01
9A11	4.73^{b}±0.06	10.000^{c}±0.13	0.833^{e}±0.03	1.429^{c}±0.01
9A12	4.81^{c}±0.03	13.056^{e}±0.05	0.833^{d}±0.01	1.865^{e}±0.01
10A	5.06^{d}±0.01	17.222^{h}±0.11	1.250^{h}±0.05	2.460^{h}±0.03
12C	5.23^{e}±0.03	17.685^{i}±0.07	1.486^{i}±0.00	2.527^{i}±0.01

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).

1C= *Rhizopus japonicus* PAP-1C, **2A=** *Aspergillus flavus* LAD-2A, **5A**= *Penicillium italicum* LAD-A5, **5B**= *R. japonicus* YP-5B, **7A**= *R. japonicus* BN-7A, **7B**= *R. japonicus* LBW-7B, **8A**=*R. japonicus* LBW-8A, **9A11**=*A. glaucus* LAD-9A11, **9A12**=*R. japonicus* LAD-9A12, **10A**= *A. flavus* LAD-10A, **12C**= *Trichosporonoides oedocephalis* FCN-12C.

Table 2. Effect of different carbon sources.

Carbon sources	Final pH	Mannanase activity (U/ml)	Protein (mg/ml)	Yield (U/g)
YP	5.09^{d} ±0.04	55.09^{f}±0.07	1.806^{g}±0.03	5.509^{f}±0.01
WB	3.29^{a}±0.05	102.78^{g}±0.05	0.694^{c}±0.02	10.278^{g}±0.00
GNS	7.15^{h} ±0.06	1.94^{a}±0.00	0.139^{a}±0.00	0.194^{a}±0.00
PKC	5.16^{de}±0.09	25.46^{c}±0.05	0.694^{c}±0.03	2.546^{c}±0.03
CP	4.03^{b}±0.10	26.85^{d}±0.05	0.278^{b}±0.01	2.685^{d}±0.01
PAP	4.71^{c}±0.19	48.15^{e}±0.01	4.953^{h}±0.03	4.815^{e}±0.01
RB	6.55^{g}±0.07	3.24^{b}±0.00	1.482^{d}±0.01	0.324^{b}±0.00
PPP	6.02^{f}±0.31	105.56^{h}±0.03	0.694^{c}±0.01	10.556^{h}±0.03
ORP	5.12^{d}±0.02	129.13^{j}±0.01	1.528^{e}±0.04	13.611^{i}±0.01
LBG (control)	5.34^{e}±0.10	141.67^{j}±0.01	1.620^{f}±0.03	14.167^{j}±0.01

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).

YP= Yam peels
WB= Wheat bran
GNS= Groundnut shell
PKC= Palmkernel cake
CP= Cassava peels
PAP= Pineapple peels
RB=Rice bran
PPP=Potato peels
ORP=Orange peels
LBG= Locust bean gum

substrates for mannanase production by *P. italicum* LAD-A5 in comparison to locust beans gum (control). *P. italicum* LAD-A5 grew well on various raw materials of commercial potential with significant differences in the rate of mannanase 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). Of all the substrates tested, orange peels were found to be the best substrate for the production of mannanase, which gave maximum mannanase activity of 129.130 U/ml. However, the value obtained for orange peels was significantly lower than that of locust bean gum (control) (141.667 U/ml). It might be due to the fact that orange peels provided adequate amount of nutrients like proteins, carbohydrates, fats, fibers, ash, calcium, magnesium, phosphorous, potassium, sulphur, various

Table 3. Effect of different nitrogen sources.

Nitrogen sources	Mannanase activity (U/ml)	Protein (mg/ml)	Yield (U/g)
YP	128.38^{h}±0.31	25.482^{e}±0.39	18.34^{f}±1.00
WH	43.472^{b}±0.15	24.537^{d}±0.28	6.21^{b}±0.98
PP	75.278^{d}±0.38	26.056^{f}±0.34	10.754^{c}±0.02
NH_4Cl	100.88^{e}±0.43	0.976^{b}±0.25	14.411^{d}±0.33
SB	117.407^{g}±0.17	24.963^{e}±0.24	16.773^{e}±0.35
UR	112.13^{f}±7.51	22.157^{c}±0.27	16.019^{e}±0.16
LB	48.704^{c}±0.46	25.417^{e}±0.38	6.958^{b}±0.40
$NaNO_3$ (control)	9.111^{a}±0.47	0.528^{a}±0.02	1.302^{a}±0.26

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).

YP: Yeast extract, **WH**: Whey, **PP**: Peptone**, SB**: Soybeans: **UR**: Urea, **LB**: Locust beans
NH_4Cl: Ammonium chloride, **$NaNO_3$:** Sodium nitrate

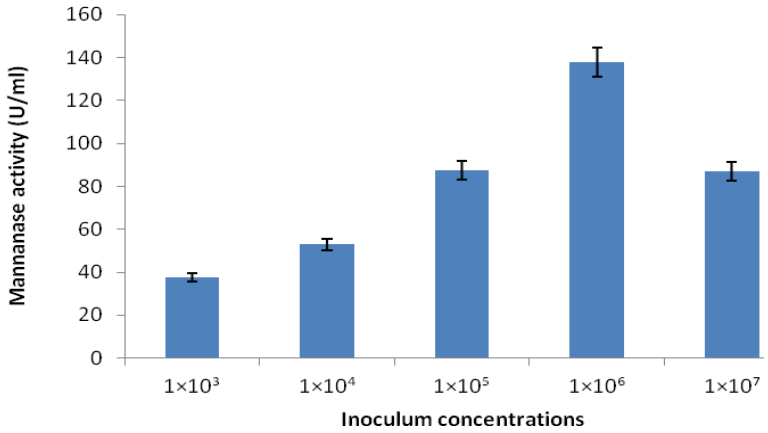

Figure 1. Effect of different inoculum concentrations (spores/ml).

amino acids and porosity for oxygen supply (Bakri *et al*., 2003; Javed *et al*., 2006).

The effect of inorganic and organic nitrogen sources

In this present work, different nitrogen sources were separately added to the fermentation medium at 0.2% concentration replacing all nitrogen sources from minimal salt medium used as moisting agent. Among all the nitrogen sources tested; soybean meal gave maximum production of mannanase (117.407 U/ml) when compared with the $NaNO_3$ (control) (Table 3). Similar result was reported by Javed *et al*. (2006) when soybeans meal was observed to be best nitrogen source for β-glucosidase production and this was attributed to the ease of mycelium to get nitrogen from it. But 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. The study is in agreement with the work done by Kansoh and Gammal (2001). They used organic nitrogen sources and reported more activity than inorganic nitrogen sources.

The effect of inoculum concentrations (spores/ml)

Inoculum concentration has profound effect on the production of mannanase and protein synthesis. The best inoculum size on mannanase production by *P. italicum* LAD-A5 was recorded at 1×10^6 spores/ml which gave highest activity of 37.562 U/ml, and as the inoculum size increased the production decreased as in indicated in Figure 1. At an increased inoculum size, the fungus might over grow and led to the phenomena where the fungus

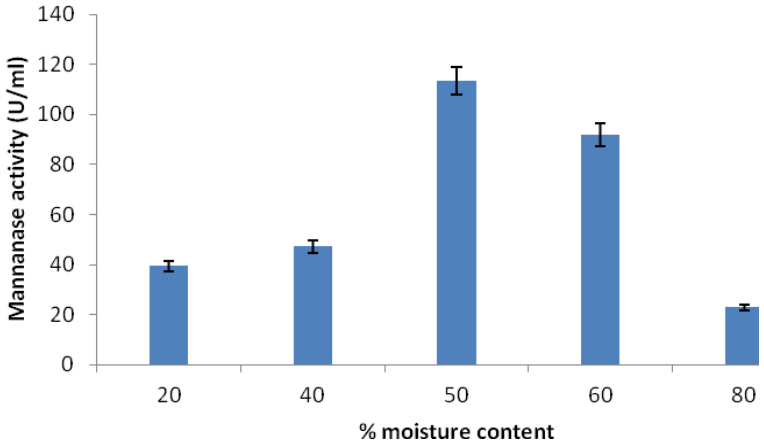

Figure 2. Effect of different percentage moisture content.

Table 4. Effect of sugar supplementation.

Sugars	Mannanase activity (U/ml)	Protein (mg/ml)	Yield (U/g)
Maltose	98.15^{g}±0.41	1.852^{bc}±0.02	19.63^{g}±0.10
Glucose	88.43^{f}±0.26	3.103^{f}±0.13	17.686^{f}±0.01
Mannose	51.39^{b}±1.82	1.852^{ab}±0.37	10.278^{b}±0.12
Arabinose	62.96^{d}±0.39	1.944^{c}±0.04	12.592^{d}±0.04
Mannitol	34.26^{a}±0.57	1.574^{a}±0.05	6.852^{a}±0.03
Lactose	80.09^{e}±0.54	2.685^{e}±0.09	16.018^{e}±0.08
Galactose	56.94^{c}±0.29	3.102^{f}±0.12	11.388^{c}±0.01
LBG (control)	115.278^{h}±0.32	2.315^{d}±0.02	28.056^{h}±0.05

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).

lacked nutrient for the production of enzyme (Abd-Aziz *et al*., 2008). It has also been reported that the inoculum 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,b; Abo-State *et al*., 2010).

The effect of moisture

"The moisture content is an important factor that influences the growth and product yield in SSF (Alam *et al*., 2005; Mrudula *et al*., 2011)". "Moisture is reported to cause swelling of the substrates, thereby facilitating better utilization of the substrate by microorganisms" (Kim *et al*., 1985; Nagendra and Chandrasekaran, 1996). The data presented in the Figure 2 clearly indicates that the yield of mannanase by *P. italicum* LAD-A5 increased with an increase in percentage moisture content from 20% to 50% with an optimum activity of 56.123 U/ml obtained at 50%. Any further increase in the percentage moisture content beyond 50% resulted in the decrease of enzyme yields may be due to clumping of solid particles which results in the decrease of inter-particle space leading to decreased diffusion of nutrients (Babu and Satyanarayana, 1996; Alam *et al*., 2005; Mrudula *et al*., 2011). In contrast, the low moisture content leads to the decrease solubility of nutrients present in the substrate thereby decreases enzyme yields.

The effect of sugar supplementation

The effect of various sugars (0.2 g/l) supplemented to the enzyme production medium containing locust bean gum in order to evaluate its induction or repression effect on mannanase production were tested (Table 4). The highest activity was exhibited by LBG (control) and the

association of additional different sugars with LBG 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).

Conclusion

In conclusion, *P. italicum* LAD-A5 is capable of producing copious titre value of mannanase activity under solid state fermentation when commercial and agro-wastes were utilized as carbon sources. The optimal nutritional factors for the production of mannanase were proposed at 1×10^6 inoculum size and 50% moisture content.

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