

Utilization Of Palm Kernel Cake For The Production Of Mannanase By An Indigenous Filamentous Fungus, *Aspergillus Niger* USM F4 Under Solid Substrate Fermentation

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Citation

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Abstract

A mannanase yielding indigenous fungal culture *Aspergillus niger* USM F4 which was isolated from decayed palm kernel cake (PKC) collected from a local oil palm processed plant was used in this study. The fungal culture produced high yields of extracellular mannanase under solid-substrate fermentation (SSF) using PKC as a substrate. The results obtained from this study revealed that after five days of cultivation period, a maximum extracellular mannanase activity was 433.84 U/ g substrate and 0.96 mg glucosamine/g substrate of fungal growth. The optimized cultural conditions and medium compositions were 10 g PKC with particle size of 0.5 mm, moisture content of 80% (v/w), inoculums size of 1×10^7 spores/ml, incubation temperature of 30°C, static (without mixing), 2% (w/w) of molasses and 4% (w/w) of ammonium nitrate. There was 53.68% increment of mannanase production after optimization compared to before optimization of cultural conditions and medium compositions.

INTRODUCTION

Malaysia as an agricultural based country produces a lot of agricultural wastes which accumulated every year. Among them are rice husks, rice straw, sugarcane baggase, cocoa pod husks, sago refuse, palm press fibers, empty fruit bunches, oil palm trunk, palm kernel cake and palm oil mill effluent. Large quantities of agricultural and agroindustrial residue are generated from diverse agricultural and industrial processes. These residues represent one of the most energy-rich resources on the globe. They are in fact one of the best reservoirs of fixed carbon in nature. Such resources are particularly attractive as they provide an inexpensive substrate and furthermore, it offers elimination of accumulation of biomass. Large amount of agrowaste usually generated a lot of environmental problems and various efforts have been made to manage this problem including open burning.

Solid-substrate fermentation (SSF) is a relatively simple, low cost technology that use up very little of water. As the moisture level is low, the volume of medium per unit weight of substrate is also low. Therefore, the enzyme activity is usually very high [1]. In SSF, bacteria, yeasts or fungi are

able to grow and utilize the solid moist substrate materials in the absence of free flowing water. However, filamentous fungi are the most suitable microorganism for SSF and this could be due to their physiological capabilities and also the mycelial (hyphal) mode of growth which can penetrate deeper in to the substrate. During their growth, fungi secrete hydrolytic enzymes and produce other useful metabolites. This included α -Mannanase (mannan endo- α -1,4-mannosidase; EC 3.2.1.78), an enzyme that catalyses the random hydrolysis of α -1,4-D-mannosidic linkages in mannan-based polysaccharides [2]. α -Mannans are α -1,4-linked polymers of mannose which are usually decorated with α -1,6-linked galactosyl residues. There are three known enzymes that participate in the complete decomposition and conversion of mannan: endo-1,4- α -mannanase (mannanase), exo-1,4- α -mannanase, and α -mannosidase [3]. The interest in mannanase is due to its major application in the food, feed, and pulp industries [4].

In this study, palm kernel cake was used as a substrate for mannanase production using an indigenous fungal isolate, *Aspergillus niger* USM F4. Palm kernel cake contains mainly linear and highly crystalline mannan with a small quantity of galactomannan in its cell wall. Therefore,

potential fungal isolates have to hydrolyze the mannan and galactomannan by producing mannanase. So, keeping this in view, the present investigation was undertaken to study the mannanase production by the use of palm kernel cake as a substrate. The cultural condition parameters such as moisture content, size of substrate, inoculum's size, substrate quantity, incubation temperature and mixing frequency were investigated. To complement the cultural condition parameters, investigation on various carbon and nitrogen sources were also investigated.

MATERIALS AND METHODS

FUNGAL CULTURE, MAINTENANCE AND INOCULUM'S PREPARATION

Aspergillus niger USM F4 obtained from Industrial Biotechnology Research Laboratory was used in this study. The fungal culture was maintained on potato dextrose agar (PDA) slants for 4 days at 30°C until sporulate, before storing them at 4°C until further used. The subculturing was performed every three weeks to assure their survival.

The inoculum was prepared by adding 5.0 ml of sterile distilled water to the agar slant and shaking vigorously. The spore suspension that obtained was adjusted to 1×10^5 spore/ml using a haemocytometer slide chamber (Neubauer, Germany) and used as the inoculums.

SOLID SUBSTRATE FERMENTATION

The agrowaste used in this study as a substrate for SSF was palm kernel cake (PKC) that was obtained locally. The PKC was dried under sunlight until constant weight. Ten grams of dried powder formed PKC with the size of 0.5 mm was placed in a 250 ml Erlenmeyer flask. Then, 80% (v/w) of sterile distilled water as a moisturizing agent [included 10% (1×10^5 spore/ml) of spore suspension] was added in to the flask. The inoculated flasks were shake to mix the inoculums and PKC before incubating them statically at 30°C for five days.

OPTIMIZATION OF CULTURAL CONDITIONS AND MEDIUM COMPOSITION FOR MANNANASE PRODUCTION

Various optimization parameters for maximal mannanase production by *A. niger* USM F4 were investigated. These included various moisture content (in the range of 40-120%; v/w), substrate size (0.5-2.0 mm), inoculums sizes (1×10^4 - 1×10^8 spore/ml), substrate quantity (5-30 g), temperature (25-40°C) and frequency of mixing (0-48

hours). The effects of supplementation of additional carbon and nitrogen sources on mannanase production were also examined using 4% of various carbon (sucrose, molasses, maltose, starch, glucose and lactose) and nitrogen (ammonium nitrate, potassium nitrate, peptone, corn steep liquor, urea and yeast extract) sources. The optimal concentrations of carbon and nitrogen sources were also determined. All experiments were carried out in triplicates and the results were presented as mean and standard deviation of the triplicates experiments.

ENZYME EXTRACTION

Distilled water was added with 0.1% (v/v) of Tween 80 and then added into the fermented SSF medium [5]. The mixture was mixed using a shaker at room temperature ($30 \pm 2^\circ\text{C}$) for 30 minutes at 150 rpm agitation speed. The mixture was then filtered through a cheese cloth, followed by Whatman filter paper No. 1. The filtrate was collected and was used as a crude mannanase source.

MANNANASE ASSAY

Mannanase activity was assayed by mixing 0.5 ml of an appropriately diluted enzyme solution with 0.5 ml of 0.5% locust bean gum in 50 mM citric acid-trisodium citrate buffer (pH 4) at 60°C for 30 min. The reaction was stopped by addition of 1.5 ml dinitrosalicylic acid [6]. After 5 minutes of boiling, the amount of reducing sugars was determined spectrophotometrically at 575 nm. Mannose was used as a standard [7]. One unit of mannanase activity is defined as the amount of enzyme that releases 1 μmol of mannose per minute under assay conditions. Mannanase production was expressed as units (U) per gram of dry weight of PKC.

FUNGAL GROWTH DETERMINATION

Fungal growth in SSF system was determined according to the presence of glucosamine after chitin hydrolysis in the cell walls of fungus [8]. Growth of the fungus was expressed as mg glucosamine per g of substrate. All experiments were carried-out in triplicates and the results were presented as mean of the triplicate experiments.

RESULTS AND DISCUSSION

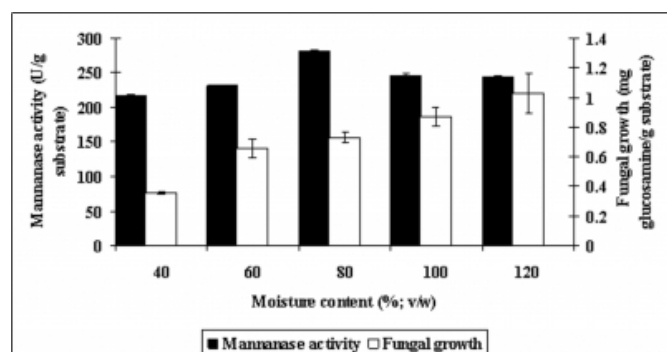
EFFECT OF MOISTURE CONTENT

This parameter is very much related to SSF definition itself; a process involving solids in absence (or near absence) of free water. The results showed that moisture level of 80% (v/w) was the optimized level for maximum mannanase

activity with 281.99 U/g substrate and fungal growth of 0.72 mg glucosamine/g substrate (Figure 1). Higher or lower moisture content than 80% produced lower mannanase activity. The lowest production was obtained at the moisture content of 40% with 216.90 U/ g substrate. For moisture content of 60%, the mannanase production was 232.33 U/ g substrate. For moisture content of 100 and 120%, the mannanase production achieved were 246.02U/ g substrate and 244.11 U/ g substrate, respectively. Moisture is very sacred in SSF system as the level of moisture determined the final metabolites production. Moisture content in SSF system can vary due to evaporation of the existing water through metabolic heat evolution, water consumption and also due to environmental factors [9]. The characteristic of substrates and microorganisms are also included. In this experiment it is important to design a suitable host and surrounding condition for fungi to interact and directly degrade the palm kernel cake. Filamentous fungi including *A. niger* have terrestrial habitats, in soil or on dead plant matters [10]. Therefore, logically the preferable of selecting growth medium is almost the same with their basal nature needs. The original habitat of fungi cannot be too soggy or dry. A perfect balance of those elements is the best condition for fungal growth. Higher level of moisture content usually give impact to the inner particles of substrate, where the porosity of the substrate reduced and the water accumulation in the substrate increased. As a result, oxygen transportation in the substrate to the fungal cells was disturbed [11]. On the other hand, lower presence of moisture content influenced the solubility of nutrient of substrate, low grade of swelling and water tenseness [12].

Figure 1

Figure 1: Effect of moisture content on mannanase production by USM F4 in SSF



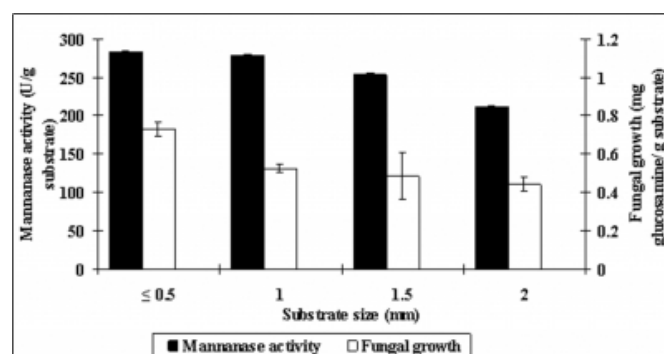
EFFECT OF SUBSTRATE SIZE

To have a complete penetration of fungal hyphae into solid substrates always become a major problem in SSF.

Therefore, the right size of substrate is important. The effect of substrate sizes ranged from 0.5 mm until 2.0 mm was examined in this experiment. Figure 2 shows the best production of mannanase by *A. niger* USM F4 was 283.58 U/g substrate with 0.73 mg glucosamine/g substrate of fungal growth. The use of particle sizes of more than 0.5 mm only contributed in low mannanase activity. As the size of substrate particle increased (1.0, 1.5 and 2.0 mm) the mannanase activity decreased with 277.85, 253.34 and 211.33 U/ g substrate respectively. Accessibility of surface area depends on substrate size which is crucial for mass transfer, microorganism attachment, growth and also final product production [13]. As the size of substrate increases, the restriction at surface area for fungal attack is getting higher. The results obtained were in agreement with the findings of Cuoto and Sanroman [14], where the preferable substrate size was smaller sizes as the size promised more advantages which not only in penetration but also nutrients absorption [14]. So, via these conditions, the period of producing maximum amount of mannanase activity will be shortened as the PKC consist a durable structure that might take a longer time to degrade the big size.

Figure 2

Figure 2: Effect of substrate size on mannanase production by USM F4 in SSF



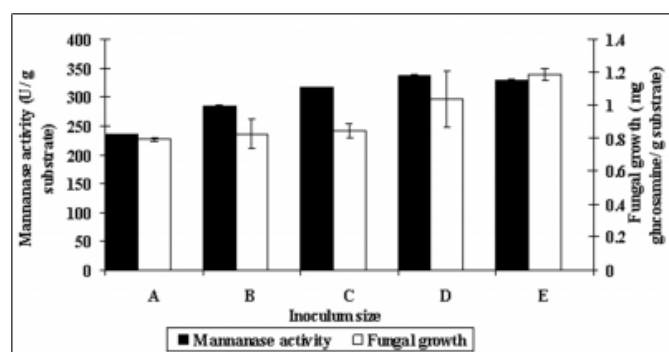
EFFECT OF INOCULUM SIZES

Figure 3 shows the mannanase production at different inoculum sizes ranged from 1×10^4 to 1×10^8 spores/ml. The maximum mannanase production was achieved at the inoculums size of 1×10^7 spores/ml with about 336.10 U/ g substrate and 1.04 mg glucosamine/ g substrate of fungal growth. Size inoculums of 1×10^4 , 1×10^5 and 1×10^6 spores/ml produced about 243.88, 283.58 and 317.00 U/ g substrate, respectively, whereas 1×10^8 spores/ml produced 329.57 U/ g substrate. A balance correlation between biomass proliferation and presence of nutrients are needed in synthesizing enzyme to a maximal level. Reduction of

inoculums size give an impetus of time prolongs during cells folding where an ample amount of cells are required to fulfill the substrate utilization and obtained the desired products [15]. On the other hand, an increment in spore concentration stimulates the proliferation and biomass synthesis in a faster rate and directly shortens the lag phase of fungal growth.

Figure 3

Figure 3: Effect of inoculum sizes on mannanase production by USM F4



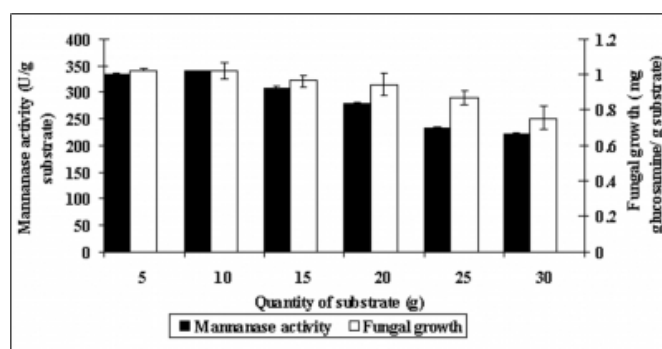
(Indicator: A: 1×10^4 , B: 1×10^5 , C: 1×10^6 , D: 1×10^7 , E: 1×10^8 spore/ml)

EFFECT OF PKC QUANTITY

The maximum mannanase production of 339.28 U/ g substrate and 1.02 mg glucosamine/ g substrate were obtained when 10 g of PKC was used (Figure 4). Lower or higher PKC quantity than the optimized one produced lower amount of enzyme production. As shown in the figure, substrate amount of 5, 15, 20, 25 and 30 g produced about 332.91, 308.72, 279.12, 233.93 and 221.19 U/ g substrate, respectively. The results also revealed that lower amount of substrate amount of 5 g produced the second highest of mannanase production compared to a higher amount of substrate. This condition indicated that too much amount of substrate in a fixed container produced a thicker substrate bed which finally reduced the substrate pore size and reduced the transferring of oxygen in between the substrate particles [12]. It also interferes with the oxygen diffusion in substrate, especially at the basement part of the flask where the substrate was not fully fermented or utilized.

Figure 4

Figure 4: Effect of PKC quantity on mannanase production by USM F4 in SSF

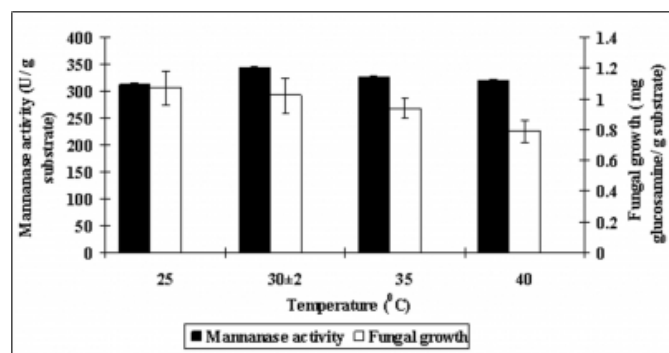


EFFECT OF TEMPERATURE

Generally, SSF process prefers mesophilic fungal growth with the temperature ranged from 25 to 40°C to be used in its system. Therefore, a suitable temperature for fungal growth is very important. Figure 5 shows that the optimized temperature for *A. niger* USM F4 to produce maximum mannanase production of 343.74 U/ g substrate and fungal growth of 1.02 mg glucosamine/ g substrate. Lower or higher temperature than 30°C produced lower enzyme production. Temperatures of 25, 35 and 40°C produced 311.91, 326.23 and 319.39 U/g substrate, respectively. Again the results agreed that *A. niger* USM F4 is a mesophilic fungal culture which suitably grow at temperatures between 30 to 40°C with significant amount of mannanase production. Higher temperatures inhibited fungal growth due to the dryness of the substrate where high temperature brought a negative impact towards metabolic activity of microorganism [16,17]. However, lower temperatures only cause a problem in fungal spore proliferation. To overcome with this temperature problem, researchers [18, 19, 20] have come out with a better solution that is by designing a proper bioreactors assisted with mathematical modeling.

Figure 5

Figure 5: Effect of temperature on mannanase production by USM F4 in SSF

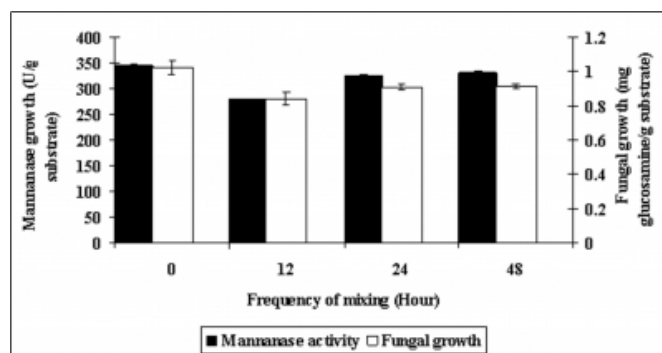


EFFECT OF MIXING FREQUENCY

Figure 6 shows that static or no mixing produced the highest mannanase activity of 345.32 U/g substrate and fungal growth of 1.02 mg glucosamine/ g substrate. The mixing frequencies of every 12 hours, 24 hours and 48 hours produced 280.08, 324.96 and 331.32 U/ g substrate, respectively. Even though mixing frequency is very much related to aeration, which essentially has two functions; to supply oxygen for aerobic metabolism and also for the removal of carbon dioxide, heat, water vapor and volatile components produced during metabolism [21], but in this case static was very much preferred by the fungus. The porosity of 10 g PKC in a flask system allowed the fungal hyphae to grow well and to use-up oxygen that available in the substrate. After all in static condition, fungal hyphae were not broken in to fragments and mature hyphae always produced significant amount of enzymes. This statement is supported by [22] who reported that a frequent mixing affects the fungal sporulation at the earlier growth stage which circuitously inhibited the enzyme production. But in other hand, a static condition only cause substrate became compress, inconsistent fungal growth, heat blockage, pH changes and also problem with substrate moisture [23]. These findings could be implemented for other substrates, but for PKC which is rich with lignocellulosic fibers, static or no mixing was the best.

Figure 6

Figure 6: Effect of mixing frequency on mannanase production by USM F4 in SSF



EFFECT OF ADDITIONAL SUPPLEMENTATION OF CARBON SOURCE ON MANNANASE PRODUCTION

Carbon sources which consist of monosaccharides (glucose and maltose), oligosaccharides (sucrose, molasses and lactose) and polysaccharides (starch) were studied. Figure 7 shows that at 4% (w/w) of various carbon sources used, molasses gave the highest mannanase production of 411.09 U/ g substrate and 0.94 mg glucosamine/ g substrate of fungal growth, followed by starch (394.16 U/ g substrate), glucose (389.14 U/ g substrate), sucrose (382.87 U/ g substrate), maltose (365.31 U/ g substrate) and lactose (313.25 U/ g substrate).

Since molasses was found as the best carbon source which produced the highest mannose production, therefore various concentration of molasses ranged from 1-10% (w/w) were further investigated. Figure 8 shows the molasses concentration of 2% produced the maximum activity of mannanase (414.44 U/ g substrate) with 0.62 mg glucosamine/ g substrate of fungal growth. Then at 4% of molasses, a slightly lower of mannanase was produced (411.40 U/ g substrate). The enzyme production decreased as the concentration of molasses increased with 343.72 U/ g substrate, 322.83 U/ g substrate and 301.95 U/ g substrate at 6, 8 and 10%, respectively.

Carbon source is important for fungal growth as it yields higher growth rates [24]. The nutrients available in the SSF substrate (in this case PKC) are derived from degradation of organic compounds present in the solid substrate [9] which may not enough to support microbial growth fully. The productivity of final products depending on the fungal growth and it is essential to boost up, mostly the initial growth by adding suitable supplement of carbon source.

Figure 7

Figure 7: Effect of the addition of various carbon sources in the SSF system on the production of mannanase by USM F4

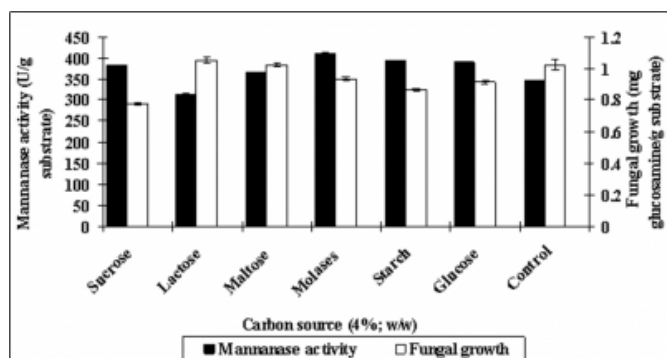
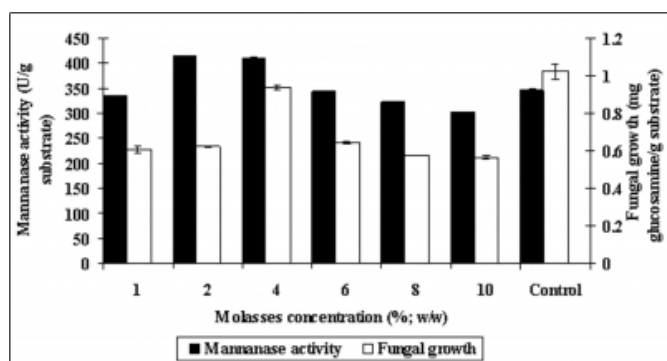


Figure 8

Figure 8: Effect of different concentration of molasses in the SSF system on the production of mannanase by USM F4



EFFECT OF ADDITIONAL SUPPLEMENTATION OF NITROGEN SOURCE ON MANNANASE PRODUCTION

Figure 9 shows the six types of organic (peptone, corn steep liquor, urea and yeast extract) and inorganic (ammonium nitrate and potassium nitrate) nitrogen sources that were applied into solid substrate with concentration of 4% (w/w). When compared to control (with the addition of molasses at 2%; w/w), it was observed that ammonium nitrate enhanced mannanase and growth production with 432.05 U/g substrate of mannanase activity and 1.21 mg glucosamine/g substrate, respectively. The other nitrogen sources such as urea gave 396.24 U/ g substrate, potassium nitrate gave 366.40 U/ g substrate and peptone gave 355.96 U/ g substrate. Moderate enzyme productions were shown by yeast extract and corn steep liquor with 324.33 U/ g substrate.

Since ammonium nitrate showed the best nitrogen source to enhance mannanase production, various concentration of ammonium nitrate ranged from 2-10% (w/w) were studied. As shown in Figure 10, concentration of 4% (w/w)

ammonium nitrate, maintained to be the most optimized condition with 432.35 U/ g substrate of mannanase with 1.91 mg glucosamine/g substrate of fungal growth. Higher and lower than 4% of ammonium nitrate produced lower of mannanase. At 2%, only 332.38 U/ g substrate mannanase was produced, whereas at 6% (428.76 U/ g substrate), 8% (321.94 U/ g substrate) and 10% (304.04%), the mannanase production decreased with the increased of ammonium nitrate concentration. Supplementation of nitrogen source is important as it plays important role in biosynthesizing cells metabolites and maintains the physiology of the cells.

Figure 9

Figure 9: Effect of the addition of various nitrogen sources in the SSF system on the production of mannanase by USM F4

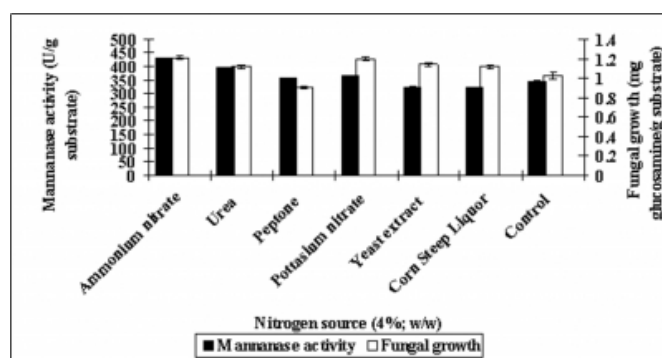
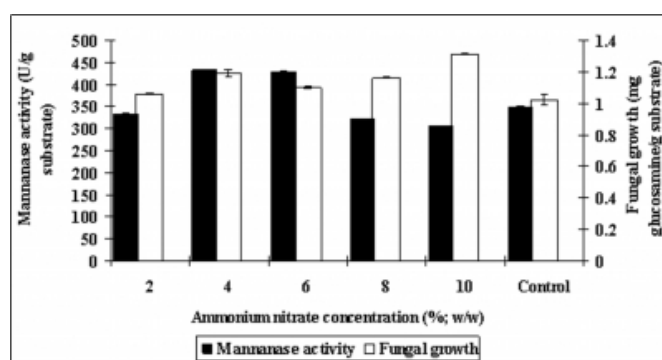


Figure 10

Figure 10: Effect of different concentration of ammonium nitrate in the SSF system on the production of mannanase by USM F4



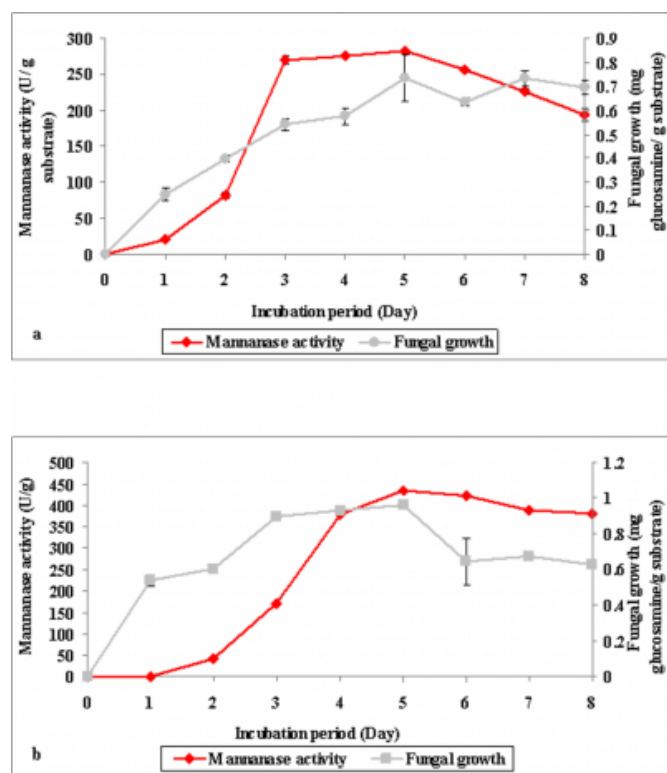
PROFILE OF MANNANASE PRODUCTION BY A. NIGER USM F4 USING PKC AS A SUBSTRATE IN SSF BEFORE AND AFTER OPTIMIZATION

Using all the optimized parameters, the SSF was carried-out for 8 days in a flask system and the results of the time course profiles before and after the optimization of cultural conditions and medium compositions are shown in Figure

11. The results obtained before optimization is shown in Figure 11a, where the maximum mannanase production achieved was 281.97 U/ g substrate and 0.73 mg glucosamine/ g substrate of fungal growth on the fifth day of cultivation. In the mean time, the results obtained after optimization is shown in Figure 11b. There were tremendous increments in mannanase activity obtained with 433.34 U/ g substrate and 0.96 mg glucosamine/ g substrate of fungal growth on the fifth day of cultivation. There was an increment of about 53.68% of mannanase production after optimization compared to before optimization.

Figure 11

Figure 11: The time coarse profiles of mannanase production by USM F4 in SSF



Profiles before and (b) Profile after optimization of cultural conditions and medium compositions

CONCLUSIONS

We are reporting the potential use of the fungal culture *A. niger* USM F4 which was isolated locally from decayed PKC as a capable producer of mannanase using PKC as substrate under SSF. The maximum mannanase production after optimization was 433.34 U/ g substrate. The mannanase production by the same fungal culture using PKC as substrate in a shallow tray system is in progress and the results will be reported elsewhere.

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