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Enhancing α-Amylase and Cellulase *in vivo* Enzyme Expressions on Sago Pith Residue Using *Bacilllus amyloliquefaciens* UMAS 1002

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Abstract: The effect of Solid State Fermentation (SSF) parameters on the production of extracellular α-amylase and cellulase (endoglucanases) by *Bacillus amyloliquefaciens* UMAS 1002 using sago pith residue hampas in shaker flasks was investigated in this study. The appropriate incubation period, temperature, pH, agitation speed, inoculum concentration, hampas concentration and additive substrate effect were optimized for enzyme production. The activity of α-amylase and cellulase was at 14.19 and 13.15 IU mL^{-1}, respectively in optimal culture medium. Maximum yield for both enzymes were achieved by employing 4% w/v hampas in 0.2 M citrate buffer at pH 6 and incubated at 40°C for 6 h with agitation speed of 100 rpm. Inoculum concentrations were found to be optimum at 3% v/v and 4% v/v for α-amylase and cellulase, respectively. Enzyme activity was 2.8 (10.80 IU mL^{-1}) and 3.2 (9.38 IU mL^{-1}) fold higher for α-amylase and cellulase respectively when 1% w/v soluble starch was applied as additive substrates with 0.5% hampas. However in optimal media that consist of 4%w/v of hampas, addition of 1% w/v soluble starch intend to inhibit both enzyme productions. Result revealed that temperature, pH and shaking condition were the most significant factors for the production of α-amylase and cellulase enzyme. Temperature influenced enzyme production by affecting the other parameters including bacterial growth, pH, Dissolved Oxygen (DO) and reducing sugars. Nevertheless, shaking condition could affect DO concentration that in turn affected bacterial growth and enzymes production too.

Key words: *B. amyloliquefaciens* UMAS 1002, α-amylase, celulase, sago pith residue hampas, solid state fermentation

INTRODUCTION

Solid State Fermentation (SSF) can be defined as insoluble substrate fermented with adequate moisture which has numerous importance compared to submerged fermentation (SmF) or liquid state fermentation (Doelle *et al*., 1992) due to simple technique applied with lower capital investment, lower level of catabolite repressions and finally for their better product recovery (Babu and Satyanarayana, 1993, 1991; Mulimani and Patil, 2000; Baysal *et al*., 2002; Ikram *et al*., 2001). According to Carmelo *et al.* (2002) effluents, especially from food processing industry, which is rich in substrates such as starch, cellulose, fats and proteins, have the potential for microbial degradation to yield products with added values.

Sago starch industry is the main food industry in Malaysia (Barrau, 1995) especially in East Malaysia, which contributes more than 90% of the country production (Bujang and Ahmad, 1999). Bujang *et al.* (1996) have estimated the three types of sago waste, which are 15.6 tons of woody bark, 237.6 tons of wastewater and 7.1 tons of starchy fibrous sago pith residue or hampas which were generated from the processing of 600 logs of sago palm per day. In the state of Sarawak, Malaysia this hampas is usually washed off into nearby streams together with wastewater, thus contributing to pollution load, or deposited in the factory's compound, which can lead to serious environmental problems. Prior studies show that sago pith waste or hampas is composed mainly of starch (41.7-65.0%) and fiber (14.8%) including a fair amount of minerals (Wina *et al*., 1986).

An attractive and proficient means in utilizing this waste is through a biotechnological approach in which microbial strains are employed to degrade the sago waste. Microorganisms such as fungi and bacteria are known to play a major role in the degradation of cellulose and starch components (Coughlan, 1985). The genus *Bacillus*, represent one of the important group of bacteria in secreting extracellular commercial enzymes such as

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proteolytic, amylolytic and also cellulolytic enzymes (Crueger and Crueger, 1982) especially when they are provided with suitable medium and growth condition.

Bacillus amyloliquefaciens UMAS 1002 strain has been reported by Apun *et al*. (2000) for their abilities to produce both amylolytic (Abante *et al*., 1999) and cellulolytic enzymes. In the later research, Apun *et al*. (2000) have affirmed that *B. amyloliquefaciens* UMAS 1002 strain that have been isolated by Universiti Malaysia Sarawak researchers could be effectively used in the bioconversation of sago pith waste due to their ability to produce both amylolytic and cellulolytic enzyme when they are cultured with the suitable media such as sago pith waste (Apun *et al*., 2000) for this bacterial strain characteristics)

Although there are many research being done in isolating and purifying the enzymes produced by *Bacillus amyloliquefaciens* UMAS 1002 but there's no published information about the optimum microbial degradation condition for this bacterial strain as well as this bacterial species for the production of α-amylase and cellulase in solid state fermentation using hampas as the substrates. Present study is important to studies related to these bacterial strains especially in enzymology studies which require a sufficient amount of good quality enzymes for enzyme kinetic studies. Besides that application of such microorganism in industrial microbial processes, also needs to determine optimum process parameter for these microbial processes so that this parameter can be tested in a fermentation reactor scale studies.

The objective of this study is to investigate the desired combination of parameters influencing α-amylase and cellulase *in vivo* enzymes expressions such as temperature, time course, inoculums concentration, agitation speed, culture pH, hampas concentration and effect of additive substrates in order to perform an optimal shake flask fermentation condition. These main parameters were chose as they are the key factors in bacterial cell growth. Culturing the cell at higher temperature to reduce contaminants and degradation of enzymes, short time period, with or without minimal additives, less energy consumptions and less labor will certainly attract the microbial industry company to utilize this strain to produce useful product from sago industry waste.

MATERIALS AND METHODS

Microorganism and laboratory work: *Bacillus* strain used in the enzyme expression came from the *Bacillus amyloliquefaciens* UMAS 1002 strain identified and isolated from sago pith waste by Universiti Malaysia Sarawak (UNIMAS) researcher and preserved in UNIMAS laboratory. The lab work for present study was conducted in UNIMAS Genetic Molecule laboratory from June 2003 till March 2004.

Sago pith waste collection: Sago pith waste media was obtained from PPES sago processing factory in the district of Mukah, Sarawak. hampas sago collected was dried at 60°C and milled using grinder into 40 mesh size before added into the medium. The medium was autoclaved before any further analysis to ensure all microorganisms are dead, so there will be no other microorganism involves in enzyme expression except for only newly introduced *B. amyloliquefaciens* UMAS 1002 that was studied.

Fermentation media: Sago hampas broth (SHB) was used as fermentation medium followed previous study by Apun *et al*. (2000). SHB was prepared by mixing exactly (0.5% w/v) of the milled sago hampas into culture medium. Culture medium used in fermentation consists of yeast extract (0.2% w/v), KH_2PO_4 (0.1% w/v), $MgSO_4$ (0.5% w/v) and sago pith waste (0.5% w/v) (Apun *et al*., 2000). The pH of this broth were modified to pH 7.2 using 2.0 M sodium hydroxide (NaOH) (Julie *et al*., 1997). This pH was used as the reference to enable the optimization of the incubation period and incubation temperature before the optimizing of pH condition took place. Hampas particle size was maintained at 40 meshes throughout this experiment. In previous study only above fermentation media was used at pH 7.2 and 37°C and suggested as the optimal condition for this culture (Apun *et al*., 2000). However in this study all the parameter involved in the cell growth were tested to optimized the production of amylase and cellulase.

Fermentation experimental design: Erlenmeyer Flasks (100 mL) containing 20 mL of sago hampas medium was inoculated with respective 1% (v/v) of overnight two-stage inocular bacterial culture (at $O.D_{600} \approx 2.0$). The entire shaker flask test for each parameter was done separately in duplicates to reduce the statistical error. Enzyme test was also done in duplicates for each replicates from particular parameters. There were all four data collected for each parameter, which were two per replicate. Two replicates, which were done per parameter, was harvested and filtered using Whatman no. 1 filter paper according to the parameters that has been set up. The filtrates were later centrifuge at 13000 rpm by using centrifuge KUBOTA 7820 at 4°C for 15 min. The supernatant was used as crude enzyme for the enzyme and protein assays.

Enzyme assay: The DNS assay (dinotrosalicyclic acid) method which was originally described by Bernfeld (1955) with some modifications was used to determine the amount of reducing sugar with glucose as standard. The reaction mixture consists of 0.5 mL crude enzyme was added with 0.5 mL of 1% (w/v) soluble starch or carboxylmethylcellulase (CMC) (in 0.02 M phosphate buffer pH 6.9, with 6.0 mM sodium chloride) for 3 min at 37°C. The reaction was stopped by addition of 1.0 mL DNS solution and placed in boiling water for 5 min. Later the mixture is added with 10.0 mL of distilled water and vortex before measuring the absorbance at 540 nm using UV-spectrophotometer (amersham pharmacia biotech model ultrospec 1100 *pro*). A standard calibration curve was constructed by using D-glucose. One International Unit (IU) of each enzyme was defined as the activity that produced 1 μmol of glucose equivalents per minute under the above assay conditions.

Protein assay: The Bradford (1976) method was used in measuring the protein concentration with Bovine Serum Albumin (BSA) as the standard. Bradford reagent was prepared by using phosphoric acid (80%), ethanol glacial and Coomassie brilliant blue G-250. Protein concentration in each crude enzyme was measured using UV-spectrophotometer with absorbance at 590 nm.

Parameter optimization: Various process parameter influencing enzyme productions during SSF was optimized. The strategy followed was to optimize each parameter, independent of the others and subsequently optimal conditions were employed in all experiments.

Time course: Time course optimization is done by using 100 mL shaker flask, containing 20 mL of fermentation media with 0.5% (w/v) hampas sago which was prepared using distilled water and adjusted to pH 7.2 using 2 M NaOH. Later this fermentation media was inoculated with 1% (v/v) of two-stage inocular bacterial culture at $O.D_{600} \approx 2.0$. Shaker flask was put in 200 rpm agitation speed at 30°C. Sample for time course was harvested as the method mentioned in fermentation design at time interval of 3, 6, 9, 12, 15 and 24 h according sequential for further enzyme and protein assay. Two replicates have been prepared for each particular time.

Temperature: Temperature effect was investigated using the same 20 mL fermentation media that was inoculated with bacterial culture. Shaker flask is put in 200 rpm agitation speed but now with different temperature value that has been set up as follows: 25, 30, 35, 40, 45, 50 and 60°C in different shaking incubator. The sample is harvested according the optimal time that has been obtained from the time course optimization earlier for the further enzyme and protein assay.

pH: Four types of different buffers with different best pH range were used in obtaining the best optimal pH condition for the fermentation. Citrate buffer was prepared using 0.2 M sodium citrate and adjusted using 1 M citric acid for following pH: 4.5, 5 and 6. Phosphate buffer was prepared using 0.2 M Na_2HPO_4 and adjusted using 1 M NaH_2PO_4 for following pH: 6, 6.5, 7 and 8. Tris-HCl buffer was prepared using 0.2 M Tris-base and adjusted using 1 M HCl for following pH: 6, 6.5, 7. 8 and 9. Acetate buffer was prepared using 0.2 M sodium acetate and adjusted using acetic acid glacial for following pH: 6, 6.5 and 7.

Best optimal pH was determined using the same fermentation media with hampas sago with buffer solutions as the moisture agent replacing distilled water and later medium was adjusted to an appropriate pH using respective solutions mentioned earlier according to the buffers. Twenty milliliter buffer containing the fermentation media was transferred into 100 mL shaker flask Culture medium was then inoculated with bacterial culture. The culture is maintained at agitation speed of 200 rpm and according the optimal temperature observed. Two replicates were prepared for each particular pH according buffers. Finally samples were harvested after the optimal time for further enzyme and protein assay.

Agitation speed: Agitation speed effect in fermentation was conducted in 3 different conditions as follows: static, 100 and 200 rpm using 100 mL shaker flask. Fermentation medium is prepared in 20 mL buffer with appropriate pH that has been optimized and inoculated with 1% (v/v) overnight two-stage inocular bacterial culture (at $O.D_{600} \approx 2.0$). All the 3 condition that has been set up for the agitation effect is run in duplicate at once using Innova 4000 shaking incubator at the optimal temperature. Finally samples were harvested after the optimal time for further enzyme and protein assay.

Inoculum concentration: Inoculum concentration effect was done at five different concentrations that were as follows: 1, 2, 3, 4 and 5% v/v. All the inoculum used in this test is from the overnight two-stage inocular bacterial culture (at $O.D_{600} \approx 2.0$). Fermentation medium in 20 mL optimized buffer with appropriate pH is prepared using 100 mL shaker flask. Then the flask is inoculated with bacterial culture according the concentration as above. Fermentation is done in duplicate for each inoculum concentration (%v/v). Samples are incubated in the optimized condition observed. Finally samples are

harvested after the optimal time for further enzyme and protein assay.

Hampas sago concentration: Different concentration (%w/v) of hampas sago was employed in fermentation media as follows: 0.5, 1, 2, 3, 4 and 5% w/v. Medium was prepared separately to obtain the correct amount of hampas for each concentration. pH of the medium was also adjusted separately after the hampas was introduced in the medium up to pH 6. For each hampas concentration two samples were prepared. All samples were run together with other optimum conditions in shaking incubator. The contents of flask were harvested and assayed after the optimum time.

Different additive substrate: Different additive substrate test was done mainly using two commercial chemicals, Soluble Starch (SS) and carboxlymethylcellulase (CMC) since α-amylase and cellulase (endoglucanase) enzymes are the major enzyme express by UMAS 1002 which enabled them to utilize this additive as medium. Additives were added in 1% w/v ratio to the fermentation medium containing optimum pH buffer. The four conditions which was set up to determine the different additive effect were as follows: hampas sago alone without additive as control, hampas sago with SS, hampas sago with CMC and finally hampas sago with CMC and SS. Samples were also inoculated with optimum inoculum concentration and run at optimum temperature and agitation speed. Finally samples were harvested after the optimal time for further enzyme and protein assay.

RESULTS AND DISCUSSION

The time course of α-amylase and cellulase production: The shaker flask fermentation was carried out using bacterial culture ($O.D_{600} \approx 2.0$) at the constant temperature of 30°C, agitation speed of 200 rpm and pH adjusted at 7.2 with the samples harvested at time interval of 3, 6, 9, 12, 15 and 24 h (Fig. 1) for enzyme assays. Under these conditions, a proper mixing, mass and heat transfer was basically satisfied. It can be spot from the graph that both α-amylase (4.51 IU mL^{-1}) and cellulase (3.46 IU mL^{-1}) yield reached highest level within 6 h and decrease soon after that. Meanwhile protein concentration generally shows constant increase within the 24 h except for the 15th h where it shows a slight decrease, which due to some protein assay error before continuing to increase later. Optimal time obtained from this study shows a contradiction with earlier study done by Adnawani (2003). which enzyme yield was highest within 9-12 h using the same medium and bacterial strain.

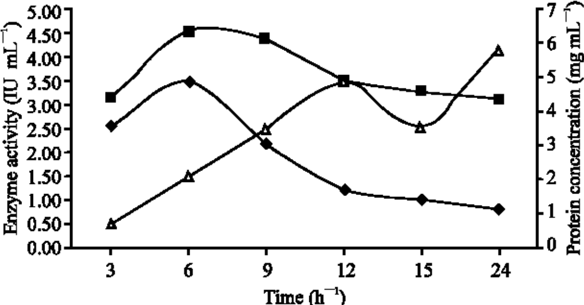

Fig. 1: The time course (hours) of α-amylase and Cellulase enzyme activity.(-■-) α-Amylase enzyme activity (IU mL^{-1}), (-◆-) Cellulase enzyme activity (IU mL^{-1}), (-▲-) Protein concentration (mg mL^{-1}). Results are means of two independent samples with two enzyme assays per sample

This might occur due to the usage of two-stage inocular *B. amyloliquefaciens* UMAS 1002 compared to single stage inocular used in many previous studies including the study done by Adnawani (2003). Julie *et al.* (1997) stated that inoculum ages that have been reported usually associated with the use of different media, conditions and strains of *B. amyloliquefaciens.* This can be even seen in simple case of single stage inocular, there was considerable variation in the inoculum ages reported. Yoon (1989) in his earlier study seem to use the youngest culture of *B. amyloliquefaciens* at 15 h while Pazlarova *et al.* (1984) used 18 h culture of *B. subtilis* for α-amylase production. Although usage of older culture seem to be uncommon but El-Leithy *et al.* (1973) and Qadeer *et al.* (1980) used 48 h culture while Therkildsen (1980) found that 72 h culture shows fastest fermentation in examining the symbiotic growth of yeast and amylase producing *B. subtilis* strain. It can be assumed that optimization of the inocular been used in previous study was not given priority but was commonly set according to the convenient of experimental scheduling (Julie *et al.*, 1997).

From the present study it was ascertain that time course of a fermentation is affected by the quality and strain of inoculum used. Calam (1976) earlier does mentioned that, old bacterial cultures seem to need a long period of time for adaptation in the culture medium followed by poorer growth compared to young culture that was used. When this is taken into account, the transfer time or age of inoculums become a crucial factor in determining the efficiency of the fermentation. The data attained from this study also has confirmed the prior study done by Julie *et al.* (1997) on usage of two-stage inocular for α-amylase production using

B. amyloliquefaciens where the optimal time for α-amylase highest production was at 6 h using two-stage inocular compared to single stage inocular bacterial culture at 28 h.

Protein concentration does show a steady increase since the enzyme that was studied was not a purified enzyme but from crude enzymes. So the protein concentration does not exactly show the concentration for α-amylase and cellulase enzyme only. *B. amyloliquefaciens* also has been reported to produce α-Glucosidases, Neutral protease, β-Glucanase (Priest and Sharp, 1989) apart from the enzyme been examined for in these study. Besides that, the protein from *Bacillus* cells itself also might interfere with other protein to show the protein concentration observed in this study since the protein assay could only identify accumulate protein in solutions then specifically. Yet, protein concentration was included in this study to look upon the production pattern of enzymes as well as the cell growth by looking straight into protein concentration as an indicator although it is not adequate to define cell growth by looking straight into total protein concentration.

Preceding study also has shown correlation between cell growth and enzyme production. It was reported that continuous culture studies of amylase and protease production by *B. amyloliquefaciens* and some strains of *B. subtilis* shows subsequent declined in enzyme production after initially exhibiting high enzyme yield in batch culture (Priest and Shrap, 1989). Extracellular enzyme synthesis in both of this species was repressed during exponential growth and derepressed during early stationary phase prior to sporulation (Priest and Sharp, 1989; Carmelo *et al*., 2002) when all the carbon source is depleted compared to *B. setearomophillus* and *B. licheniformis* which were derepressed during exponential phase of cell growth (Priest and Sharp, 1989). It can be concluded that two-stage inocular achieved faster cell growth in order to exhaust the carbon sources compared to single stage inocular as the enzymes were only derepressed at stationary phase.

Effect of temperature on α-amylase and cellulase production: In order to investigate the effect of temperature on α-amylase and cellulase enzyme expression, various temperatures was set as shown in Fig. 2 independently. The fermentation was done under same condition as the time course experiment except for the harvesting time was now fixed after 6 h for all the temperature tested. Temperature was noted to affect fermentation performance more significantly than other parameters. Highest yield of amylase and cellulase enzyme was obtained at 40°C with enzyme activity at 5.06 and 2.92

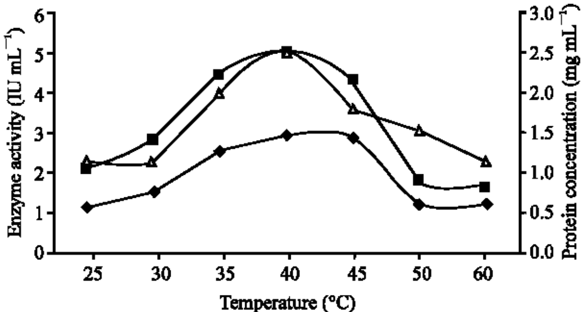

Fig. 2: Effect of temperature (°C) on α-amylase and Cellulase production. (-■-) α-amylase enzyme activity (IU mL^{-1}), (-◆-) Cellulase enzyme activity (IU mL^{-1}), (-▲-) Protein concentration (mg mL^{-1}). Results are means of two independent samples with two enzyme assays per sample

(IU mL^{-1}), respectively as shown in Fig. 2. Protein concentration (2.52 mg mL^{-1}) does show in identical optimum temperature at 40°C and decrease soon after that. The results obtained for both enzymes also does indicated that rapid decrease in enzyme activity as the temperature rises from 45-50°C. The enzymes were only stable at temperature between 35-45°C. Both enzymes and other proteins expressed tend to deactivate because they seem to denature after the optimum temperature as observed in Fig. 2.

Earlier in relating study by Kainuma (1986) using crude amylase from *Chalara paradoxa* fungi in degrading the raw starch from sago palm shows that optimum temperature was at 45°C and significantly decreases at 55°C. They also determined that raw starch degrading amylase produced by *Chalara paradoxa* was totally different from the conventionally studied raw starch digesting amylase attain from *Aspergillus* sp. or *Rhizopus* sp. In different research done by Mei and Yu (1997) showed that α-amylase from *B. amyloliquefaciens* strains shows better stability at 60°C compared to 37°C which was normally used to obtained better cell physiology and growth. This study again has confirmed that different strain that been applied on a particular medium has different physiological properties of enzyme been expressed although using same medium as carbon sources in expressing α-amylase and cellulase enzymes.

The study also discovered that *Bacillus* strain applied in this study came from moderate thermophile strain. Industrial usually prefer thermophilic enzymes compared to normal enzymes such as α-amylase from thermostable *B. amyloliquefaciens* strain which requires temperature of 70-85°C (Gerhartz, 1990), while *B. licheniformis* has a temperature optimum between

90-105°C (Fogarty and Kelly, 1990). The same seen in cellulase production by *B. stearothermohilus*, *B. coagulans*, *B. licheniformis* strains which grows well at 50-60°C (Fogarty and Kelly, 1990). Enhanced thermostability in themophilic α-amylase compared to a mesophilic enzyme was found to be due to extra two or three salt bridge of specific lysine residues which stabilized the enzyme with electrostatic interactions when heat induces unfolding of enzymes (Tomazic and Klibanov, 1988).

This enabled the use of recombinant DNA technology to produce a stable α-amylase of *B. amylolilquefaciens* by considerably improved, if amino acid residues 88, 253 and 385 were replaced by lysine or arginine residues (Fogarty and Kelly, 1990). Temperature was also become a major factor in determining the enzyme production in fermentation process in previous studies. From this study again it can be concluded that both α-amylase and cellulase enzymes were growth associated enzymes since the cells need to reach stationary phase before expressing the enzymes (Carmelo *et al*., 2002).

Effect of pH on α-amylase and cellulase production: The influence of fermentation pH was depicted in Fig. 3a and b. Fermentation was done at 40°C and 6 h as harvesting time while fixing all other parameter as before. Effect of pH was done using 4 various organic buffers adjusted to a range of pH from 4.5-9 according to their buffering capacity. Buffer was chosen to study pH effect since organic and in organic acids, such as acetates, citrate, tris-base and phosphate have better buffering capacities (Takashi, 2003) compared to conventional medium which uses water as the moisture in fermentation medium to assess pH effects. It can be observed from Fig. 3 that sodium-citrate buffer at pH of 6.0 shows the highest yield at 4.58 and 3.58 (IU mL^{-1}) for both enzymes α-amylase and cellulase, respectively.

The result obtained from this study shows in an agreement with the previous data collected by many other researchers for α-amylase production with an optimum activity at pH 5.9 (Suckling, 1990; Priest and Sharp, 1989). Besides that several studies done on cellulase production using *Bacillus* species does shows in variety of optimum pH for enzyme activity such as *B. licheniformis* at pH 6.1 (Dhillon *et al*., 1985), *B. pumilus* at pH 10 (Kawai *et al*., 1988) and *B. subtilis* at pH 7.0 (Chundakkadu, 1998). Results also show great stability in the enzyme within pH range of 6.0-7.0 for optimal α-amylase and cellulase activity. The data obtained again strengthen the fact that enzyme production in *B. amyloliquefaciens* was growth associated since it can be detected in the reduction of protein concentration at pH lowered than 6 and increased than 7, which indicate inhibition of cell growths (Data not shown in Fig. 3). Several studies also indicated the importance of fermentation media type used since pH of culture may change in response to microbial activities and nitrogen sources (Doelle *et al*., 1992). So a buffering agent used in this study could minimize the initial pH change in order to scrutinize pH effect.

As observed in Fig. 3 each buffer has different optimal pH condition for α-amylase and cellulase production. Sodium-citrate buffer shows the best buffer compared to phosphate, tris-base and sodium-acetate buffers at the pH of 6. This could be due to the different ion components in different buffers. Many literature reviews have mentioned the importance of phosphorus ion in increasing the enzyme production

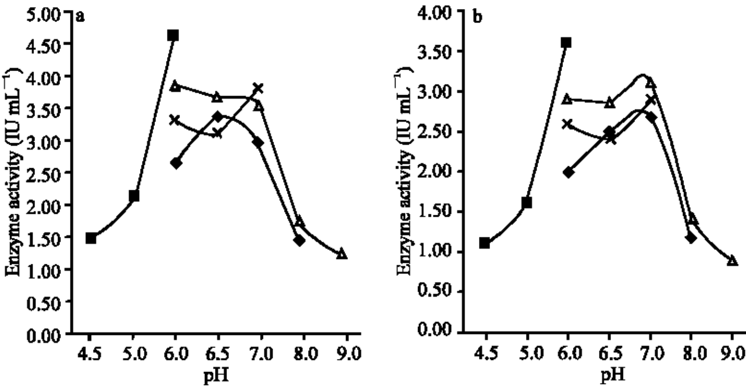

Fig. 3: Effect of pH (± 0.02) on (a) α-Amylase and (b) Cellulase production. (■) Citrate buffer; (◆) Phosphate buffer; (▲) Tris-base buffer; (✱) Sodium-acetate buffer. Results are means of two independent samples with two enzyme assays per sample

when carbohydrate was used as the carbon sources (Priest and Sharp, 1989). However this study shows in disagreement since the *Bacillus* strain applied in this study does not show in high yield in phosphate buffer, which in contrast shows lowest enzyme activity for both enzymes compared to other buffers. This might be due to the excessive phosphate ions supplemented by phosphate buffer since there already phosphate ions in fermentation medium form such as potassium phosphate at 0.1% (w/v). Besides that it was also identified 0.1 M of phosphate concentration was sufficient for optimum enzyme yield (Priest and Sharp, 1989). This does explain why enzyme yield was lowest in phosphate buffer in this study for respective same pH in other buffer and supported that sodium citrate buffer consist better ions which enhance enzyme yield at pH 6.

Effect of agitation speed on α-amylase and cellulase production: With operating temperature and pH maintained at 40°C and 6, respectively, the effect of agitation speed was investigated by comparing the performance of the agitation rate at three agitation rates namely 0 rpm (static), 100 and 200 rpm. Result shows a remarkable increase in fermentation medium under shaking condition compared to static condition (Fig. 4). It was observed more than 2 fold higher enzyme activity in shaking condition 3.96 and 2.97 IU mL^{-1} compared to non shaking condition at only 1.75 and 1.38 IU mL^{-1} for amylase and cellulase activity, respectively. There were not much difference in enzyme activity produced at agitation speed of 100 and 200 rpm. However there were slight reductions in enzyme activity at 200 rpm compared to 100 rpm. Amylase production was at 3.96 and 3.84 IU mL^{-1} in 100 and 200 rpm, respectively, while cellulase production was at 2.97 and 2.89 IU mL^{-1} in 100 and 200 rpm, respectively.

From the data obtained it is best supported the importance of agitation in solid state fermentation to facilitate the maintenance of homogenous conditions, especially with respect to temperature and the gaseous environment (Hesseltine, 1977). Agitation serves to replenish the interparticle spaces with fresh air. This could not be achieved in static culture since only upper most substrate was in contact with air while others were not. Such condition can lead to reduction of oxygen in interparticle spaces at limiting levels and the carbon dioxide can rise to inhibitory levels (Suckling, 1990). Nevertheless carbon dioxide that dissolved in fermentation medium might also cause pH decrease that in turn inhibits cell growth. In different cases, agitation does play important role in preventing and encouraging the agglomeration of solids (Suckling, 1990).

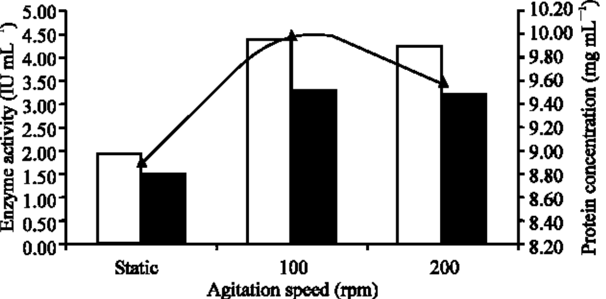

Fig. 4: Effect of agitation (rpm) on α-amylase and Cellulase production. (□) α-amylase enzyme activity (IU mL^{-1}), (■) Cellulase enzyme activity (IU mL^{-1}), (—▲—) Protein concentration (mg mL^{-1}). Results are means of two independent samples with two enzyme assays per sample

Dissolved oxygen also plays an important role in the way that there was critical dissolved oxygen concentration, below which metabolism pathways might be changed (Atkinson and Mavituna, 1983). Ingle and Boyer (1976) and Milner *et al.* (1996) have reported that critical value, as much as 20% saturation of dissolved oxygen was needed for enzyme production by *Bacillus* species. This gave a better explanation on the drastic reduction of enzyme yield under static condition compared to shaking condition. Dissolved oxygen in fermentation media also became important since *B. amyloliquefaciens* is an aerobic respiration species (Fogarty and Kelly, 1990). This also does elaborate on the importance of oxygen for *Bacillus* growth in fermentation media for enzyme expressions.

As outlined earlier agitation plays an important role in increasing the amount of dissolved oxygen and dispersion of macromolecules in the medium. It might therefore, contribute to the greater growth and better enzyme production noted in this study. However, the shearing effect induced by the higher agitation speed on the cells and enzyme activity may contribute negatively towards cell growth and enzyme stability (Suckling, 1990). Similar observation in the later study of higher level of agitation shows that high agitation speed might have aggravated cell damage which in turn led to enzyme inactivation (Shioya *et al.*, 1999; Feng *et al.*, 2003). Reduction in cell growth can be observed in protein concentration at higher agitation conditions as explained. This in fact gave a better understanding in this research on the cause of slight reduction of enzyme activity as the shaking speed that was increased from 100 to 200 rpm. The study has indicated the importance of agitation as the significant factor in enzyme expressions with 100 rpm as

the optimum agitation speed for α-amylase and cellulase production.

Effect of inoculum concentration on α-amylase and cellulase production: In order to identify the effect of inoculum concentration, fermentation media was set up using citrate buffer adjusted to pH 6.0 at 40°C and agitation speed of 100 rpm with various inoculum concentration of 1, 2, 3, 4 and 5% (v/w) as shown in Fig. 5. Bacterial cultures were prepared before using two-stages of inocular at $O.D_{600} \approx 2.0$ and sample were harvested after 6 h. There were no meaningful differences observed in the different inoculum concentration applied in this study. However, it can be spotted that the highest enzyme activity was found at 3% v/w (4.39 IU mL^{-1}) and 4% (3.25 IU mL^{-1}) for amylase and cellulase activity respectively. Although there was not much difference ($p>0.05$) the result obtained by the present study does agree with previous study done by Baysal *et al.* (2002) since reduction of enzyme activity was observed at higher inoculum that exceeds 3 and 4% for amylase and cellulase activity, respectively. This does indicate the incompatible condition of enzyme expression at higher inoculum concentration.

It can be observed from Fig. 5 that protein concentration does not shows in any specific trend at differences inoculum concentration since there were also less meaningful differences in enzyme production. Highest level of protein was observed at both the lowest and highest inoculum concentration. This does not indicate for any clear evidence in showing the highest or lowest cell growth rate in the culture medium at any inoculum concentration since it is hard to interpret the result obtained.

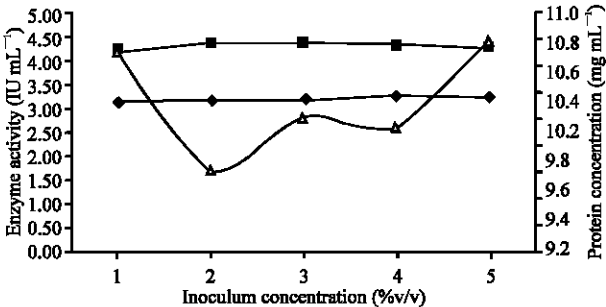

Fig. 5: Effect of inoculum concentration (%v/v) on α-amylase and cellulase production. (-■-) α-amylase enzyme activity (IU mL^{-1}), (-◆-) Cellulase enzyme activity (IU mL^{-1}), (-▲-) Protein concentration (mg mL^{-1}). Results are means of two independent samples with two enzyme assays per sample

This study shows parallel results with the study done earlier by Baysal *et al.* (2002) using *B. subtilis* on wheat bran and rice husk in producing α-amylase. They also mentioned that this situation might occur due to the cell numbers that are close to each other in different inoculum concentration that has made in no meaningful differences in enzyme expressions. This might be due to the inoculum concentration tested in this study was done by only increasing the volume of inoculum (%v/v). However the bacterial culture O.D that was taken for all the difference inoculum concentration was still remains the same at $O.D_{600} \approx 2.0$. Besides that the present study was also done using shaker flask fermentation or batch fermentation at the volume of 20 mL. This might be one of the reasons why less meaningful differences occurred in batch fermentation that made the bacterial culture grows almost equally although different inoculum concentration was applied.

In related study using banana waste as the main carbon source, Chundakkadu (1998) has shown that maximal cellulase production by *B. subtilis CBTK 106* strain, occurred with 15% inoculum size while El-Nawwi and El-Kader (1996) shown about 4% v/v as the optimal inoculum for *Aspergillus terreus* in hydrolyzing sugarcane bagasse Although many study has shown different needs of inoculum concentration but this concludes that different microorganism strains have different needs of inoculum concentration condition in enhancing extracellular enzyme production. Type of fermentation (batch, fed batch or continuous) also does influence the inoculum concentration used since different bacterial strain produced optimum enzymes at different fermentation type (Priest and Sharp, 1989).

As mentioned earlier a fall in enzyme activities with the increase of the inoculum concentration could be observed. The data obtained from this study also supports the previous study on inoculum effect done by many researchers. (Babu and Satyanarayana, 1996; Baysal *et al.*, 2002; Nutan *et al.*, 2002; Uyar and Baysal, 2004). The production of enzyme declined with the increase in inoculum level due to the exhaustion of nutrients in the fermentation mash. Nevertheless, Baysal *et al.* (2002) has mentioned that higher inoculum concentration will increase the moisture content to a significant level. They have indicated that this excess liquid which derived from the bacterial activity will be in an unabsorbed form which will therefore give rise to an additional diffusional barrier together with that imposed by the solid nature of the substrate and lead to a decrease in enzyme production and growth (Nutan *et al.*, 2002). In the related subject Babu and Satyanarayana (1996) has stated the importance of the water content of a medium

that has a profound influence on growth and the production of products by microorganism.

In addition, Muniswaran and Charyulu (1994) have also reported similar result on the effect of inoculum volume which was only marginal cellulase enzyme production using *Trichoderma viride* NCIM 1051 on coconut coir. In addition, they have indicated that the higher inoculum size used in solid state fermentation will increase the moisture content to a significant extent which at the same time will be detrimental to growth and production apart from adding to the fermentation cost. All this declaration has given a brief idea on the grounds of the reduction of enzyme activity after exiting higher inoculum concentration in the present study.

Effect of hampas concentration on α-amylase and cellulase production: To understand how the presence of hampas sago affects the amylase and celulase production, different hampas sago concentrations (%w/v) as follows: 0.5, 1.0. 2.0, 3.0, 4.0 and 5.0% (w/v) was added to the fermentation medium independently. The fermentations were set using citrate buffer adjusted to pH 6.0 at 40°C and agitation speed of 100 rpm but using only 1% of inoculum concentration since there were not much meaningful differences were obtained in different inoculum concentrations applied before. Among the concentration of hampas sago tested, enzyme activities were rapidly increased when 4% (w/v) of hampas was added to the fermentation medium as shown in Fig. 6. This has resulted amylase and cellulase activities as high as 14.19 and 13.15 IU mL^{-1}, respectively. However a gradual decrease for both of the enzyme formation could be observed when the amount of hampas concentration increased from the optimal concentration.

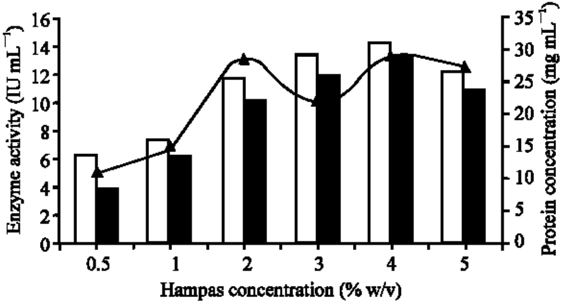

Fig. 6: Effect of hampas concentration (%w/w) on α-amylase and Cellulase production. (□) α-amylase enzyme activity (IU mL^{-1}), (■) Cellulase enzyme activity (IU mL^{-1}), (—▲—) Protein concentration (mg mL^{-1}). Results are means of two independent samples with two enzyme assays per sample

Reduction in enzyme activity after a certain tolerance towards substrate concentration was probably due to the compromise between the mass transfer and shear stress that can only tolerate till a certain limit (Liu *et al.*, 2003). Beside that in the allied study by Ikram *et al.* (2001) on α-amylase production by *Bacillus licheniformis* found that the α-amylase production on wheat bran was maximized at 1.25%w/v and starts to decrease with any further increase in the concentration. Reduction of enzyme activity on high concentration has been explained by Ikram *et al.* (1998) was due to the thickness of fermentation media that resulted in gradual reduction in agitation and poor assimilation of air which has was the major factor for the growth of the organism and also enzyme production. Similar results were also noticed by other authors (Mulimani and Patil, 2000; Liu *et al.*, 2003) and agreed with the explanation since it was also batch fermentation.

Although in the present study moisture content was not one of the parameter being tested but it was indirectly correlated with the hampas concentration test done in this study. As mentioned earlier, the entire fermentation medium contains 20 mL of moisture content. However at higher hampas concentration the ratio of substrate dissolved to free moisture becomes less. This can be taken as the indicator that at higher hampas concentration the free moisture content becomes less. Babu and Satyanarayana (1993) as used the same technique in evaluating the effect of different moisture level by varying the ratio (w/v) of wheat bran to the moistening agent. This has given another reason in describing the reduction occur at higher hampas concentration which consist of lower ratio of moisture content The initial moisture content in the fermentation medium was another major factor in the success of the solid state fermentation (Lonsane *et al.*, 1985; Doelle *et al.*, 1992; Baysal *et al.*, 2002).

Authors also have mentioned previously that a decrease in enzymes production could be observed when the moisture level is higher or lower from the optimal level obtained. This does gave a strong explanation on the reduction of enzyme activity at higher and lower level of hampas concentration then the optimal concentration. It also reported that higher substrate moisture in SSF resulted in only half of the optimal product formation due to reduced mass transfer process such as diffusion of solutes and gas to the cells during fermentation process take place which can be observed at lower hampas sago concentration. In another hand, lowering moisture content will resulted in minimized solubility, heat exchange, oxygen transfer and low availability of nutrients for the cells to growth which can be described as the condition

at higher hampas concentration. Finally this condition will influence the microbial activity and results in decrease of enzyme productivity (Hesseltine, 1979; Carrizales *et al*., 1981). Beside these Doelle *et al.* (1992) has also revealed that the optimum moisture levels raged from 22.4 to 75% and depends on the nature of the product, the substrate and the choice of the organism used in the fermentation process.

Beside the reasons that was given by the authors who were mentioned earlier, Babu and Satyanarayana (1993) added that the influence on the biosynthesis of bacterial α-amylase have been attributed to the interference of moisture in the physical properties of the solid properties. Babu *et al.* (1993) pointed out that the higher moisture content shown a decrease in porosity, changes wheat bran particle structure, promotes development of stickiness, reduces gas volume and exchange the diffusion which has resulted in lowered oxygen transfer and enhanced formation aerial mycelium in fungi. Lower moisture content on the other hand will contribute to the reduction in the solubility of nutrients of the solid substrate, a lower degree of swelling and higher water tension. The same could be applied to hampas sago as the substrate since it also has given the same result at different hampas concentration or moisture content.

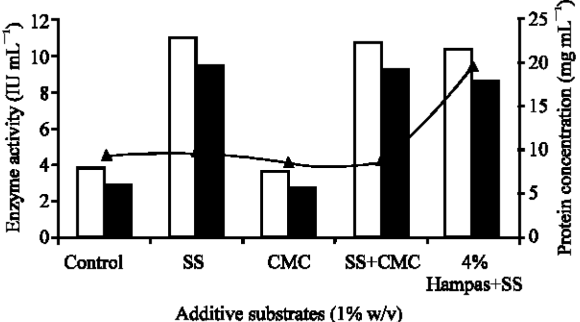

Fig. 7: Effect of additive substrates (1% w/v) on α-amylase and Cellulase production. (□) α-amylase enzyme activity (IU mL^{-1}), (■) Cellulase enzyme activity (IU mL^{-1}), (-▲-) Protein concentration (mg mL^{-1}). Results are means of two independent samples with two enzyme assays per sample. * SS = Soluble starch + 0.5% w/v hampas; CMC = Carboxylmethylcellulase + 0.5% w/v hampas; SS + CMC = Addition of soluble starch and carboxylmethylcellulase + 0.5% w/v hampas; control = only 0.5% w/v "Hampas sago without additives; 4% hampas + SS = Addition of 4% w/v hampas + soluble starch 1%w/v

Effect of additives on α-amylase and cellulase production: Two important locally and commercially available substrates, soluble starch (SS) and carboxylmethylcellulase (CMC) were employed at 1% (w/v) as additives substrates in order to study their effect on α-amylase and cellulase production by *Bacillus amyloliquefaciens* UMAS 1002. The fermentation was carried out for 6 h and other parameters were kept at their optimum level that has been observed earlier. In order to investigate the effect of the additives 4 combination of medium were prepared. In each medium only the substrate used were change and manipulated. It can be shown in Fig. 7 that the best additive for both enzyme production were medium containing only SS which yield highest at 10.80 and 9.38 IU mL^{-1} for amylase and cellulase activity respectively. Although the medium containing SS with CMC gives nearly the same yield as medium containing only SS but it was not considered as the alternative best medium because it increased the fermentation cost by the addition of CMC since a better yield could be obtained using SS alone.

Addition of SS as the additive substrate in 0.5% w/v hampas has increased 2.8 and 3.2 fold higher in amylase and cellulase activity respectively compared to hampas alone which was set as the control in this test. The data obtained from this study also indicates that CMC did not exert any impact as the inducer or repressor since it does not differ much in cultures activity compared to control. So this does again support that the addition of SS itself enough in improving enzyme yield rather than adding CMC too in basal medium. The data obtained was in agreement with Priest and Sharp (1989) that only SS could be the major carbon source as nutrient for *B. amyloliquefaciens* in α-amylase production although in this study cellulase also expressed by this *Bacillus* strain. Priest and Sharp (1989) also has mentioned that starch also could be replaced by lactose for α-amylase production.

Several studies highlighted the importance of starch as the substrate in promoting amylase production in *Bacillus*. Priest and Sharp (1989) has mentioned that Srivastava and Baruah (1986) have identified the importance of starch on inducing amylase synthesis in *B. stearothermophilus*. They also have indicated that amylase production was induced with maltotriose, maltotetraose, maltopentaose and maltohexaose applied as the substrates. In addition, Sumitra *et al.* (2004) also have show the importance of starch as additive substrate for coconut raw material SSF in stimulating α-amylase production. In addition they also mentioned that, since carbon source was the energetic source that is obtainable for the growth of the cells so it could be concluded that the enzyme production was growth associated.

Chundakkadu (1998) on the other hand mentioned the importance of glucose as the inducer in the cellulase production, although several authors have mentioned that glucose was the carbon repressor (Priest and Sharp, 1989; Chambliss, 1993) in amylase production. However from the data obtained from this study cellulase production was correlated with amylase production since any stimulation in amylase production does also promote cellulase production as well.

Since supplementation of SS resulted in marginal increased activities in comparison with CMC, SSF was carried out with 4% w/v hampas as in Fig. 7. However the same changes were not observed when 1% w/v soluble starch was applied with 4% w/v "hampas" (10.28 and 8.55 IU mL^{-1} for amylase and cellulase, respectively). Results indicate a drop for both enzyme productions with addition of SS in 4% w/v "hampas" if compared to enzyme production in 4% w/v "hampas" alone (14.19 and 13.15 IU mL^{-1} for amylase and cellulase respectively). Reduction in enzyme production might be due to excess carbon sources, which inhibit the enzyme production. Sumithra *et al.* (2003) also mentioned that the higher concentration of starch above 0.5% w/v has resulted in the inhibition of amylase enzyme synthesis. This shows that amylase production could only tolerate up to a certain level of carbon sources.

In general it can be concluded that composition of a medium is a critical factor in obtaining optimum enzyme yield and it does varies according to *Bacillus* species (Priest and Sharp, 1989). Carbon sources such as starch, lactose, maltose, glycogen, galactose and others also ware reported in inducing amylase production in *Bacillus* (Priest and Sharp, 1989). Besides that many studies also stated that amylase production was generally repressed in *Bacillus* sp. with the presence of glucose (Priest and Sharp, 1989; Chambliss, 1993). Result obtained from this study does suggest that additive substrate was not important in inducing enzyme production since it will only increase the fermentation cost although it gave significant increase at lower level of hampas concentration.

CONCLUSIONS

The results from this studies has designated the suitability of *Bacilllus amyloliquefaciens* UMAS 1002 in using cheap and abundantly available sago pith residue or sago waste hampas as the carbon source for large scale production of α-amylase and cellulase in solid state fermentation, thereby lessen the high cost when other substrates and chemical are used in the same enzymes production. The study also revealed the bacterial strain that was used in this study was moderate thermopile. It has been identified that temperature, pH and agitation has a significant importance in both enzyme production and cell growth since the enzymes produced in this study were growth associated. Enzyme activity observed for both of this enzymes were 6.2 fold and 20.9 fold higher for amylase and cellulase respectively compared to the optimum enzyme expression reported by Apun *et al.* (2000) for amylolytic (2.30 IU mL^{-1}) and cellulolytic (0.63 IU mL^{-1}) activity. Optimum properties identified from this study were summarized in Table 1.

Table 1: Summary of optimal properties for α-amylase and cellulase enzyme expression by *Bacillus amyloliquefaciens* UMAS 1002

Optimum properties	α-Amylase	Cellulase
Time course	6 h[a]	6 h[a]
Temperature	40°C[a]	40°C[a]
pH	6.0[a]	6.0[a]
Agitation speed	100 rpm[a]	100 rpm[a]
Inoculum concentration	3% (v/w)[b]	4% (v/w)[b]
Hampas concentration	4% (w/v)[a]	4% (w/v)[a]
Additives effect at 1% (w/v)	Soluble starch[a]	Soluble starch[a]
Optimum yield	14.19 IU mL^{-1}	13.15 IU mL^{-1}

[a]letter indicates results obtained with significant differences at ($p<0.05$)
[b]letter indicates results obtained with no significant differences at ($p>0.05$)

Several other parameters such as different ion effect, aeration rate, different moisture level effect, other plant waste (wheat bran, maize bran, rice husk) and cell density can be included in the future studies since this parameters also have been pointed out by many authors as the important parameters in enzyme production. Beside that genetic engineering work could also be done on UMAS 1002 in order to improve the enzyme activity with better physiological properties mainly on their sustainability at higher temperature that will enable their enzymes application in industries. Finally with all the information obtained from this shaker flask study, solid state fermentation process in a bioreactor could be done for large scale of enzyme production. This is important because maximum utilizations of this waste can also contribute to efficient solid waste management, where continuous accumulation of agricultural wastes poses a serious environmental problem.

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