Effect of Nitrogen Addition on the α-Amylase Production by *Aspergillus niger*, *Rhizopus oligosporus* and *Neurospora crassa* in media contained Sargassum and Rice Seed on Solid State Fermentation
(Pengaruh Penambahan Nitrogen pada α-Amylase yang Diproduksi oleh *Aspergillus niger*, *Rhizopus oligosporus* dan *Neurospora crassa* pada Media yang Mengandung Biji Sargasum dan Beras pada Kondisi Fermentasi)

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ABSTRACT

Amylase is one of the hydrolytic enzymes which is commercially important and represent about 25–33% of the world enzyme market. Amylase production is affected by substrate for enzyme productions include particle size, initial moisture content and nutrient addition. The present study describe the effect of N-species addition on alpha amylase production by *Aspergillus niger* Km1*, Rhizopus oligosporus* Km2 and *Neurospora crassa* Km3 in medium contained sargasum and rice seed on solid state fermentation. Various ration of media composition contained dried sargassum and rice seed were studied. The effect of particle size of sargassum, initial water content on α-amylase production were evaluated. The best media composition was then augmented with N-species include sodium nitrate, yeast extract, and peptone on solid state fermentation. Best media composition was 60:40 (w/w) of sargassum and rice seed respectively, with initial moisture content was 60%. Increase 5 times of amylase activity was obtained when sodium nitrate (0,5% w/w) was added to production medium. N-species significantly affect Alpha amylase production on sargassum and rice seed with maximum alpha amylase production was 36,66 unit by *Rhizopus oligosporus.*The economic value of Sargassum can be increased through alpha amilase production.

Keywords: Amylase, Nitrogen, solid state fermentation, *Rhizopus oligosporus*, *Neurospora crassa*

ABSTRAK

Amilase adalah salah satu kelompok enzim hidrolisis yang menguasai sekitar 25-33 % dari pasar dunia. Produksi enzim amilase pada sistem fermentasi padat dipengaruhi oleh ukuran partikel, kadar air awal, dan nutrisi tambahan. Penelitian ini bertujuan mengetahui pengaruh sumber N terhadap produksi enzim α-amilase oleh *Aspergillus niger* Km1*, Rhizopus oligosporus* Km2 dan *Neurospora crassa* Km3 pada media sargassum, gabah pada sistem fermentasi fase padat. Optimasi dilakukan terhadap kadar air awal terhadap produksi enzim α-amilase. Selanjutnya dipelajari pengaruh sumber nitrogen (sodium nitrat dan ekstrak khamir) pada media dengan kadar air optimum. Komposisi media yang paling optimum adalah yang mengandung sargsasum dan gabah padi dengan perbandingan 60:40 (b/b), dengan kadar air awal 60 %. Terjadi kenaikan aktivitas α-amilase 5 kali pada penambahan sodium nitrat 0,5 % (b/b). Penambahan sumber nitrogen sangat berpengaruh terhadap produksi enzim α-amilase. Maksimum aktivitas enzim α-amilase adalah 36,66 unit yang diperoleh oleh inokulan *Rhizopus oligosporus.* Nilai ekonomi sargassum dapat ditingkatkan melalui produksi α-amilase.

Kata Kunci: Amilase, nitrogen, fermentasi fase padat, *Neurospora crassa, Rhizopus oligosporus*

INTRODUCTION

Enzyme production systems are interest of many scientists (Apar & Özbek 2005). Amylase is one of the hydrolytic enzymes that is commercially important and represent about 25–33% of the world enzyme market (Sahnoun *et al.*2012; Nater *et al.* 2007). Amylases that cleave the glycosidic linkage in starch. Two important groups of amylases are glucoamylase and α-amylase (Steyn *et al*. 1995). α-Amylases (α-1, 4-glucan-4-glucanohydrolase, EC 3.2.1.1) are widely present in growing seeds, animal tissues, and microorganisms (Xiao *et al.* 2006)). They randomly catalyze the hydrolysis of the α-(1→4) glucosidic linkages of polysaccharides, such as starch and other polysaccharides of various sizes (Apar & Özbek 2005). The most intensive studies have been carried out on fungal and bacterial amylases (Djekrif-Dakhmouche *et al.* 2006). Production of amylase using microorganism offer several

advantages which include higher enzymes production (Ugwuanyi *et al*. 2004), and easier fermentation-technology manipulation (Pothiraj *et al*. 2006).

Aspergillus and *Rhizopus* are intensively studied to produce fungal α-amylase *(*Bhavsar *et al*. 2012; Schmoll *et al*. 2010; Shiraga *et al*. 2005; Maas *et al*. 2008) . Manipulation of environmental and cultural conditions is common strategy to obtain higher production of -α-amylase (Singhania *et al*. 2010). *Aspergillus niger* ML-17 and *Rhizopus oligosporus* ML-10 produce optimal α-amylase when grown at initial pH of 5,0, and incubation temperature at 30° C, grown for 96° h (Pothiraj *et al*. 2006).

To reduce cost of amylase production, scientist proposed the use of low cost fed material and nutrient augmentation (Ugwuanyi *et al*. 2004; Bertagnolli *et al*. 2014). Though which mechanism N-supplement affects enzyme production, scientist noted N-type augmentation increase enzyme production (Anto *et al*. 2006a). Sargassum is a member of brown algae (Phaeophyceae) widely produced by local farmer in the Northern of Java, Indonesia. Due to the price of raw sargassum exported for alginate production fluctuated, utilization of this polysaccharide can offer alternative to increase their economic value. This present study aims to evaluate the effect of N-supplement on alpha amylase production on media contained sargasum and rice seed.

MATERIALS AND METHODS

Fungi were isolated from soil, oncom and tempe. To isolate the fungi from the sample, 1.0 g of sample was diluted in 9 mL sterilized water and vortex-mixed. One-tenth of a milliliter of successive decimal dilutions was spread on acidified Dichloran Rose Bengal agar chloramphenicol agar (OXOID, Cat.1076012). This selective medium was used because growth of bacteria is prevented, and spreading of molds is suppressed.

Plates were incubated for 5 days at room temperature. Strain purification was done at least twice by selecting one of each type of fungi colony and streaking twice for single colonies The plate were incubated at 27°C for 3 days. Representative colonies were picked, purified and maintained on Potato Dextrose Agar (OXOID, Cat.CM 0139).

Fungi DNA template was prepared from freshly-grown cells on the Potato Dextrose Broth and used for extracted the DNA (Butinar *et al*. 2005). PCR amplification of the partial Internal Transcribed Spacer (ITS) ribosomal subunit with primers ITS 4: 5`– TCC TCC GCT TAT TGA TAT GC – 3`and Primer ITS 5: 5`– GGA AGT AAA AGT CGT AAC AAG G –3`(White *et al.* 1990; O`Donnell 1993) using GoTaq master mix (Promega, M7122) . PCR products were visualized on 2% agarose and sequenced with both primers using Big Dye terminator v3.1. Cycle Sequencing Ready Reaction Kit (Applied Biosystems) following the manufacturer's instructions. The partial 26S sequences determined in this study were compared to those in the EMBL/ GenBank/DDBJ databases using the nucleotide Basic Local Alignment Search Tool (BLASTn) (Altschul *et al*. 1997).

Fungi screening for α-amylase production was conducted following the protocol described by (Saleem & Ebrahim 2014). Fungi were cultured on solid starch yeast extract agar medium (soluble starch, 5.0 g; yeast extract, 2.0 g; KH_2PO4, 1.0 g; $MgSO_4.7H2O$, 0.5 g and agar, 15 g). Fungal isolates were tested for amylase production by starch hydrolysis. Starch agar medium (peptone, 0.5 g; beef extract, 0.15 g; yeast extract, 0.15 g; NaCl, 0.5 g; starch, 1 g; agar, 2 g; distilled water, 100 ml) was inoculated with fungi and incubated at 30° C, then flooded with iodine solution (iodine, 0.2 ml; potassium iodide, 0.4 ml; distilled water, 100 ml). A clear zone around fungal growth indicated the production of amylase (Naidu & Saranraj 2013). On this basis, *Aspergillus niger*, *Rhizopus oryzae*, and *Neurospora crassa* were selected for further studies on amylase production.

The culture of *Aspergillus niger*, *Rhizopus oryzae*, and *Neurospora crassa* were grown and maintained on potato dextrose- agar (PDA) slants. The slants were stored at 4°C and sub-cultured fortnightly. Five-day-old fully sporulated slant was used for inoculant preparation. For this, 10 ml sterile distilled water containing 0.1% Tween-80 was added to the slant and spores were scraped with a sterile needle. The inoculant obtained contained 4.7 x 10^7 spores per ml.

Sargassum spinosum (SS) and rice seed (RS) obtained from local company were used as substrates for the α-amylase production. Five grams of the dried substrate taken in a cotton plugged 250 ml Erlenmeyer flask were supplemented with 6.0 ml of salt solution containing (%) NH_4NO_3 0.5, $MgSO_4.7H2O$ 0.1 and NaCl 0.1. Media for α-amylase

production were contained percentage a mixture of SS/RS as the following: 100/0; 90/10; 80/20; 70/30; 60/40; 50/50 and 60/40.

To study the effect of particle size of *Sargassum spinosum* (SS) on α-amylase production, the particle size was adjusted using blender and passed through siever with a size of 10, 25 and 40 mesh. The *Sargassum spinosum* (SS) was then autoclaved.

To estimate the effect of initial moisture on α-amylase production, the moisture was adjusted to the required level by adding distilled water. Substrates were sterilized at 121°C and 15 psi for 15 min, cooled and inoculated with 1.0 ml spore suspension (4.8 x 10^7 spores per ml) of fungal strain. The flasks were incubated at 30°C for 96 h unless otherwise mentioned. All experiments were carried out in 2 replicates.

To determine the effect of temperature the solid state fermentation was conducted at 25°, 30° and 35° C. The α-amylase activities was then measured after 5 days incubation.

To determine the effect of nitrogen augmentation, to fermentation medium 0.5 % (w/w) of either sodium nitrate, yeast extract, urea and peptone were added. The α-amylase activities was then measured after 5 days incubation.

Enzyme extraction was carried out using distilled water with 0.1% Tween-80. Known quantities of fermented substrates were mixed thoroughly with the required volume of distilled water (so that the final extraction volume was 100 ml) by keeping the flasks on a rotary shaker at 180 rpm for one hour. The suspension was centrifuged at 8000 g for 20 min and the clear supernatant obtained was assayed for α-amylase activity which determined follows protocol of (Sahnoun *et al*. 2012).

α-amylase activity was assayed by measuring the amount of inorganic phosphorus released from sodium phytate solution using the method of Harland & Harland (1980). One unit of enzyme activity was defined as the amount of α-amylase required to release one micromole of inorganic phosphorus per minute under the assay conditions.

Soluble protein content of the crude samples was determined spectrophotometrically according to the method described by Lowry *et al*. (1951) using bovine serum albumin as standard.

Fungal biomass estimation was carried out by determining the N-acetyl glucosamine released by the acid hydrolysis of chitin present in the cell wall of the fungi (Sakurai *et al*. 1977). For this, 0.5 g (dry wt) of fermented matter was mixed with concentrated sulphuric acid (2 ml) and the reaction mixture was kept for 24 h at room temperature (30°C). This mixture was diluted with distilled water to make a 1 N solution, autoclaved for 1 h, neutralized with 1 N NaOH and the final volume was made up to 100 ml with distilled water. The solution (1 ml) was mixed with 1 ml acetyl acetone reagent and incubated in a boiling water bath for 20 min. After cooling, ethanol (6 ml) was added followed by the addition of 1 ml Ehrlich reagent and the resulting mixture was incubated at 65°C for 10 min. Once cooled the optical density of the reaction mixture was read at 530 nm against a reagent blank. Glucosamine (Sigma) was used as the standard. The results obtained are expressed as mg glucosamine per gram dry substrate (gds).

RESULTS

Amylolytic fungi

Our present study evaluate 12 isolates of fungi and yeast which include Ascomyecetous fungi (*Rhizopus oligosporus*, *Aspergillus niger*, *Neurospora crassa*, and *Trichoderma*), and Ascomyceteous yeast (*Saccharomyces* sp) and *Imperfect* yeast (*Candida* sp). Among other *Aspergillus* was the prominent amylolytic fungi (Figure 1 and Table 1).

The effect of particle size on α-amylase production by fungi is presented in Figure 2. All fungi evaluated respond similarly. Best particle size was 25 mesh. Lower or higher particle size than 25 mesh for sargassum appear not good for α-amylase production.

Table 1. Amylolytic properties of fungi and yeast evaluated using starch medium grown at 30° C, for 2 days

Species	Halo zone (cm)	Ratio
Aspergillus niger	5.2	2.6
Rhizopus oryzae,	4.4	2.2
Neurospora crassa	4.2	2.1
Saccharomyces sp	2,0	1.0
Candida sp.	3.8	1.8
Mucor sp.	3.2	1.6
Rhizopus sp.	3.2	1.6
Trichoderma sp.	2.4	1.2
Trichoderma sp.	2.1	1.1
Trichoderma sp.	2.1	1.0
Trichoderma sp.	2.0	1.0

Substrate composition

Solid state fermentation offered several advantages over submerge fermentation for enzyme production and other secondary metabolites. The substrate composition influences the enzyme production (Chen & He 2012). Polysaccharide contained substrate such as sargassum can be used as substrate. Nutrient addition was to complement necessary nutrient for cell growth and activities (Apar & Özbek 2005). The effect of nutrient composition on α-Amylase production can be shown in Figure 3 which is shown that substrate composition contained sargassum and rice seed at ration of 60:40 w/w is the best substrate composition for α-Amylase production.

Initial moisture content

Initial moisture content has various effect on alpha amylase production (Figure 4). Best biomass growth and enzyme production was obtained by 60% moisture content. Increasing moisture affect negatively on biomass growth and enzyme production.

Effect of temperature

Figure 3 showed the effect of incubation temperature (25-35ºC) on the production of α-amylase by *Aspergillus niger*, *R. oligosporus* and *Neurospora crassa* using SSF. Maximal enzyme production (6.87 U ±0.02 IU) was obtained in SSF incubated at 30º C after the conidial inoculation of *R. oryzae*. Whereas *N. crassa* produced higher alpha amylase when was grown in 35ºC than 25ºC. Alpha amylase production by fungi is related to the growth which sequentially depends upon the incubation temperature.

Effect of nitrogen sources

The effect of nitrogen sources is shown in Figure 6. Sodium nitrate stimulated the α-amylase production by more than 5 times for *R. oligosporus* followed by *Aspergillus niger*. In the case of *N. crassa* enzyme production was slightly increased by addition of nitrogen sources. Yeast extract, urea and

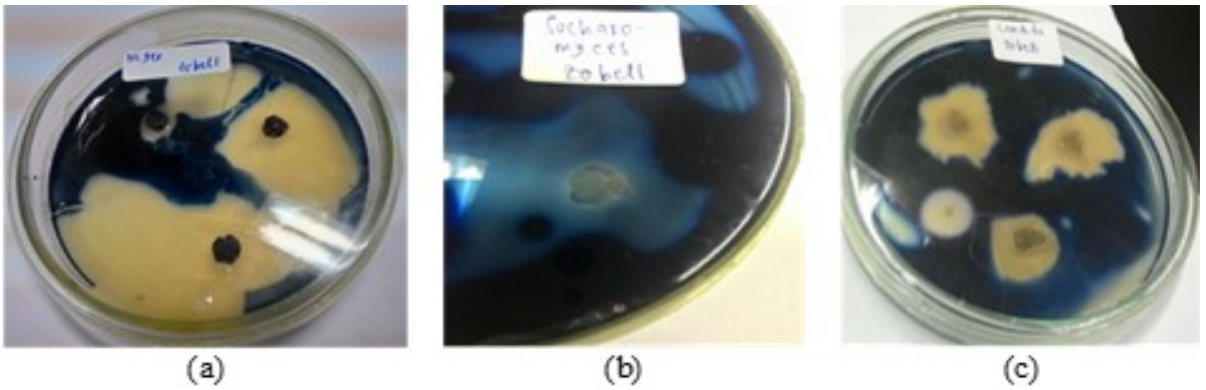

Figure 1. Amilolytic fungi *Aspergillus niger* (a), *Saccharomyces* sp, and *Candida* sp. grown on Zobell E

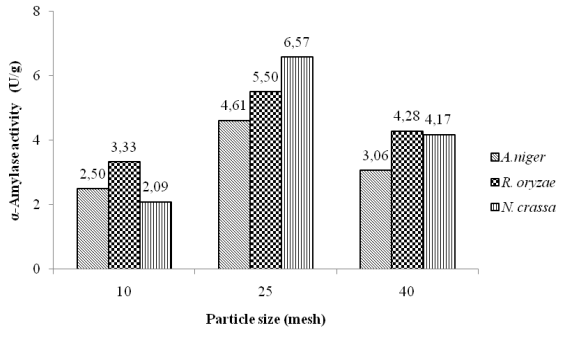

Figure 2. The effect of particle size on the α-Amylase activities, which was measured after 5 days incubation at 30°C

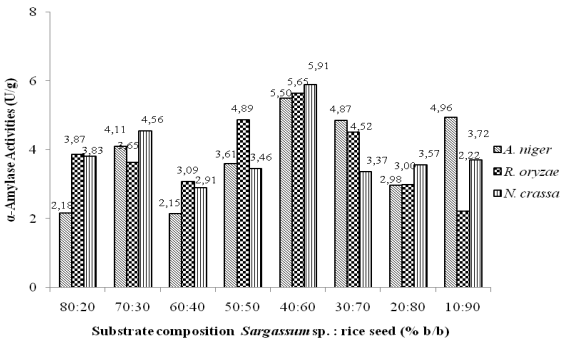

Figure 3. The effect of substrate composition on α-Amylase activities, which was determined after 5 days incubation at 30°C

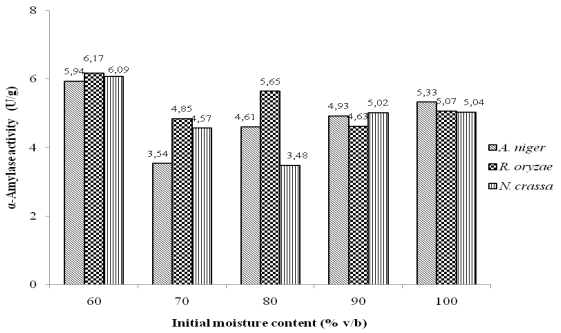

Figure 4. Effect of initial of moisture content on α-Amylase activity

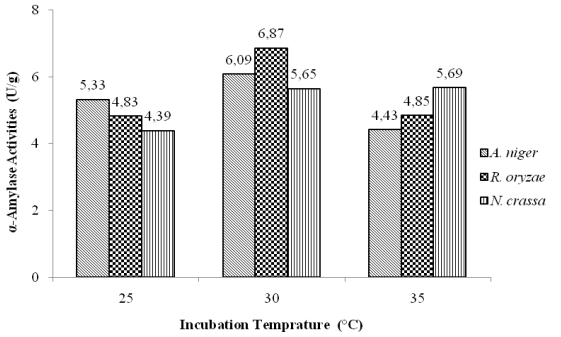

Figure 5. Effect of incubation temperature on α-Amylase activity

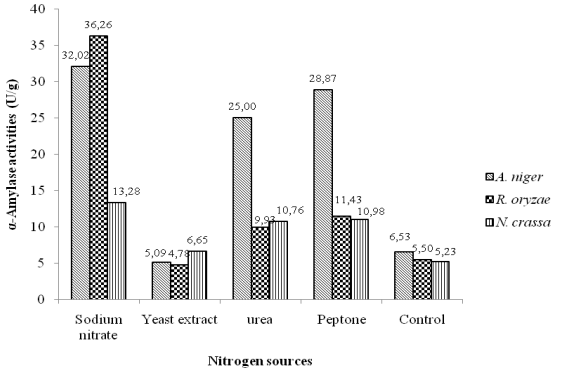

Figure 6. Effect of nitrogen sources (0.5 % w/w) on α-Amylase activity

peptone behave differently. Urea was good as nitrogen sources for *Aspergillus niger*, but not for *Neurospora crassa*. While yeast extract appear not to have effect on α-amylase production (Figure 6).

DISCUSSION

Three isolates (*Aspergillus niger, Rhizopus oligosporus* and *Neurospora crassa*) formed high clear zone which implies that the isolates produce amylase. *Aspergillus niger* as α-amylase producer was reported by (Pothiraj *et al*. 2006). Not many scientist observed high production of α-amylase by *Neurospora crassa*, but our present study shows that *Rhizopus oligosporus* and *Neurospora crassa* are good α-amylase producer.

The reaction rate on solid state fermentation is greatly affected by substrate homogeneity, water retention ability and volumetric heat capacity and thermal conductivity (Chen & He 2012). These parameters are associated with particle size. Particle size clearly affect α amylase production by fungi (Fig. 2), best particle size was 25 mesh. The particle size and the specific area of the substrate is of importance in SSF (Singhania *et al*. 2010). Usually better mycelia growth is obtained when fungi grown on smaller size (Fukuda *et al*. 2008), but with smaller particle, the surface area for growth was greater but the inter-particle porosity was less. With the larger size, the porosity was greater but the saturated surface area was less. These two opposing factors probably interacted to give the value corresponding to optimum growth and product formation (Djekrif-Dakhmouche *et al*. 2006). The influence of substrate particle size, which determines the accessible surface area to the micro-organisms on product formation.

Temperature for SSF is other main factor affect α-amylase production. Best temperature for alpha amylase production that *Aspergillus niger*, *Rhizopus oligosporus* and *Neurospora crassa* for was 30°C. When the temperature lower or higher the growth of mycelia was inhibited and this was concomitant with the α-amylase production, which implies that enzyme production is growth associated (Djekrif-Dakhmouche *et al*. 2006). Many other researchers have also reported 30°C as optimum temperature for the fungal growth and enzyme production. This is because the enzyme production is growth associated and 30°C is optimum temperature for fungi and subsequently α-amylase production (Shafique *et al*. 2009, Dakhmouche *et al*. 2006).

Initial moisture content is important environmental factor that influence the growth and enzymes production by fungi (Anto *et al*. 2006 b). Initial moisture content affected α-amylase production (Figure. 4). Mostly better growth of fungi was observed in moist substrate but when substrate was

too wet than the surface area for growth through inter -particle porosity was less, which further determines the accessible surface area for mycelia growth and product formation. Hence it is importance to optimize particle size, surface accessibility and water content.

Initial moisture content not only important for producing α-amylase, but also for the activity of cellulase for increasing lipid accumulation by *Microspha-eropsis* sp (Yen & Zhang 2011; Peng & Chen 2008). They found 75% initial moisture content is optimal.

Nitrogen sources affected α-amylase production (Figure 6). Sodium nitrate was the nitrogen sources for α-amylase production (Fig 6). Variability effect of N-source on α amylase production has been noted by many scientist, and each fungal species respond differently to nitrogen sources. Not only carbon sources, nitrogen supplement also affected α-amylase production by microorganism (Ugwuanyi *et al*. 2004). In the case of *Bacillus stearothermophilus* additional C-sources increase amylase production in order starch > dextrin > glycogen > cellobiose > malto-hexaose-malto-peptaose > malto-tetraose and malto-triose. Monosaccharides however repressed the enzyme production, whereas inositol and D-sorbitol increased amylase production.

α-Amylase production also respond positively to organic and inorganic salts addition in the order of KCI > sodium malate > potassium succinate. Amino acids such as isoleucine, cysteine, phenylalanine, and aspartic acids, were crucial for amylase synthesis. Medium containing $CaCl_2$ $2H_2O$ enhanced amylase production over that on Ca-deficient medium. The detergents Tween-80 and Triton X-100 increased biomass but significantly suppressed amylase synthesis (Thippeswamy *et al*. 2006).

Nguyen *et al*. (2000) proposed selection of carbon and nitrogen sources is crucial for α-amylase production. They found *Thermomyces lanuginosus* grown on starch, malto-dextrin, dextrin, maltose, amylopectin, glucose and dextran with asparagine as nitrogen sources, pH adjusted to 4.9 produce good α-amylase (92–125U/mL) and gluco-amylase (6–13 U/mL) activities.

Production of α-amylase under solid-state fermentation by *Bacillus cereus* MTCC 1305 showed different behavior. Using wheat bran and rice flake manufacturing waste as substrates. This bacteria produced α-amylase about (94 2) U/g). Addition of glucose (0.04 g/g) enhanced enzyme production (122 5) U/g). Supplementation of different nitrogen sources (0.02 g/g) showed decline in enzyme production (Anto *et al*. 2006b).

Our present study showed that *Aspergillus niger*, *Rhizopus oligosporus* and *Neurospora crassa* are important fungi for alpha amylase production, and optimizing particle size, moisture content, nitrogen sources are crucial to enhance alpha amylase production. Media contained sargassum and rice seed at ratio of 60:40 was the best for alpha amylase production.

CONCLUSION

Aspergillus niger Km1, *Rhizopus oligosporus* Km2 and *Neurospora crassa* Km3 were good α-amylase producer on solid state fermentation using sarrgassum and rice seed as fermentation media. Higher α-amylase production obtained through addition of sodium nitrate and particle size adjustment. Production of α-amylase using sargassum as fermentation media can be alternative to increase its economic value.

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