

Characterisation of Amylase Produced by Local Isolate *Aspergillus flavus* NSH9 Undergone Solid-state Fermentation on Rice Husks

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Amylases are a group of important hydrolytic enzymes that have been applied in a vast variety of industries and processes around the world. Amongst the different hydrolases, amylases possess the highest potential for use of biopharmaceutical and medicinal purposes. These starch-degrading enzymes are omnipresent glycoside hydrolases that can be found in a variety of organisms which include animals, plants, a few yeasts, bacteria and fungi. However, fungi have been shown to possess a list of advantages over other organisms in producing amylase. Mesophilic fungi such as *Aspergillus* spp. are reported to be one of the dominant amylase-producing species. Despite many species of *Aspergillus* have been identified, only two species have been extensively studied and applied commercially for amylase production. Thus, amylase obtained from a local isolate *Aspergillus flavus* NSH9 which undergone solid-state fermentation on rice husks without supplement was studied as an attempt to outsource potential local industrial competent amylases. Biochemical analyses such as effects of temperature, pH, incubation period, metal ions, soluble starch concentration and different reagents were performed as to investigate the nature of the enzyme. Also, molecular analyses were conducted which include Native-PAGE, SDS-PAGE and zymography to identify the types of amylases present.

Keywords: amylase; *Aspergillus flavus*; solid-state fermentation; biochemical analyses; molecular analyses

I. INTRODUCTION

Around the globe hydrolytic enzymes have gradually replacing chemicals such as acid in the industrial processes due to their numerous advantages. The substitution of harmful chemicals with enzymes not only makes the process less toxic and eco-friendly but also reducing the consumption of natural resources such as water and costs associated with the production process while improving quality of the products. Amylase that hydrolyses starch and its related polysaccharides is one of the important hydrolytic enzymes utilised in the industrial processes and it represents approximately 30% of the global enzyme production (Vaidya *et al.*, 2015). It is an important enzyme as starch digestion by amylase is the basis for a vast range of industrial applications and processes such as food and beverages industries, textile

and paper making, detergent industry, ethanol production, medicine, pharmaceutical, cosmetics, and other Biotechnology-related areas (Gupta *et al.*, 2003; Chi *et al.*, 2009; Das *et al.*, 2011; Singh *et al.*, 2011; Mosjov, 2012; Mamatha *et al.*, 2012; Mobini-dehkordi & Javan, 2012; Vaidya *et al.*, 2015; Gopinath *et al.*, 2017). Their vast applications and potentials in a variety of fields and processes have made the demand for amylase becomes even greater. In the beginning, researchers were focused on bacteria in obtaining the amylases, later as fungi was found to possess a list of advantages over bacteria, it has become the centre of attention in outsourcing for more competent enzymes. The mesophilic fungi are reported to be the principal amylases producers, and *Aspergillus* members appear to be one of the dominant producing species (Ali *et al.*, 2015).

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The filamentous *Aspergillus* spp. have been widely studied and applied commercially for the industrial enzymes production. Despite over than 200 species have been identified, only two species, namely, *Aspergillus niger* and *Aspergillus oryzae* have been extensively studied and applied commercially for amylase production (Lubertozzi & Keasling, 2008; McNeil *et al.*, 2013). Hence, a local isolate *Aspergillus flavus* NSH9 was applied in this study as a search for potential amylases that could be developed for the local industrial demand with a low production cost by applying solid-state fermentation (SSF) process which utilised agro-waste rice husks as the fermentation substrate. Several biochemical analyses were performed on the obtained amylase as to investigate the nature of the enzyme and subsequently, to reveal its characteristics. Crucial biochemical parameters which affecting or influencing the activity of an enzyme such as temperature, pH, period of incubation, concentration of the soluble starch, metal ions and other agents were studied. In further validating the presence of amylase(s) as well as to identify the types of amylase present, several molecular approaches were applied which include native polyacrylamide gel electrophoresis (PAGE), Zymogram, SDS-PAGE and MALDI ToF.

II. MATERIALS AND METHOD

A. Sub-culturing of *A. flavus* NSH9

Local isolate *A. flavus* NSH9 stock culture applied in this study was obtained from the fungal collections of Molecular Genetics Laboratory at the Faculty of Resource Science and Technology, UNIMAS (Saat *et al.*, 2025). This stock culture was used in the subsequent fungal sub-culturing whereby the fungus was sub-cultured on potato dextrose agar (BD Difco, USA) containing 50 µg/ml of ampicillin (Nacalai Tesque, Japan) under a sterile condition. Incubation was then conducted at room temperature for about 5 to 6 days until full fungal growth and mature state were achieved. The culture was then maintained at 4 °C prior to use.

B. SSF of *A. flavus* NSH9

1. SSF substrate

Rice husks used as the SSF substrate were obtained from the rice milling factories around Samarahan division in Sarawak, Malaysia.

2. Pre-treatment of the substrate

Prior to use for SSF process, the rice husks were mechanically pre-treated by grinding into smaller size particles using a cutting mill (Retsch SM 100, Germany). The pre-treated rice husks were then stored in an air-tight container, at room temperature before use.

3. SSF process

SSF process with an optimal condition was conducted based on the method by Saat and colleagues (2025). The SSF process started with the addition of ultra-pure water (Barnstead water purification system, ThermoFisher Scientific) onto 10 mg of rice husks (in an autoclaved 500 ml Erlenmeyer conical flask) to achieve initial moisture content of 70%. The added water was let to be absorbed by the rice husks at room temperature. Once ready, inoculation of 6 plugs of fungus (approximately 0.5 cm² in size which were cut out from PDA) onto the substrate was performed under a sterile condition. Subsequently, incubation was performed at 30 °C under a static condition in in an incubator (Taitec Bioshaker BR-43FL) for 144 hrs (6 days) continuously.

C. Crude Enzyme Extraction

The crude enzyme extraction was performed based on the method described by Saat *et al.* (2025). A volume of 40 ml of cold sodium acetate buffer (0.1 M; pH 5.8) was added into the flask (containing substrate with the fungus) upon completion of the SSF process and left shaken (Taitec Bioshaker BR-43FL) at 120 rpm for 30 mins at room temperature. The homogenised cultured was then filtered through a muslin cloth. Subsequently, the filtered fluid was centrifuged at 6,000 rpm for 30 mins at 4 °C (Himac CR 21G High-Speed Refrigerated Centrifuge, Hitachi, Japan). The collected supernatant was then filtered through Whatmann paper No.1 on ice for twice. The filtrate collected was used as the crude enzyme.

D. Partial Purification of Crude Enzyme

The obtained crude enzyme was then re-filtered using V1 Filtration glass set (Rocker, Taiwan R.O.C) through cellulose acetate filter paper. The process was performed on ice. The enzyme was then further purified and concentrated using Vivaspın20 (MWCO of 10,000 kDa) with polyether sulfone as the membrane (Sartorius Stedim, Germany) according to the manufacturer's protocol. The concentrated enzyme was then kept at 4 °C prior to further analyses.

E. Measurement of Enzymatic Activity

The enzyme activity of extracted enzymes was assayed using a reaction consists of 1% (w/v) of soluble starch (Sigma-Aldrich) and extracted enzyme (1:1). The mixture was then incubated at 37 °C for 30 mins. Total reducing sugars released from the assay was determined using standard DNSA method (Miller, 1959) with glucose as the standard. The enzyme activity was calculated using the following formula:

Enzyme activity (U)

$$= \frac{\text{concentration of glucose release} \times \text{total assay volume} \times \text{dilution factor}}{\text{volume of enzyme used} \times \text{diameter of cuvette} \times \text{incubation time}}$$

F. Biochemical Analyses

The concentrated partial purified enzyme was subjected to biochemical analyses. The effects of several parameters and agents which include temperature, incubation period, pH, different soluble starch concentration, metal ions, surfactants and chelator on the amylolytic activity of the enzyme were studied.

1. Effect of temperature

The enzyme activity was assayed using a reaction consisted of partial purified enzyme and 1 % (w/v) of soluble starch (1:1). The assay was incubated at 25 °C, 30 °C, 37 °C, 40 °C and 45 °C (Taitec bioshaker br-43fl), separately for 30 mins at pH 5.8, at a static condition. The total reducing sugars released was determined using standard DNSA method, and the measurement of enzymatic activity for each temperature was performed as described in section E.

2. Effect of incubation period

A reaction consisted of partial purified enzyme and 1 % (w/v) soluble starch (1:1) was applied to assay the enzyme activity. The assay was incubated for different period of 25 mins, 30 mins, 40 mins and 45 mins, separately, at 37 °C and pH 5.8 (Taitec bioshaker br-43fl), at a static condition. The total reducing sugars released was determined using standard DNSA method, and the measurement of enzymatic activity for each period of time was performed as described in section E.

3. Effect of pH

The enzyme activity was assayed using a reaction consisted of partial purified enzyme and 1 % (w/v) soluble starch in 0.1 M sodium acetate solution (1:1) with different pH of 5.0, 5.5, 6.0, 6.5, 7.0 and 7.5, separately, and incubated at 37 °C in an incubator (Taitec bioshaker br-43fl) for 30 mins at a static condition. The total reducing sugars released was determined using standard DNSA method, and the measurement of enzymatic activity for each different pH was conducted as described in section E.

4. Effect of soluble starch concentration

A reaction consisted of partial purified enzyme and soluble starch (1:1) was used for the enzyme activity assay. The concentrations of soluble starch studied were 0.5 % (w/v), 1.0 % (w/v), 1.5 % (w/v), 2.0 % (w/v) and 2.5 % (w/v). Each assay was incubated at 37 °C and pH 5.8 for 30 mins at a static condition (Taitec bioshaker br-43fl). The total reducing sugars released was determined using standard DNSA method, and the enzymatic activity for each concentration assay was measured as described in section E.

5. Effect of metal ions

The enzyme activity was assayed using a reaction consisted of partial purified enzyme and 1 % (w/v) soluble starch (1:1) with the addition of different metal ions. The metal ions studied were 5 mM of calcium (II) chloride (Merck), 5 mM of copper (II) chloride (Sigma Aldrich), 5 mM of ferum (II) chloride (Sigma Aldrich), 5 mM of kalium chloride (Emsure), 5 mM of magnesium (II) chloride (Amresco), 10 mM of natrium chloride (J.T. baker) and 1 mM lithium chloride

(Amresco). The assay of each metal ion was incubated at 37 °C and pH 5.8 for 30 mins (Taitec bioshaker br-43fl). The total reducing sugars released was determined using standard DNSA method, and the enzymatic activity for each different pH was measured as described in section E.

6. Effect of different reagents

The different reagents studied were surfactants [0.1 % (v/v) Triton x-100 (Pro Pune) and 0.1 % (v/v) of Tween-20 (Merck)], denaturing agent of 0.1 % (v/v) β -mercaptoethanol (Merck) and chelator of 1 mM EDTA (Humb G Chemicals). The enzyme activity was assayed using a reaction consisted of partial purified enzyme and 1 % (w/v) soluble starch (1:1) with the addition of each reagent, separately. Each assay was incubated at 37 °C and pH 5.8 for 30 mins (Taitec bioshaker br-43fl), at a static condition. The total reducing sugars released was determined using standard DNSA method (Miller, 1959), and the measurement of enzymatic activity for each different agent assay was performed as described in section E.

G. Molecular Analyses

Several approaches were applied in validating the presence and types of amylases, namely, Native PAGE, Zymogram SDS-PAGE, SDS-PAGE and MALDI ToF.

1. Native PAGE analysis

A discontinuous native PAGE system (Bio-RAD) was applied with a separating gel of 8 % [incorporated with 0.5 % soluble starch (Sigma-Aldrich) or 0.3 % amylopectin (Sigma-Aldrich)] and a stacking gel of 4 %. Preparation of each gel was performed according to the manufacturer's protocol (Bio-RAD). Samples were prepared by diluting them with sample buffer without β -mercaptoethanol (1:4) and without heating. Electrophoresis of the samples was conducted on ice with 1x running buffer at 90 V, 15 mA until the blue dye reached near bottom of the gel. Then, the gel was briefly rinsed with ultra-pure water at room temperature and subsequently, soaked in the sodium acetate solution (pH 5.8; 0.1 M) for 30 mins, at room temperature and at a static condition. Upon completion of the process, the solution was discarded and the gel was briefly rinsed again with ultra-pure water at room

temperature. Staining of the gel was then performed using Lugol's iodine by shaking at 55 rpm for 5 mins at room temperature.

2. Zymography analysis

A discontinuous Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) system (Bio-RAD) was used with a separating gel (12 %) incorporated with soluble starch (Sigma-Aldrich) of 1 % and a stacking gel of 4 % (which included 10 % of SDS). Preparation of each gel was performed according to the manufacturer's protocol (Bio-RAD). Samples were prepared by diluting them with sample buffer without β -mercaptoethanol (1:4) and without heating. Electrophoresis of the samples was conducted on ice with 1x running buffer at 90 V and 15 mA, until the blue dye reached near bottom of the gel. The gel was then washed with 2.5 % of Triton X-100 for about an hour by gentle shaking (on ice). The solution was replaced with fresh one for every 20 mins. After washing had completed, Triton X-100 was discarded, and the gel was rinsed with sodium acetate solution (0.1 M; pH 5.8) by shaking at 63 rpm for 5 mins at room temperature. Subsequently, the gel was soaked in a fresh sodium acetate solution (0.1 M; pH 5.8) for about 30 mins, at room temperature and at a static condition. Then, the sodium acetate was discarded, and the gel was briefly rinsed with ultra-pure water at room temperature. Followed with staining of the gel using Lugol's iodine and shaken at 55 rpm for 5 mins at room temperature.

3. SDS-PAGE analysis

Discontinuous SDS-PAGE system (Bio-RAD) was applied with a separating gel of 12 % and a stacking gel of 4 % (which include 10 % of SDS). Preparation for each gel was performed according to the manufacturer's protocol (Bio-RAD). Samples were prepared by diluting them with sample buffer (1:4) and subsequently heated on a heat block at 95 °C for 4 mins before loading into the gel system. Electrophoresis of the samples was performed at 100 V and 20 mA, with 1x running buffer. The process continued with the staining of the gel with 0.1 % of Coomassie blue R-250 in fixative (40 % of methanol and 10 % of acetic acid) for about half to an hour by gentle shaking (Bioshaker BR-43FL, Taitec). De-staining of the gel was then performed with 40 % of methanol /10 % of

acetic acid solution for about 1 to 3 hours by gentle shaking (Bioshaker BR-43FL, Taitec).

4. Viewing and image capturing of the gels

Images of all the gels from sections G.1, G.2 and G.3 were viewed and captured using the imaging system ImageQuant 400 (GE HEALTHCARE, USA).

5. Drying of the gels

Selected gels from sections G1, G2 and G3 were dried as to document them apart from capturing their images. The drying process was conducted at room temperature, for about 12 hours by using GelAir drying system (BIO-RAD).

6. Protein sequencing and analyses

Protein bands in the SDS-PAGE gels which corresponded to the localised activity of the enzyme in the zymogram gels were cut out. In ensuring the amount of protein was sufficient for protein sequencing, a pool of bands of the same molecular size was collected in a 1.5 ml centrifuge tube. The protein samples were sent to 1st BASE (Malaysia), and the sequencing was performed via peptide mass fingerprinting using MALDI-ToF by Proteomics International PTY Ltd (Western Australia). The samples were also sent to BGI Tech (Hong Kong) for MALDI-ToF analysis to reveal the enzyme's protein sequence as well to annotate the protein.

Bioinformatics analyses were performed on the results obtained from the MALDI-ToF analyses as to identify the proteins. Mascot software version 2.3.02 was applied by BGI Tech as the search tool for protein identification, and the protein databases that were searched against are such as NCBI nr, Swiss Prot and UniProt as well as other species genome or protein databases. As for 1st BASE, Mascot search tool with Ludwig NR protein database were applied by the Proteomics International PTY Ltd. Additional Bioinformatics analysis own search was performed on the obtained proteins' sequences using SmartBLAST and BLAST (National Center for Biotechnology Information, NCBI: <https://www.ncbi.nlm.nih.gov/>) against several databases which include non-redundant protein sequence (nr) of NCBI database, UniProtKB/Swiss-Prot, reference sequence protein

(refseq_protein) of NCBI database, protein data bank (pdb) and landmark database of NCBI.

III. RESULT AND DISCUSSION

A. Biochemical Analyses

There are several crucial factors or parameters which affecting or influencing the activity of an enzyme such as temperature, pH, period of incubation, concentration of the soluble starch, metal ions and other agents. These biochemical parameters were studied as to investigate the nature of the obtained enzyme and subsequently, its characteristics can be revealed.

1. Effects of temperature on enzymatic activities

Temperature is the most crucial factor which can influence not only the fungal growth, but also the activity of enzymes secreted by the fungus. *A. flavus* NSH9 can be found in nature at the temperature of between 12 °C and 48 °C, and its optimal growth at the temperature ranges from 28 to 37 °C (Amaiike & Keller, 2011; Sriram *et al.*, 2012). Therefore, temperatures of between 25 °C and 45 °C with an interval of 5 °C were investigated for the enzyme activity performance and 37 °C was included instead of 35 °C. The enzyme was observed to be active at the temperature of between 25 °C and 45 °C whereby the highest amylolytic activity (0.350 U) was achieved at 40 °C and the lowest enzyme activity (0.201 U) was at 25 °C (Figure 1). At 45 °C, the enzyme activity was observed to decline slightly (Figure 1). This result was different from the optimal temperature observed for growth of *A. flavus* NSH9 on rice husks during the SSF which was at 30 °C. This data suggested that there was no direct correlation between optimal growth and the maximum activity of the enzyme produced, although the optimal temperature for amylase activity is said to be related to the growth of microorganisms (Saranraj & Stella, 2013).

The obtained result is similar with many previous research findings, including by Yasmin *et al.* (2016a). The optimal temperature for *A. flavus* (KIBGE IB-34) growth on rice husks (supplemented with mineral salt) via SSF was different with the optimal temperature for the highest amylolytic activity whereby the maximum enzyme activity was observed at 50 °C. Also, the secreted enzyme was found to be active

over a range of 40 to 80 °C, suggesting that the enzyme was thermophilic in nature (Yasmin *et al.*, 2016a). Optimal temperature of 50 °C for maximum amylase activity was also observed for enzyme secreted by *A. flavus* which was isolated from soil sample of cassava waste dump site that undergone SSF process on millet pomace (Famotemi *et al.*, 2015). Different range of optimal temperature for maximum activity of amylase from *A. flavus* could also be observed from other previous research findings. The lowest optimal temperature reported was 30 °C using SSF method (Sidkey *et al.*, 2011). The highest optimal temperature was at 80 °C which was observed in amylase obtained from rhizosphere isolate of *A. flavus* (Padmini *et al.*, 2012). Other optimal temperatures reported were 35 °C which was shown by the amylase obtained from *A. flavus* var *columinaris* via SSF using Nile hyacinth as the substrate (El-Safey & Ammar, 2004), 45 °C (Oyewale, 2012) and 55 °C which was observed in amylase obtained from *A. flavus* isolated from mangrove (Bhardwaj *et al.*, 2011). The variation in optimal temperature for maximum amylase activity observed may be due to the different isolate, strain and species as well as the source, nature and physiological activities of the organism involved, and also the substrate used (Oyewale, 2012).

Based on the enzyme activity observed, the amylase secreted by *A. flavus* NSH9 isolate may be mesophilic. The ability of amylases secreted by *A. flavus* NSH9 isolate to work actively in a considerably wide range of temperature suggesting a potential usage in a variety of industrial applications. As 40 °C was the optimal temperature for the maximum amylolytic activity whereby the pancreatic amylase has the same optimal temperature, suggesting it could be applied also in the pharmaceutical industry.

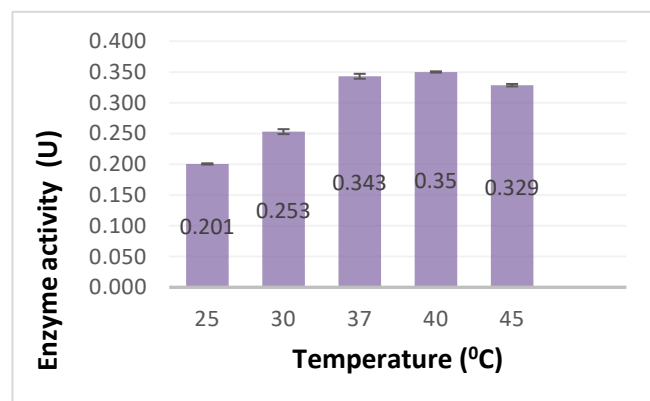


Figure 1. Enzyme activity at different temperature.

One unit of enzyme activity (U) is defined as the amount of enzyme that released 1 mg/ml of glucose per minute from a soluble starch under the standard assay condition.

2. Effects of incubation period on enzyme activities

The duration for an enzyme to withstand process environment is one of the crucial factors for the enzyme to be viable for industrial applications, especially, industrial processes which require long operation duration. Although many characterisation studies were conducted on amylases produced by fungi and bacteria, studies on the effect of incubation time on enzyme activity assayed with starch as the substrate were very few or lack, especially, amylase from *A. flavus*. Thus, in this study, a range of incubation period of 25 to 45 minutes (with 5 minutes interval) was investigated. The performance of the enzyme on 1 % (w/v) soluble starch at 37 °C for each incubation time was observed. The results obtained indicated that the enzyme was active throughout the tested incubation time with the maximum enzyme activity (0.281 U) achieved at 30 minutes (Figure 2). Based on the amylolytic activity observed, the enzyme may be still active if the incubation time is extended up to an hour. This finding suggests that the enzyme has a promising potential to be applied in diverse industrial processes, especially, moderate to long operation duration of processes.

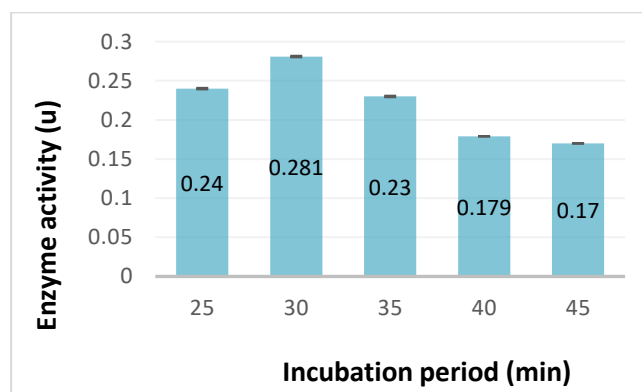


Figure 2. Enzyme activity at different incubation periods. One unit of enzyme activity (U) is defined as the amount of enzyme that released 1 mg/ml of glucose per minute from a soluble starch under the standard assay condition.

3. Effects of pH on enzyme activities

Generally, enzymes are found to be stable over a wide range of pH 4 to 11. However, amylases produced by most bacteria and fungi were shown to have optimal pH in the range of acidic to neutral (Gupta *et al.*, 2003). Thus, the effect of pH values of between 5.0 and 7.5 on the amylolytic activity of the enzyme was studied. As indicated in Figure 3, the enzyme was active in the pH range of acidic, neutral and slightly alkaline conditions with two peaks; one acidic (pH 5.5) and one neutral (pH 7.0). This observation suggested there were two optimal conditions for enzyme activity; acidic and slightly alkaline, indicating the presence of at least two types of amylases in the enzyme extract whereby they may act synergistically in the starch degradation. Nevertheless, the acidic condition was favoured with pH 5.5 was observed to give the highest amylolytic activity. This suggests that the enzyme would be useful in processes which require a wide range of pH change from acidic to slightly alkaline and vice-versa. The observation is similar with other findings obtained by Alva *et al.* (2007) and Yasmin *et al.* (2016a). They also reported two peaks in the enzyme activity. Acidic (pH 5.8) and basic (pH 9.0) peaks were observed in the amylase secreted by *Aspergillus* sp. JGI 12 (Alva *et al.*, 2007). Whereas in the other study, two peaks were exhibited at pH 7.5 and pH 9.0 for the amylase obtained from *A. flavus* KIBGE IB-34 strain which was undergone SSF process on rice husks supplemented with minerals (Yasmin *et al.*, 2016a).

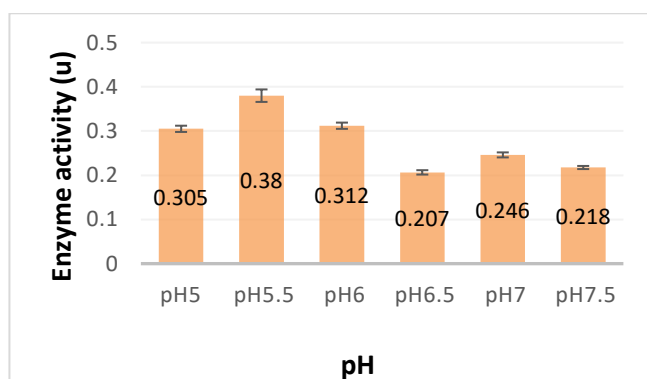


Figure 3. Enzyme activity at different pH.

One unit of enzyme activity (U) is defined as the amount of enzyme that released 1 mg/ml of glucose per minute from a soluble starch under the standard assay condition.

4. Effects of soluble starch concentration on enzymatic activities

Amylases are a group of enzymes that able to hydrolyse starch and related polysaccharides to produce low molecular weight dextrins and sugar (Nirmala & Muralikrishna, 2003). As starch hydrolysis by amylase is the basis for a wide range of processes and industrial applications (Mobini-dehkordi & Javan, 2012), it is also important to look at the effect of starch concentration. Different processes may require different optimal concentration of starch. Therefore, four different concentrations of soluble starch were investigated, namely, 0.5, 1.0, 1.5, 2.0 and 2.5 % (w/v) as to determine the best concentration for maximum amylolytic activity.

The enzyme exhibited good activities over the five concentrations with the enzyme activity was at its peak at 2.0 % (Figure 4) which indicates that the enzyme can be applied in processes of a wide range starch concentration including the moderate high starch concentration. The obtained result was in parallel with findings reported by Oyewale (2013), whereby amylase from *A. flavus* grown on the agricultural wastes in Nigeria via SSF process showed that with 2.0 % soluble starch gave the highest enzyme activity. Nevertheless, it is contradicted with many previous reported findings, whereby 1% of soluble starch was shown to be the optimal concentration for maximum activity of amylase obtained from *A. flavus* (Bhardwaj *et al.*, 2011; Bhattacharya *et al.*, 2011; Famotemi *et al.*, 2015).

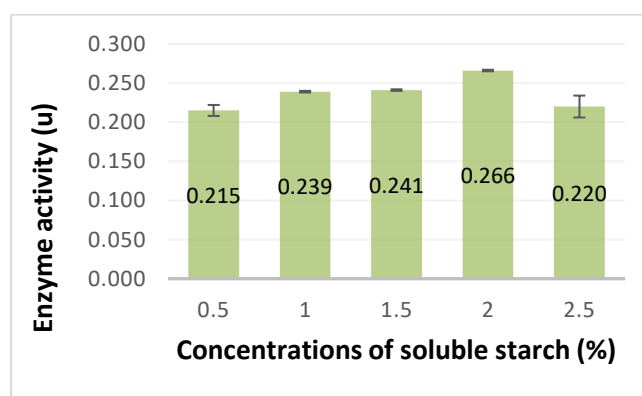


Figure 4. Enzyme activity at different concentrations of soluble starch. One unit of enzyme activity (U) is defined as the amount of enzyme that released 1 mg/ml of glucose per minute from a soluble starch under the standard assay condition.

5. Effects of metal ions on enzyme activities

Metal ions may play a key role in protein folding or in catalysis, thereby metal binding to an enzyme plays a significant role in the activation and stabilisation of an enzyme (de Oliveira *et al.*, 2010; Prakash *et al.*, 2011). These ions are important in the enzyme action as their presence or absence regulates the enzyme activity by inhibiting or enhancing (Prakash *et al.*, 2011; Jha *et al.*, 2013). Most of amylases is reported to be metal ion dependent enzymes which require especially the divalent ions such as Mg^{2+} , Mn^{2+} , Ca^{+2} , Zn^{2+} and Fe^{2+} (Sivaramakrishnan *et al.*, 2006; Koç & Metin, 2010; Prakash *et al.*, 2011).

In this study, eight different metal ions which include divalent and monovalent ions were investigated. Overall, all divalent ions with concentration of 5 mM were observed to exhibit elevation in the enzyme activity, on the other hand, two monovalent ions of Na^+ and Li^+ (10 mM and 1 mM of concentration) were shown to have inhibitory effect (Figure 5, Table 1). Presence of divalent metal ions of Mg^{2+} , Mn^{2+} , Ca^{2+} , Fe^{2+} and Cu^{2+} as well as monovalent K^+ in the enzyme was observed to enhance the amylolytic activity with varying percentage (Table 1). Mn^{2+} exhibited the highest elevation of enzyme activity of 6.1 % and followed by Mg^{2+} (Figure 5, Table 1). The results indicate that the enzyme may require metal ions, especially divalent Mn^{2+} and Mg^{2+} , for enzyme activation. Other previous findings suggested similar results such as amylase obtained from mangrove isolate of *A. flavus* also exhibited higher enzyme activity enhancement with Mn^{2+} compared to Ca^{2+} (Bhardwaj *et al.*, 2011) and also, amylase of *A. flavus* HBF34 strain was reported to be activated by Mn^{2+} and Ca^{2+} , with Mn^{2+} showed the highest stimulation amongst the metal ions investigated (Koç & Metin, 2010). However, a similar study by Famotemi *et al.* (2015) showed that the amylase of *A. flavus* fermented on millet pomace via SSF was activated in the presence of Ca^{2+} and Mn^{2+} , with Ca^{2+} exhibited the highest enhancement of enzyme activity amongst the metal ions studied. Several other studies have reported otherwise which Ca^{2+} was shown to inhibit amylase activity (Ramachandran *et al.*, 2004; Koç & Metin, 2010; Jha *et al.*, 2013). There are also reports on which Ca^{2+} has no effect on the enzyme activity especially of the bacterial amylases (Saranraj & Stella, 2013). Based on the findings of this present study and previous studies, it appears

that the action of metal ions on amylase vary not only between species and microorganism but even within the same species of different isolates.

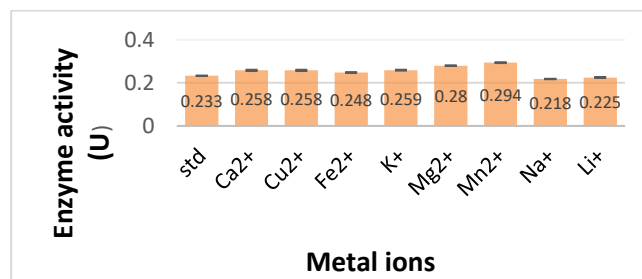


Figure 5. Enzyme activity in the presence of different metal ions. Std: enzyme without any reagents (control). One unit of enzyme activity (U) is defined as the amount of enzyme that released 1 mg/ml of glucose per minute from a soluble starch under the standard assay condition.

Table 1. Summary of the enzyme activity results in the presence of metal ions, compared to control (enzyme activity of enzyme without any metal ions was taken as 100 %).

Metal ion	% Increase	%Decrease
5 mM Mn^{2+}	6.1	-
5 mM Mg^{2+}	4.7	-
5 mM K^+	2.6	-
5 mM Ca^{2+}	2.5	-
5 mM Cu^{2+}	2.5	-
5 mM Fe^{2+}	1.5	-
10 mM Na^+	-	1.5
1 mM Li^+	-	0.8

6. Effect of different reagents on the enzyme activity

Reagents such as surfactants, denaturing agent and chelator can be applied to characterise an enzyme. The characteristics of an enzyme can be revealed by looking at the effect of these reagents on the enzyme activity such as stimulatory or inhibitory.

Chelating agents or chelators can be organic or inorganic compounds that capable of binding metal ions to form a complex ring-like structure called 'chelate'. Chelators possess "ligand" binding atoms that form either two covalent linkages or one covalent and one co-ordinate or two co-ordinate linkages in the case of bidentate chelates (Flora & Pachauri, 2010). One of the common chelators used is EDTA which is a chemical used for both industrial and medical purposes

(Flora & Pachauri, 2010) as well as in molecular studies. EDTA is known as a strong chelating agent for heavy metals, the alkaline earth metals such as calcium and magnesium (Bowen & Kerwin, 1954; Sivaramakrishnan *et al.*, 2006). It can chelate out the divalent metal cations from the enzyme molecule and thereby, inhibit the enzyme activity. The reduction in enzyme activity indicates that the enzyme is a metallo-enzyme which requires a certain metal ion as co-factor for its activity (Yasmin *et al.*, 2016a). In this study, the amylolytic activity was observed to reduce significantly at 1 mM of EDTA with 6.4 % of decrease (Figure 6, Table 2) suggesting that the enzyme obtained from *A. flavus* NSH9 is a metallo-amylase which requires metal ions in its active site for activity. The result obtained confirms the observation of enzyme activity enhancement in the presence of most divalent metal ions which indicate that the produced enzyme is a metal-dependent amylase. The finding is similar with other previous findings such as by Yasmin *et al.* (2016b) whereby the activity of amylase from *A. flavus* isolate was also decreased significantly in the presence of EDTA. However, the concentration used was 10 mM and the inhibitory effect was by 50 %. Also, inhibitory effect of EDTA was shown in amylase produced by *A. flavus* of mangrove isolate (Bhardwaj *et al.*, 2011) as well as in amylase produced by *A. flavus* HBF34 (Ö Koç & Metin, 2010).

In native proteins such as enzymes, generally all peptide bonds are in the trans-configuration. Partial or complete protein denaturation can be accomplished by a variety of agents. One of the most broadly used denaturing agents is β -mercaptoethanol. Treatment of proteins with this thiol compound can cleave the disulfide bonds or bridges (Bano *et al.*, 2014). In general, disulfide bonds play important roles in the activity and stability of an enzyme. Disulfide bridges are said to enhance rigidity of the protein molecules as to prevent unfolding or developing ionic interactions at their active sites (Bostrichidae *et al.*, 2008). The formation and reduction of the disulfide bonds are suggested to function as an on-off switch to regulate enzyme activity in response to stress (Siddiqui *et al.*, 2005). In the presence of 0.1 % β -mercaptoethanol, the enzyme activity was observed to be reduced significantly (Figure 6, Table 2), indicating that disulfide bonds are essential for its enzyme activity. The finding is similar with result reported by Koç and Metin (2010)

whereby reduction of enzyme activity was shown in amylase produced by *A. flavus* HBF34 with the presence of β -mercaptoethanol. It has been suggested that secreted enzymes such as amylases may be stabilised by maintaining disulfide bonds. Removal of disulfide bonds has been shown to cause reduction in enzyme activity, loss of enzyme activity and reduction in stability (Siddiqui *et al.*, 2005). Nevertheless, there are reported findings on the increase of enzyme activity of amylases from different organisms such as bacteria and beetle, in the presence of β -mercaptoethanol (Siddiqui *et al.*, 2005; Bostrichidae *et al.*, 2008; Shafiei *et al.*, 2011).

The term surfactant derived from a combination of surface-active agent, and it is a substance which reduces surface tensions. Surfactants are among the most versatile products of chemical industry, applied in a variety of industries and products such as house-hold cleaning, personal care, textile, automobile-related, pharmaceuticals, and its application has extended to advance or high technology areas such as biotechnology processes (Rosen & Kunjappu, 2012). Based on the charges that the surface-active portion bears, surfactants are classified into four categories, namely, cationic, anionic, non-ionic and zwitterionic. Triton X-100 and Tween-20 are the examples of non-ionic surfactants which the surface-active portion bears no apparent ionic charge. Triton X-100 is one of the most widely used non-ionic surfactants in the laboratory as detergents, emulsifiers, wetting agents, de-foaming agents, removing SDS from SDS-PAGE gels prior to renaturing the proteins within the gel as well as for lysing cells to extract protein. Its chemical name is octoxinol (Koley & Bard, 2010; Triton X-100 PubChem). TWEEN 20 is a brand name for polysorbate 20. It is a non-ionic detergent widely used in biochemical applications, and it has been used as an emulsifying agent for the preparation of stable oil-in-water emulsions. Also, TWEEN 20 has been applied in pre-extraction of membranes to remove peripheral protein (TWEEN 20 Sigma-Aldrich; Tween 20 PubChem). The effect of these two non-ionic surfactants on the amylase was studied. The results showed that both surfactants elevated the enzyme activity with TWEEN-20 gave higher activity than Triton X-100 (Figure 6, Table 2).

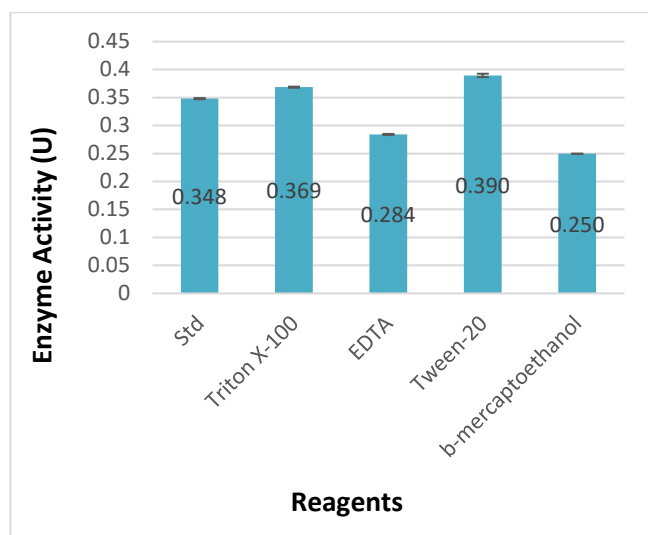


Figure 6. Enzyme activity in the presence of different reagents.

Std: enzyme without any reagents (control). One unit of enzyme activity (U) is defined as the amount of enzyme that released 1 mg/ml of glucose per minute from a soluble starch under the standard assay condition.

Table 2. Summary of the enzyme activity in the presence of different reagents, (enzyme activity of enzyme without any reagents and additives was taken as 100%).

Reagents	Increase (%)	Decrease (%)
Tween-20 (0.1 %)	4.2	-
Triton X-100 (0.1 %)	2.1	-
β-mercaptoethanol (0.1 %)	-	9.8
EDTA (1 mM)	-	6.4

B. Molecular Analyses

In further validating the presence of amylase(s) as well as to reveal its identity, several molecular approaches were applied. The approaches were native PAGE, Zymogram, SDS-PAGE and MALDI ToF.

1. Native PAGE analysis

Native PAGE was performed as to identify and localise the enzyme activities on the gel incorporated with substrates as this technique allows separating proteins in their native conformation with the absence of denaturing and reducing agents (SDS and β-mercaptoethanol) in all buffers and gels. Furthermore, this electrophoresis allows subunit interaction,

biological activity and its native charge density to be maintained and preserved (Walker, 2002; Ramachandran *et al.*, 2004).

In this analysis, two different substrates of starch and amylopectin were incorporated into two separate gels. Two clear bands were localised on the starch-incorporated gel as well as the amylopectin-incorporated gel after staining with Lugol's iodine (Figure 7 and Figure 8). The appearance of bands in the starch-incorporated gel suggested the presence of amylolytic activity. A group of enzymes that is known to hydrolyse starch and related polysaccharides to produce sugar and low molecular weight dextrins is amylases (Nirmala & Muralikrishna, 2003). Positive result obtained with amylopectin-incorporated gel suggested that there is a possibility that the enzyme extract may contain enzyme(s) of exoamylase group or/and debranching group. Exoamylases and debranching amylases are known to be able to hydrolyse amylopectin as they capable to hydrolyse α-1,6 glucosidic bonds in addition to α-1,4 glycosidic linkages (Benjamin *et al.*, 2013; Djekrif *et al.*, 2016). The two bands observed in both substrates may indicate there were at least two types of amylases present in the enzyme extract.

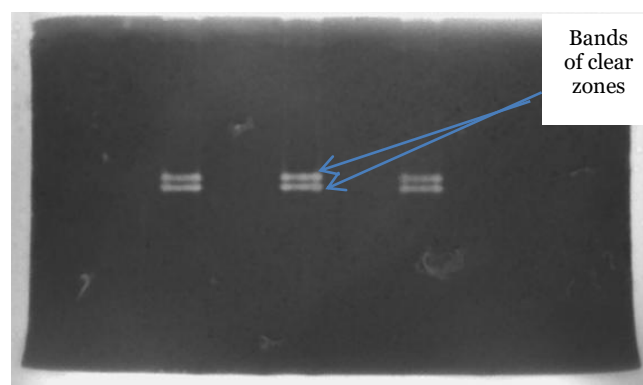


Figure 7. An image of native PAGE gel (with 0.5 % soluble starch) documented using imaging system ImageQuant 400. The intense deep blue colour was captured as black colour and the clear zone as in white.

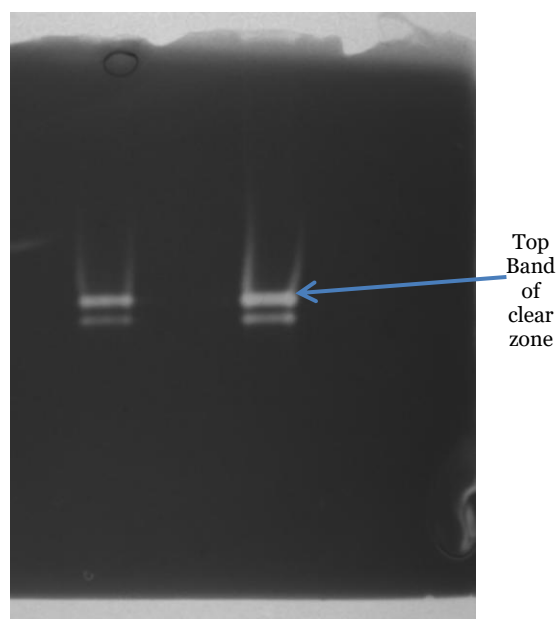


Figure 8. An image of native PAGE gel (with 0.3% amylopectin) documented using imaging system ImageQuant 400. The intense deep blue colour was captured as black colour and the clear zone as in white.

2. Zymography analysis

Following the native PAGE analysis, zymography was performed on the enzyme extract as to estimate the molecular mass of the proteins that have been localised on the native gels. This technique is commonly applied for studying hydrolytic enzymes such as amylase on the basis of substrate degradation and has been widely used for fungal fermentation extracts (Dojnov & Vujčić, 2012; Vandooren *et al.*, 2013). Zymography yields both quantitative and qualitative information; for instance, the molecular mass and the presence of different hydrolases in a sample (Vandooren *et al.*, 2013).

The areas of starch digestion were made visible by gel staining with Lugol's iodine. Based on the areas of digestion, amylolytic activities were localised at three different positions in the zymographic gel with different molecular masses (Figure 9). Two slim bands of hydrolysis areas observed to be located at around 35 kDa and 40 kDa (Figure 9) in zymographic gel, instead of one band appeared on the top area of the native gel (Figure 8). The difference observed is due to the presence of SDS in the zymographic gels whereby SDS will cause the protein samples to denature and become inactive in order to separate them by molecular mass (Bio-Rad Bulletin, 2012). Whereas in the native gels, denaturing

agent SDS and reducing agent β -mercaptoethanol were absent that allowed proteins to be separated on gel in their native conformation. These observations may suggest presence of two forms of amylases with close molecular masses.

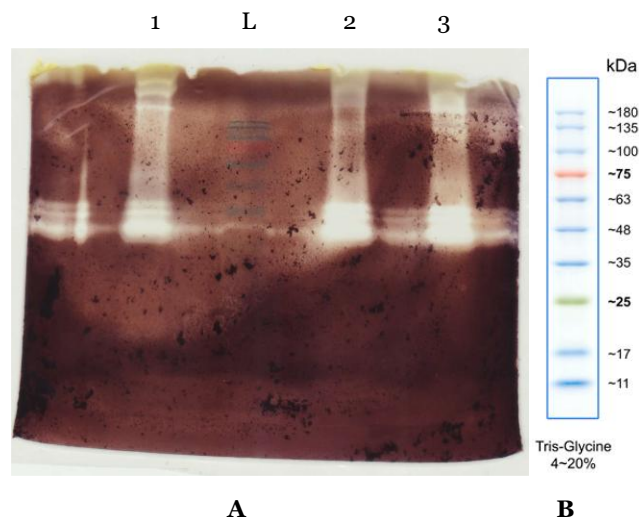


Figure 9. Zymogram analysis (with 1 % starch as the substrate). A. A scanned image of a dried zymogram gel with protein ladder. Lane L: protein ladder, Lane 1, 2 and 3: partially purified enzyme. B. BlueRAY pre-stained protein ladder (GeneDireX, Inc).

2. SDS-PAGE analysis

As to prepare for protein sequencing analysis, SDS-PAGE was performed whereby the complex tertiary structure of the enzyme can be transformed into primary structure which is suitable for sequencing. SDS-PAGE was performed as to identify protein bands that corresponded to amylolytic activity localised on the zymogram gels. Protein bands in the SDS gel (non-heated samples/without β -mercaptoethanol. Figure 10) which corresponded to the localised activity of the enzyme in zymogram gel (Figure 9) were identified and cut. The pools of cut bands were sent to 1st BASE (Malaysia) and MALDI-ToF (BGI Tech, Hong Kong) for protein sequencing.

Mass spectrometry (MS) was applied for this purpose, and it is a universal method for high throughput annotation of anonymous proteins (Strachan & Read, 2004). There are three different approaches that can be used to annotate a protein by MS, one of them is peptide mass fingerprinting (PMF) which was applied in this study. The enzyme sample protein mixture was digested with trypsin generating a collection of tryptic peptides. These peptides were then

subjected to MALDI- ToF (Strachan & Read, 2004; Moran *et al.*, 2006). The spectra of mass collected was used to identify and annotate the enzyme samples by correlating the experimentally determined masses with those predicted from databases of protein sequences.

Protein annotation by both Proteomics International PTY Ltd through 1st BASE (Malaysia) and BGI Tech revealed that the extracted enzyme suggested to contain both α -amylase and glucoamylase. Additional own search from other protein databases using information generated by both companies fore mentioned suggested that the proteins are from *A. flavus*. The suggested annotated proteins are in parallel with the results obtained from native PAGE analysis (Figure 7 and Figure 8) as well as zymography analysis (Figure 9) whereby there were more than one band area of amylolytic activity localised which indicated the presence of more than one type of amylases. Furthermore, glucoamylase which was identified to be one of amylases present in the extract fits the observation of amylolytic activity localised in the amylopectin-incorporated gel (Figure 8). Glucoamylase belongs to exoamylase group and able to hydrolyse α -1,4- and/or α -1,6 glycosidic linkages from the non-reducing ends of amylose as well as amylopectin in starch, producing only β -D-glucose (Nahar *et al.*, 2008). Whereas α -amylase is endoamylase which randomly cleaves the α -1,4 glycosidic linkages internally between adjacent glucose units in the linear amylose chain in starch and bypass α -1,6 glycosidic linkages yielding low molecular weight products such as glucose, maltose and maltotriose (Alkando & Ibrahim, 2011). The co-occurrence of α -amylase and glucoamylase generally is observed in the fungal fermentation extracts, especially, filamentous fungi such as *Aspergillus* spp. whereby glucoamylases rarely occur without α -amylase. Other amylases, such as α -glycosidase, are also likely to be produced concomitantly (Dojnov & Vujčić, 2012).

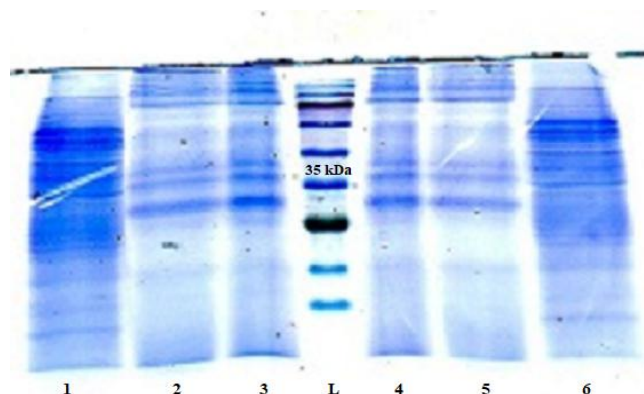


Figure 10. A scanned image of a dried SDS gel. Lane L: BlueRAY pre-stained protein ladder. Lane 1 & 6: Heated protein samples in sample buffer contained SDS and β -mercaptoethanol. Lane 2 & 5: Non-heated protein samples in sample buffer contained SDS and β -mercaptoethanol. Lane 3 & 4: Non-heated protein samples in sample buffer contained SDS without β -mercaptoethanol.

IV. CONCLUSION

The α -amylase and glucoamylase identified in the partially purified enzyme extract obtained from *A. flavus* grown on the rice husks alone are of important industrial enzymes with diverse applications. Glucoamylase is one of the most important enzymes applied in the food industries, particularly, in the production of glucose and fructose syrup from liquefied starch, due to its ability to completely convert starch into glucose. Molecular and biochemical profiles suggested a promising application of these secreted hydrolytic enzymes in a wide variety of industries and processes including detergents, pharmaceutical, food, textile, syrup and glucose, and biotechnology related processes. The findings are important, particularly, to the rice producing countries such as Malaysia, and can be used in future work for the development of local industrial importance enzymes such as amylases by utilising the under-utilised rice husks to replace the use of an expensive synthetic substrate in SSF process.

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