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Production of Reducing Sugars by *Trichoderma* sp. KUPM0001 during Solid Substrate Fermentation of Sago Starch Processing Waste *Hampas*

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Abstract: *Trichoderma* sp. KUPM0001 showed good growth during solid substrate fermentation (SSF) of sago pith residue known as *hampas*, supplemented with 10% (v/w) of mineral salts solution containing 0.5% (w/v) (83.3 mM) urea as nitrogen source and an initial moisture content of 80% (v/w). Mycelium suspension of 10% (v/w) density was used as initial inoculum and SSF was carried out at 25±2°C in static condition over a period of 120 h. The parameters optimized included the initial moisture content of the substrate, mineral salts solution, urea concentration, inoculum density, incubation temperature and incubation time. Without optimized condition, the maximum reducing sugar obtained was 24 mg mL^{-1} compared to 46 mg mL^{-1} substrate during optimized SSF after 96 h incubation. The optimum parameters obtained were 80% (v/w) of initial moisture; 10% (v/w) of inoculums size; 1.0% of urea in 20% (w/v) of mineral solution and incubated at 30±2°C. The enzyme activities using optimized condition gave maximum α-amylase, glucoamylase, carboxymethyl cellulase, filter paperase and β-glucosidase of 3.19, 2.22, 1.66, 1.11 and 1.48 U mL^{-1}, respectively.

Key words: *Trichoderma* sp. KUPM0001, Optimization, solid substrate fermentation

INTRODUCTION

In Malaysia, the use of sago starch has been increasing and it is presently being used for the production of glucose. Sago starch represents an alternative cheap carbon source for fermentation processes that is attractive out of both economic and geographical considerations (Abd-Aziz, 2002). Production of fermentable sugars from the hydrolysis of starches normally carried out by an enzymatic process that involves two reaction steps, liquefaction and saccharification that requires the use of an expensive temperature control system and a complex mixing device.

The sago starch processing industry produces three major types of by-products viz, bark of sago trunk, fibrous pith residue, commonly known as *hampas* and wastewater. Sago *hampas* (the fibrous pith residue) obtained after starch extraction from the rasped sago pith consists of about 66% starch, 15% crude fiber and 1% crude protein on a dry weight basis. On dry basis, sago *hampas* contains 60- 70% starch, trapped within its fibers (Robertson *et al*., 2006). The fibers have to be degraded first to release the starch. *Bacillus subtilis* has been reported to be successful in saccharifying starch within the fibers in cassava solid wastes by using α-amylase and glucoamylase. The resultant

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glucose-syrup was used as a feedstock for ethanol production. Therefore, sago *hampas*, which have similar constituents, may be amenable to the same treatment in microbial degradation.

In the utilization of agricultural materials by SSF, process parameter, such as initial moisture content, mineral salts solution, inoculum density, urea concentration, incubation temperature and time are important to enhance the successful of SSF. Therefore the emphasis of this study was to convert a locally-available agro-waste material, *hampas* to soluble sugars by isolated fungal *Trichoderma* sp. KUPM0001 and optimization of parameters via solid substrate fermentation.

MATERIALS AND METHODS

Microorganism

The locally isolated fungus, *Trichoderma* sp. KUPM0001 was isolated from the decayed sago *hampas* obtained from the collection ramp of the sago mill. Pure cultures were maintained on potato dextrose agar (PDA-Difco) slants and stored at 4±2°C.

Substrate

Sago *hampas* was collected from Hup Guan Sago factory in Johor Darul Takzim, Malaysia. The substrate was air-dried and sieved through a 1.0 mm sieve and stored at room temperature prior to use.

Inoculum Preparation

Trichoderma sp. KUPM0001 was grown on Potato Dextrose Agar (PDA) plate for seven days at 30±2°C. The spore suspension was harvested using 10 mL of sterile distilled water containing 0.1% (v/v) Tween 80 and then inoculated into 50 mL Potato Dextrose Broth (PDB) in 250 mL Erlenmeyer flask. The inoculated flasks were incubated at 200 rpm at 30°C for 48 h. The vegetative inoculum was aseptically blended at low speed for 10 sec in a Waring blender. A 10% (v/w) of this blended mycelium culture was used as inoculum in each SSF unless otherwise stated.

Solid Substrate Fermentation (SSF)

Solid substrate fermentation cultures were developed in 250 mL Erlenmeyer flask containing 5 g of sago *hampas*. The substrate was autoclaved at 121°C, 15 psi for 20 min. Prior to cooling, 1 mL of mineral salts solution containing 0.2% (w/v) KH_2PO_4, 0.05% (w/v) $MgSO_4.7H_2O$ and filtered sterilized 1.0% (w/v) urea w/v) as nitrogen supplement was added. The content of the flask with a moisture content of 60% was then thoroughly mixed with sterile spatula and allowed to stand for 1 h. Each flask was aseptically inoculated with 10% (v/w) of 48 h old mycelial suspension of *Trichoderma* sp. KUPM001. To increase aeration, the content of the flask was gently mixed using sterile L stick every 24 h. The cultures were incubated at 30±2°C in a static condition for 5 days (120 h) unless otherwise stated. Experiments were done in triplicate.

Optimization of Reducing Sugars Production

The Effect of Initial Moisture Content

To investigate the influence of the initial moisture content on the sugar production the fermentation was carried out at various moisture levels (60, 65, 70, 75 and 80%) contents. The moisture content (v/w) was adjusted with a total volume inclusive of distilled water, mineral salts solution and inoculum suspension. Fermentation was carried out at 30±2°C for 120 h. The optimum initial moisture content was maintained for subsequent experiments.

The Effect of Mineral Salts Solution

Mineral salts solution consisted of 0.2% (w/v) KH_2PO_4, 0.05% (w/v) $MgSO_4.7H_2O$ and filter sterilized urea (1.0% w/v) as nitrogen supplement was used. The different volume of salts solution

to weight of substrate (v/w: 10, 20 and 30%) was assessed. Fermentation was carried out at 30±2°C for 120 h. The optimum volume of the mineral salts solution for solid substrate obtained was maintained for subsequent experiments.

The Effect of Varying Concentrations of Urea

Urea supplementation at different levels of (w/v: 0.5, 1.0 and 2.0%) in the substrate on sugar production was studied. The optimum level of the urea concentration required for solid substrate was used for subsequent experiments.

The Effect of Inoculum Density

The effect of various inoculum levels (v/w: 10, 20 and 30%) on reducing sugar production was studied. The optimum inoculum level obtained was then fixed for subsequent experiments.

The Effect of Incubation Temperature

The fermentation was carried out at various temperatures such as 25, 30, 35, 40 and 45°C and the effect of the varying temperature on reducing sugar production was studied. The optimum incubation temperature obtained was fixed for subsequent experiments.

The Effect of Incubation Time

Different incubation periods (0, 12, 24, 36, 48, 60, 72, 84, 96, 108 and 120 h) were investigated for their effect on reducing sugar production. The cultivation was carried out at 30±2°C for 120 h keeping other conditions at their optimum levels. The optimum incubation period achieved by in this experiment was then fixed for subsequent experiments.

Extraction Procedure

Extraction was done by adding 50 mL of 0.01M, phosphate buffer (pH 7.0) to the contents of each flask. The solid culture was broken down into smaller particles and then homogenized at 8000 rpm for 3 min at room temperature. The culture slurry was centrifuged at 8000 rpm at 4°C for 20 min (Conti *et al*., 2001). The supernatant was filtered through 0.45 μm filter and kept in 1.5 mL microcentrifuge tubes at -20°C for 24 h prior to assay for reducing sugar and enzymes. Assays were performed in triplicates and the results for all the values were expressed as a mean of triplicate values.

Sample Assays

The extracellular soluble protein was quantified using the dye-binding method with crystalline bovine albumin as standard (Bradford, 1976). Total nitrogen was determined by Kjeldahl method. The dinitrosalicylic acid (DNS) method was employed for reducing sugar determination (Miller, 1959). The absorbance was then translated into glucose equivalent using a standard graph obtained by plotting glucose against absorbance.

Enzyme Assays

Alpha-amylase activity was assayed by incubating the crude enzyme with 1.0 mL (1% w/v) cooked soluble starch along with 0.01 M phosphate buffer, pH 6.9 for exactly 6 min at 37°C (Bernfeld, 1955). The amount of reducing sugar was determined using the DNS method and a standard of pure maltose was used. Glucoamylase activity was assayed by incubating the crude enzyme with 500 μL of 1% (w/v) raw soluble starch in 0.1 M sodium citrate buffer (pH 4.0) at 60°C for 1 h. Reducing sugar produced was determined by DNS method (Bradford, 1976) and glucose was used as standard. Carboxymethyl cellulase (CMCase) activity was determined by adding 1.8 mL substrate

solution and 0.2 mL of enzyme sample and the resulting solution was incubated at 40°C for 30 min in a water bath with moderate shaking (Mandels *et al*., 1974). Filter paper hydrolysis activity was measured by adding 0.2 mL of supernatant and 1.8 mL of 0.05 M sodium citrate buffer (pH 4.8) into test tubes containing 25 mg Whatman No. 1 filter paper strips (1.0×3.0 cm). The substrate used for the determination of ß-D-Glucosidase activity was p-nitrophenol- β-D-glucopyranoside (Sigma).

Statistical Analysis

Analysis of variance was done with data of 96 h incubation when maximum reducing sugar productivity was recorded and the fermentation profile of SSF after optimization was compared with the fermentation profile before optimization.

RESULTS AND DISCUSSION

Solid Substrate Fermentation (SSF)

Significant growth of *Trichoderma* sp. KUPM0001 was observed on sago *hampas*. The first sign of growth was seen after 12 h of inoculation. Visual observation every 24 h revealed a spreading white mycelia web on the surface of the substrate and covered it entirely by time. As the culture grew older the colour of the mycelia change from white to dark green and by 96 to 120 h of solid substrate fermentation complete colonization by the fungus was observed. The initial pH of the supplemented culture on 0 h was 5.54 (Fig. 1a). There was not much variation of the pH in the crude culture extract during the fermentation period, the pH ranging from 5.54 to 5.60. The slight decreased in pH of the substrate may be correlated directly with the decomposition activity of the fungus. In the present experiment, it was found that pH range between 5 and 7 to be suitable for the growth of *Trichoderma* sp. KUPM001 on sago *hampas*.

The concentration of soluble protein in 0 h sample was 0.54 mg mL^{-1} and the extractable protein reached 1.97 mg mL^{-1} after the first 48 h of fermentation (Fig. 1b). The increased in protein was due to the secretion of extracellular enzymes responsible for the degradation of *hampas*. The soluble protein content can be used to relate the growth of fungus. The soluble protein content was used as an indirect assessment of fungal biomass and changes in soluble protein correlated with the morphogenesis of the fungus.

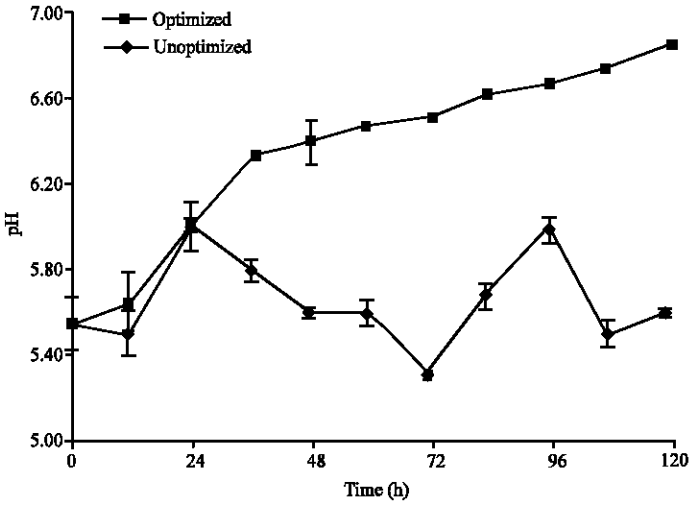

Fig. 1a: Profiles of pH during SSF of sago *hampas* by *Trichoderma* sp. KUPM0001

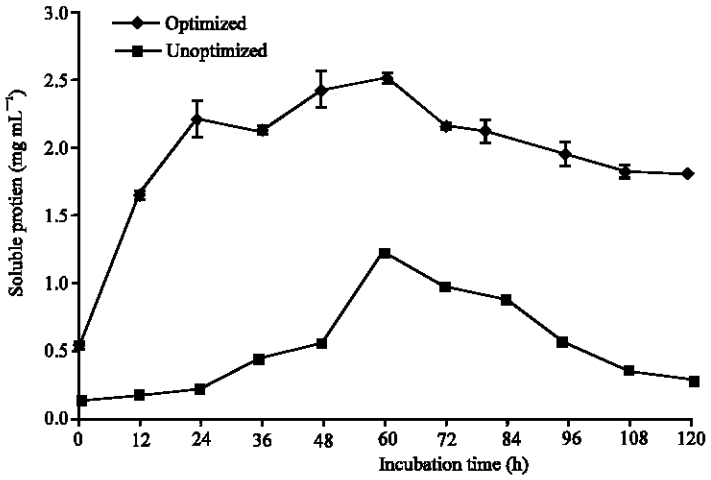

Fig. 1b: Profiles of soluble protein during SSF of sago *hampas* by *Trichoderma* sp. KUPM0001

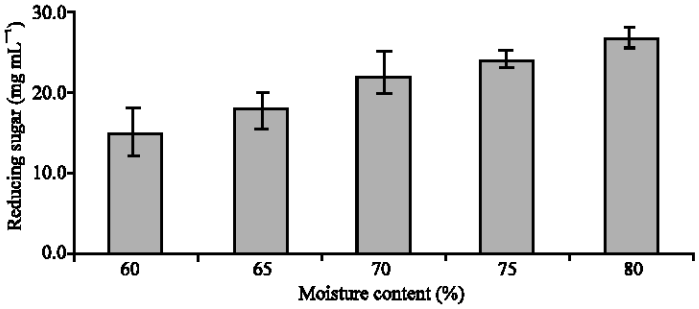

Fig. 2: Effect of different percentage of initial moisture content of solid substrate medium on reducing sugar production by *Trichoderma* sp. KUPM0001 via SSF

Effect of Initial Moisture Content on Reducing Sugar Production

The effect of initial moisture content of the substrate on reducing sugar production is presented in Fig. 2. In the present study, the highest reducing sugar production obtained was 27 mg mL^{-1} at 80% (v/w) initial moisture content. During the course of fermentation, the content of the flask was gently mixed using sterile L stick at 24 h intervals to increase the aeration and to evenly distribute water availability to the fungus. A decrease in reducing sugar production was observed when the moisture level was set at levels higher or lower than the 80%. The results showed that the levels of reducing sugar increased significantly ($p<0.05$) with incubation time. Higher substrate moisture in SSF resulted in suboptimal product formation due to reduced mass transfer such as diffusion of solutes and gas to cell during fermentation while, lower moisture level minimized heat exchange, oxygen transfer and low availability of nutrients to the culture (Rahardjo *et al*., 2005). These conditions affected microbial activity and resulted in decreased productivity.

Effect of Mineral Salts Solution on Reducing Sugar Production

The effect of mineral salts solution on reducing sugar production was studied by altering the volume of salts solution to the weight of the substrate. In this study, the results indicated a positive relationship between levels of salts solution used and reducing sugar production from sago *hampas*

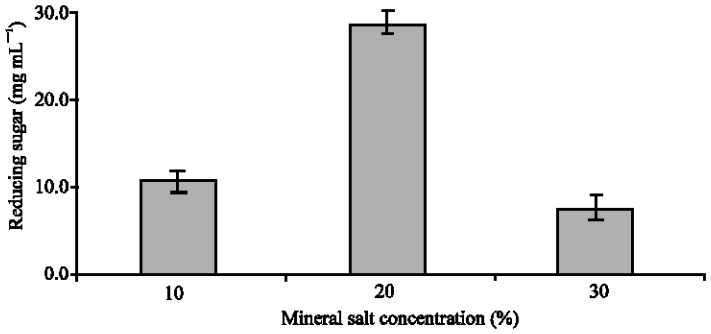

Fig. 3: Effect of different concentration of mineral salt solution on reducing sugar production by *Trichoderma* sp. KUPM0001 via SSF

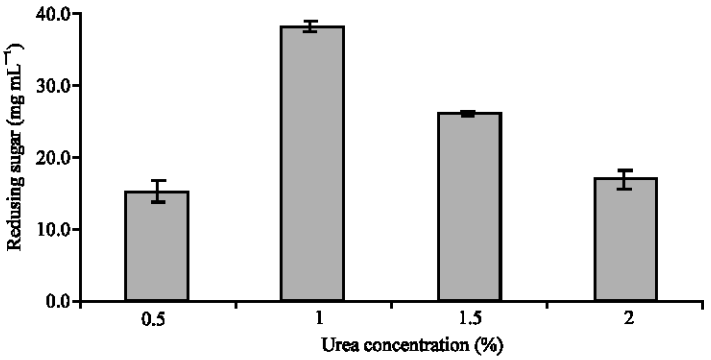

Fig. 4: Effect of different concentration of urea on reducing sugar production by *Trichoderma* sp. KUPM0001 via SSF

using *Trichoderma* sp. KUPM0001 via SSF. On lower and further enhancement in mineral solution level inversely influenced the production of reducing sugar significantly. The maximum sugar obtained was 29 mg mL^{-1} (Fig. 3), significantly ($p<0.01$) when mineral salts solution concentration was at 20% (v/w), compared to lower and higher levels whereby the productions were much lower. The high and low concentration of salts had a significant effect on the metabolic activities of the organism. Basically, the addition of nutrients to the solid medium improved the growth of organism and thus the production of the desired products. Supplementation of sago *hampas* with minerals also stimulated the production of relevant enzymes by *Trichoderma* sp. KUPM0001 and as the result good production of reducing sugar was obtained.

Effect of Urea Concentration on Reducing Sugar Production

Based on the results obtained, among the different concentration levels of urea tested, 1.0% (w/v) gave significant increased ($p<0.01$) on reducing sugar production (38 mg mL^{-1}) compared to 0.5, 1.5 and 2.0% (Fig. 4). In this study, supplementation of 1.0% (w/v) urea was 48.32% of the total nitrogen measured in the *hampas*. Urea has been reported to stimulate fungal growth when cultures were supplemented up to 40-50% (v/v) of the total nitrogen in the substrate. Furthermore, from ecological investigation it was known that the degradation of lignocellulose containing substrates was

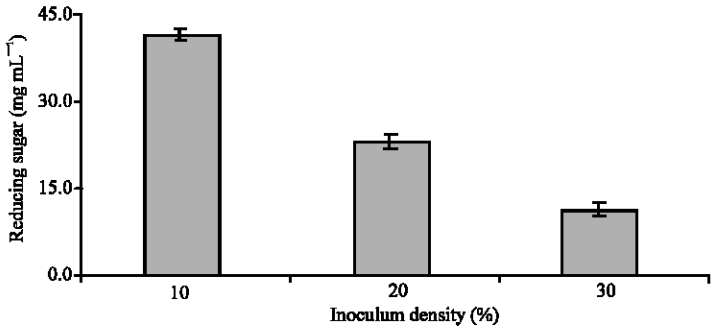

Fig. 5: Effect of different inoculum densities on reducing sugar production by *Trichoderma* sp. KUPM0001 via SSF

enhanced by supplementation of nitrogen. Addition of nitrogen sources to the substrate enhanced the consumption of the easily degradable components especially cellulose, leading to a reduction of *in vitro* digestibility. It was shown that addition of urea to the solid medium, enhanced the production of reducing sugar. The use of urea as inorganic nitrogen source also supported good fungal growth and protein production, which was attributed to the increase in enzyme protein. However, further increase in the concentration levels led to a sharp decline in sugar yield. This may explain the decrease in levels of reducing sugar when higher concentrations of urea were used.

Effect of Inoculum Density on Reducing Sugar Production

To evaluate the effect of inoculum level on reducing sugar production, different cell density (10%, 20 and 30% v/w) were added. Fermentations were carried out for 120 h and results are shown in Fig. 5. A 10% (v/w) of inoculum density gave maximum reducing sugar (41 mg mL^{-1}) which was significantly higher ($p<0.01$) when compared to higher inoculum densities. It has been shown that inoculum-to-substrate ratio (v/w) had a significant impact on enzymes production in SFF hence effecting the production of reducing sugar. This was because at higher inoculum levels, biomass production was increased leading to poor product formation (Nutan *et al*., 2002). Further with the increase in inoculum levels, the production of enzymes declined due to the exhaustion of nutrients in the fermentation mash. In addition, free excess liquid present in an unabsorbed form would pose an additional diffusion barrier together with that imposed by the solid nature of the substrate leading to decrease in cell growth and enzymes production.

Effect of Incubation Temperature on Reducing Sugar Production

Different incubation temperatures (25, 30, 35, 40 and 45°C) were used on reducing sugar production. The fermentation was carried out for 120 h. The results are presented in Fig. 6. Maximum reducing sugar production (37 mg mL^{-1}) was obtained at 30°C. A decreased in the production was observed when the incubation temperature was higher or lower than the optimum incubation temperature. Higher temperatures were found to have adverse effect on the metabolic activities of the microorganisms and it has been reported by various researchers that the metabolic activities of the microorganisms become slow at lower than the optimum temperature. Incubation temperature and its control in SSF process is crucial as the heat evolved during SSF processes is accumulated in the medium due to poor heat dissipation in solid medium. This phenomenon resulted in reduced microbial activity, thereby decreasing the product yield. The significance of temperature in development of biological process was such that it could determine the protein denaturation, enzyme inhibition, promotion or suppression of the production of a particular metabolite, cell viability and death. At higher temperatures, the heat may destroy the catalytic activities of the enzymes and the growth was inhibited.

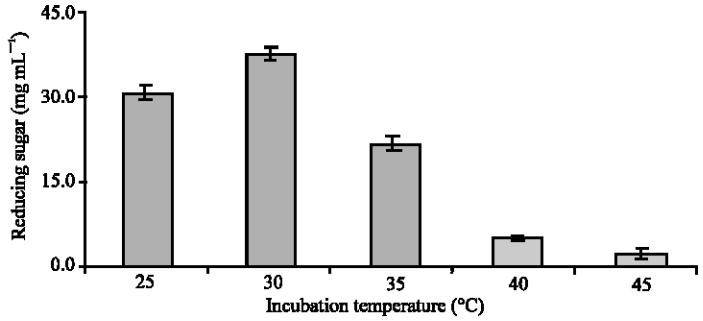

Fig. 6: Effect of incubation temperature (°C) on reducing sugar production by *Trichoderma* sp. KUPM0001 via SSF

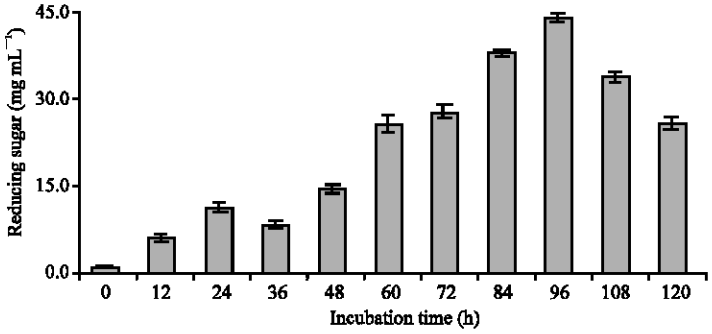

Fig. 7: Effect of different incubation time on reducing sugar production by *Trichoderma* sp. KUPM0001 via SSF

Effect of Incubation Time on Reducing Sugar Production

The reducing sugar production showed growth relatedness as the incubation period progressed and maximum sugar was obtained (46 mg mL^{-1}) after 96 h (Fig. 7). During the first 24 h of fermentation, *Trichoderma* sp. KUPM0001 was at its log phase, which extended up to further 96 h followed by the period when it was gradually decreased. The pre-germinated mycelial inoculum ensured the rate of production was very rapid and incubation beyond the optimum time showed rapid decline in sugar levels (25 mg mL^{-1}) at 120 h. A prolonged fermentation time beyond this period did not increase significantly the production of reducing sugar. The production of reducing sugar was very much associated with the enzyme production, which was highest when the fungus was at its peak log phase. The growth-associated production of amylases in a recombinant *A. oryzae* proved that the specific amylases production was closely coupled to the growth of the fungus. This suggests that the growth of the mycelium is crucial for high production of extra-cellular protein. Incubation beyond the optimum time was undesirable as this resulted in decreased of reducing sugar production which indicated that the fungus was at its stationary phase from 96 to 120 h of incubation.

Solid Substrate Fermentation with Optimum Conditions

Solid substrate fermentation was conducted and optimal parameters evaluated were applied such as 80% moisture content; 20% (v/w) mineral salts solution; 1.0% (w/v) urea concentration; 10% (v/w) inoculum density at 30°C for 120 h. Reducing sugar, enzymes activities, soluble protein and pH involved were determined. In the present study, it was shown that as the enzymes (amylases)

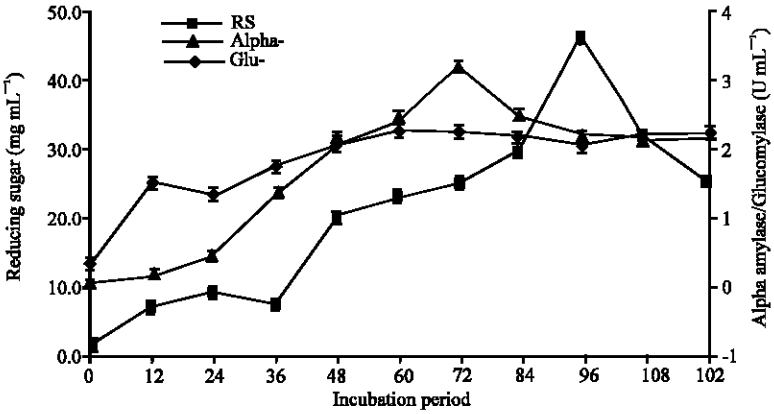

Fig. 8: Relationship between reducing sugar yield and amylases activities at optimized parameters; 80% moisture content; 20% (v/w) mineral salt solution; 1% (w/v) urea concentration; 10% (v/w) inoculum density and incubated at 30°C, 120 h via SSF

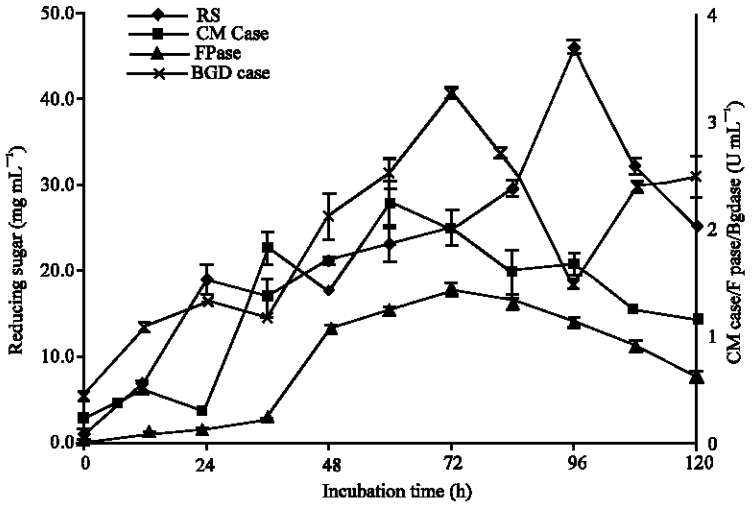

Fig. 9: Relationship between reducing sugar yield and cellulases activities at optimized parameters; 80% moisture content; 20% (v/w) mineral salts solution; 1% (w/v) urea concentration; 10% (v/w inoculum density at 30°C, 120 h via SSF

increased, the reducing sugar yield increased proportionately. The maximum alpha-amylase produced was at 72 h (3.19 U mL^{-1}) for the optimum production of reducing sugar at 46 mg mL^{-1} (Fig. 8). The correlation between the alpha-amylase and reducing sugar yield was positively correlated ($r = 0.767$; $p = 0.006$) and significant at $p<0.01$ (2-tailed). However, maximum production of glucoamylase was at 72 h (2.24 U mL^{-1}). The correlation between the glucoamyalse and reducing sugar yield was positively correlated ($r = 0.730$; $p = 0.011$) and significant at $p<0.05$ (2-tailed). The correlation between the amylases was significant $p = 0.01$ (2-tailed) level of significant ($r = 0.928$; $p = 0.001$).

Fungi are in direct contact with their nutrients in the environment. Smaller molecules (simple sugars and amino acids) in the solution in the watery film surrounding the hyphae can be directly absorbed by the hyphae. Larger insoluble polymers such as cellulose, starch and proteins must undergo

a preliminary digestion before they can be used. Molecules that are too large to be absorbed by the fungus are attacked by extracellular enzyme. The ability to utilize large molecules ultimately depends on the ability of the fungus to digest them, which in turn depends on the enzymes with which the fungus is equipped.

There are two major classes of starch degrading enzymes identified in the fungi: α-amylase and glucoamylase. A single species may secrete both. The amylases activity generally increased as fermentation proceeded. Amylases activities are known to be regulates by catabolite repression.

Extracellular enzyme was commonly controlled by catabolite repression, in the presence of rapidly metabolized carbon source, generally glucose, which is little or no synthesis of the enzymes. Some authors have suggested that the absence of catabolite repression in SSF system is due to several factors collectively, including the slow and low processes of diffusion in SSF cultures due to the low water activity. However, all SSF system described as resistant to catabolite repression were developed using wheat bran as substrate and in this present study sago *hampas* acts the same way. During the active growth, cellulose is often cell or substrate bound. They only appear in medium as the growth rate slow down. This was probably associated with a decrease on the area of substrate available for adsorption of enzymes and/or cells. Hence at the end of growth enzymes are desorbed from residual substrate and released from the surface on non-growing cells only if catabolite repressor was present in the medium.

Cellulases may not be produced until the repressors are mostly utilised and the growth rate has slowed down, i.e. late in the growth of the culture. Data of comparable enzymes activities were shown in Fig. 9. In the present study there were no significant difference ($p = 0.314$), ($p = 0.799$) and ($p = 0.712$) in CMCase, FPase and BGDase activity, respectively. Like cellulases produced from other sources, cellulases from KUPM001 are known to be catabolite sensitive. The CMCase activity of *Trichoderma* sp. KUPM0001 on sago *hampas* may be inhibited by high reducing sugar concentration in the culture.

There was no catabolic repression was observed in sago *hampas* during solid substrate fermentation and maximum production of reducing sugar and the relevant enzymes was obtained from sago *hampas* by fermentation by KUPM001 after 96 h of cultivation. However, prolonged incubation time after the optimal period caused a reduction in the production of both amylases and cellulases. After 96 h of incubation, the reducing sugars levels decreased and this may be due to the consumption of fungal cells.

The starchy fibrous residue hampas from sago starch processing may be a potential renewable resource for the production of reducing sugars via SSF. The sugars can then be a cheap feedstock for bioethanol production. The results of this study indicate the potential and further scale-up studies are warranted.

CONCLUSION

Production of reducing sugars by *Trichoderma* sp. KUPM0001 during solid substrate fermentation of sago starch processing waste *hampas* were successfully optimized. The optimum parameters obtained were 80% (v/w) of initial moisture; 10% (v/w) of inoculums size; 1.0% of urea in 20% (w/v) of mineral solution and incubated at 30 h±2°C which gave the enzyme activities for α-amylase, glucoamylase, carboxymethyl cellulase, filter paperase and ß-glucosidase of 3.19, 2.22, 1.66 , 1.11 and 1.48 U mL^{-1}, respectively. It was suggested that sago *hampas* is a suitable substrate for conversion to reducing sugar by local fungal. The recovered sugar could be further utilized as fermentation feedstock or converted into bioethanol.

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