Production of Phytase, Amylase and Cellulase by *Aspergillus niger*, *Neurospora crassa,* and *Rhizophus oryzae* on Sargassum and Rice Bran Under Solid State Fermentation

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Abstract

The objective of study was to produce amylase, cellulase and phytase on sargassum and rice bran on solid state fermentation using *Aspergillus niger, Neurospora crassa and Rhizophus oryzae*. Media for solid state fermentations composed of dried sargassum and rice bran. The effect of particle of sargassum, initial moisture content on phytase, amilase, and cellulase were evaluated. Optimum enzyme activity of phytase, amylase and cellulase were obtained after 4 days fermentation at 30°C, and initial moisture content was adjusted to 60%. The optimum particle size of dried sargassum attaining the highest enzyme activity was 25 mesh. Best formula for enzymes production was at the ratio of 4:6 (w/w) of *Sargassum spinosum* (SS) and rice bran (RB) respectively. At this formula highest phytase activity was obtained by *Aspergillus niger*, cellulase by *Rhizopus oryzae*, and amylase by *Neurospora crassa*. Media composed of sargassum and rice bran can be used for phytase, amylase and cellulase production.

Keywords: amylase, celluase, phytase, rice bran and sargassum

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Introduction

The introduction of enzyme in cattle feed is one alternative to increase feeding efficiency. Enzyme production cost however is still high due to many factors (Singh *et al*., 2013). Sargassum is renewable resources produced in coastal area and recently is being cultivated by farmer. However the price of sargassum is still low and unstable. It is therefore necessary to explore the utilization of this renewable resource for other economically valuable product i.e. hydrolytic enzyme production. Sargassum has been used for cattle feed (Rey-Crespo *et al*., 2014; García-Casal *et al*., 2009) and source for mineral. Sargassum contained carbohydrate, protein, lipid and plant growth hormone (Williams & Feagin, 2010) which not only good for poultry feed but also suitable as medium for enzyme production. Hydrolytic-enzyme supplement in ruminal and monogastric animal feed is to increase nutrient availability and hence reduce production cost. Sargassum and seaweed are potential resources for cattle feed. The rare breed of primitive sheep on North Ronaldsay, Orkney (Scotland) survives under extreme conditions on the beach shore of North Ronaldsay with seaweed as virtually their sole feed source, mainly brown kelps (*Laminaria digitata* and *Laminaria hyperborea*) (Hansen *et al*., 2003). They found dry matter degradation (DMD, 71.7%, at 48 h) and organic matter digestibility (OMD, 79.6 %).

Fermentation technology has been proposed to improve the contents of protein and polysaccharide in seaweed waste, and proved to be an available method to improve the nutritional value of animal feed produced from seaweed waste. Additives were necessary to increase nutrient value of fermented sea weed.

Seaweed solid wastes obtained after the extraction of κ-carrageenan was used for bioethanol production through separate hydrolysis (SHF) and fermentation process and simultaneous saccharification and fermentation process (SSF). For the SHF process, enzymatic hydrolysis was conducted by varying three process variables, substrate

concentration, pH and temperature, but a constant enzyme dosage was maintained. The highest glucose yield of 99.8 % was obtained at pH 4.8, at temperature of 50 °C and a substrate concentration of 2 % (w/v) seaweed solid wastes. With subsequent fermentation, a bioethanol yield of 55.9 % was obtained. In contrast, for the SSF process, a yield of 90.9 % bioethanol was obtained. From these results, it was determined that the SSF of seaweed solid wastes with *Saccharomyces cerevisiae* has several advantages over SHF because the former is a simple one-step procedure that can save time, cost and energy consumption while achieving a high yield of bioethanol (Tan & Lee, 2014).

Reducing sugar produced through hydrolyses of seaweed waste is due to the activity of several hydrolytic enzyme include amylase and cellulose (Zhang *et al*., 2004). Phytase is necessary to increase phosphorous availability in animal feed. Therefore additional of additive substrate is necessary to produce phytase using sargassum as the main carbon sources. Solid state fermentation has been successfully introduced for efficient enzyme production system (Roopesh *et al*., 2006). Many factors however should be optimized to produce hydrolytic enzymes in solid state fermentation (Pandey *et al*., 2000). The objective of this study was to evaluate hydrolytic enzymes (cellulase, amylase and phytase) production using sarggasum and rice bran in solid state fermentation system.

Materials and Methods

Inoculum Preparation for SSF. 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. 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 4.7×10^7 spores per mL.

Substrates preparation for SSF. *Sargassum spinosum* (SS) and rice bran (RB) obtained from local company were used as substrates for the phytase 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, $MgSO_4.7H_2O$ 0.1, and NaCl. Media for phytase production were contained percentage a mixture of SS/RB as the following: 100/0; 90/10; 80/20; 70/30; 60/40; 50/50 and 60/40.

Particle size. To study the effect of particle size of *S. spinosum* (SS) on phytase production, the particle size was adjusted using blender and passed through siever with a size of 10, 25 and 40 mesh. The *S. spinosum* (SS) was then autoclaved ad used as substrates for the phytase production.

Moisture optimation. To estimate the effect of initial moisture on phytase 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 (4.8×10^7 spores per mL) of fungal strain. The flasks were incubated at 30 °C for 96 hours. All experiments were carried out in 2 replicates.

The effect of nitrogen supplement. Four of additional nitrogen sources were selected to study the effect of additional nitrogen sources on phytase, amylase and cellulase production. Additional nitrogen sources evaluated were peptone, yeast extract sodium nitrate and urea at the concentration of 0.5 %.

Enzyme extraction. 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 8,000 g for 20 min and the clear supernatant obtained was assayed for phytase activity.

Phytase assay. Phytase activity was assayed by measuring the amount of inorganic phosphorus released from sodium phytate solution using the method of Harland and Harland (1980). One unit of enzyme activity was defined as the amount of phytase required to release one micromole of inorganic

phosphorus per minute under the assay conditions.

Amylase assay. Amylase activity was determined following the methods described by Raul *et al*. (2014) by determining the amount of reducing sugar produced (µMol) per hour by 1 mL enzyme.

Cellulase assay. Cellulase activity was determined following the methods previously described by Ferrari *et al*. (2014**)** by determining the amount of reducing sugar produced (µMol) per hour by 1 mL enzymes using carboxymethyl cellulose as substrate for enzymes.

Results and Disscussion

The effect of particle size.

Particle size affect production of phytase, amylase and cellulase. The optimum particle size for all enzyme production was 25 mesh (Figure 1-3). Each fungi produced their specific enzyme. *Aspergillus niger* was better for phytase production (Figure 1), *Rhizophus oryzae* was better for cellulase (Figure 2), and *Neurospora crassa* was good for amylase production (Figure 3). Maximum production of phytase by *A. niger* was 5.1 unit/g.ds, while maximum amylase production by *N. crassa* was 3.8 unit/g.ds. Production of cellulase by *R. oryzae* was 4.2 unit/g.ds)

Particle size greatly affect the enzyme production on solid state fermentation (Bhargav *et al*., 2008). Particle size affect aeration, substrate diffusion, humidity, and mycelial growth (Lakshmi *et al*., 2009). We observed slower mycelia growth on smaller particle size, and this similar to previous observation on the growth of *A. niger* for production of glucoamylase using corn as substrate (Pandey, 1991). It is therefore importance to optimize the particle size before solid state fermentation especially when using dry substrate.

Production of cellulase by *A. niger*, *N. crassa* and *R. oryzae* also affected by particle size. Best particle size was 25 mesh. The inoculant used was heterothophic aerobic fungi, which their growth and hydrolytic enzyme production were affected by aeration methods and substrate diffusion profile (Kumar *et al*., 2003).

Many fungi produce amylase under solid state fermentation (Sivaramakrishnan *et al*., 2007; Kunamneni *et al*., 2005). Solid state fermentation for enzymes production is more effective then submerge fermentation (Yu *et al*., 2008).

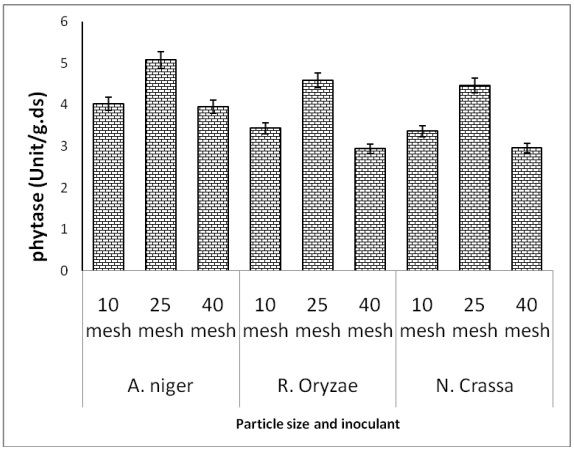

Figure 1.Comparison of phytase production base on the effect inoculant type and particle size of substrate. The enzyme activity was determined at 4 days incubation period, the SSF was at 30 ºC, and the initial moisture content was 60 %.

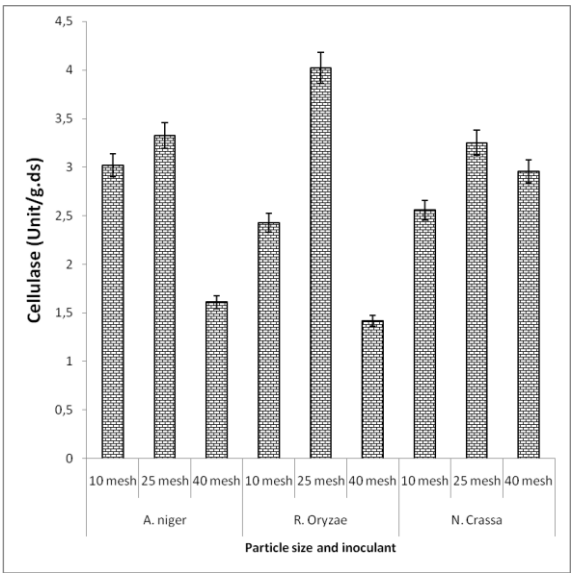

Figure 2. Comparison of cellulase production base on the effect inoculant type and particle size of substrate. The enzyme activity was determined at 4 days incubation period, the SSF was at 30 ºC, and the initial moisture content was 60 %.

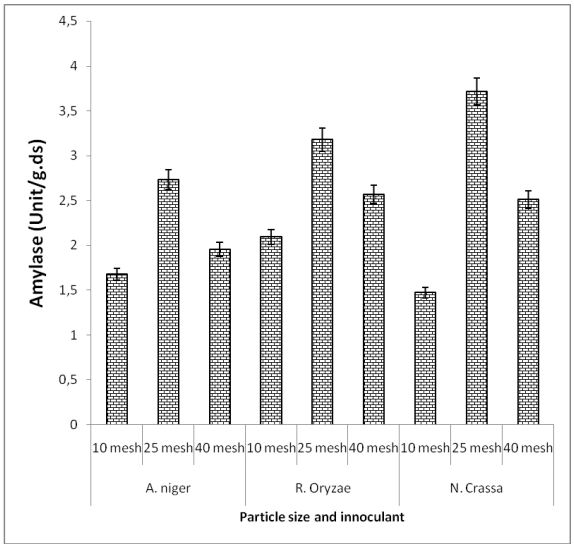

Figure 3. Comparison of amylase production base on the effect inoculant type and particle size of substrate. The enzyme activity was determined at 4 days incubation period, the SSF was at 30ºC, and the initial moisture content was 60 %.

The effect of substrate composition

Media composition and inoculant type affect hydrolytic enzyme production. Best formula media for enzymes production was Sargasum (SS):Rice bran (RB) (4:6) for all isolated tested. Best phytase producer was *R. oryzae*. This isolate produce about 30 % phytase higher than other isolates (Figure 4). *R. oryzae* is well known fungi has been used widely in traditional fermented food such as Tempe. It is quite reasonable that this species has been used in tempeh fermentation for centuries. Higher rice bran portion result in higher phytase production. Rice bran has been effectively used for phytase production (Wang *et al*., 2008 ; Chang *et al*., 2008).

N. crassa produced slightly higher amylase than the other isolates (Figure 5), but all isolates produce almost similar quantity of amylase. *N. crassa* has been effectively produce amylase using, coffee cherry husk, silver skin, spent coffee and mixtures of these coffee wastes (Murthy *et al*., 2009).

The sequence for cellulase production was *A. niger*, *N. crassa*, and *R. oryzae.* At the same formula *A. niger* produce cellulase 20 % higher than other isolates. While *N. crassa* produce slightly higher cellulase than *R. oryzae.*

A. niger is powerful fungi for cellulase production on various substrate which include corn stover (Ghori *et al*., 2011), *Jatropha curcas* seed cake (Ncube *et al*., 2012), saw dust (Acharya *et al*., 2008), sugar cane bagasse (Cunha *et al.*, 2012), ground nut and soyabean mill (Sathyavrathan & Krithika, 2013). Hence this fungi has been intensively studied for many of industrial product (Gamarra *et al*., 2010).

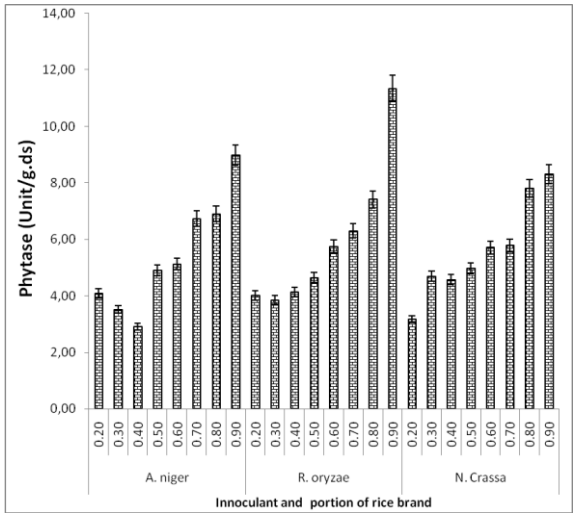

Figure 4. Comparison of phytase production as affected by media composition and inoculant type. The enzyme activity was determined at 4 days incubation period, the SSF was at 30 ºC.

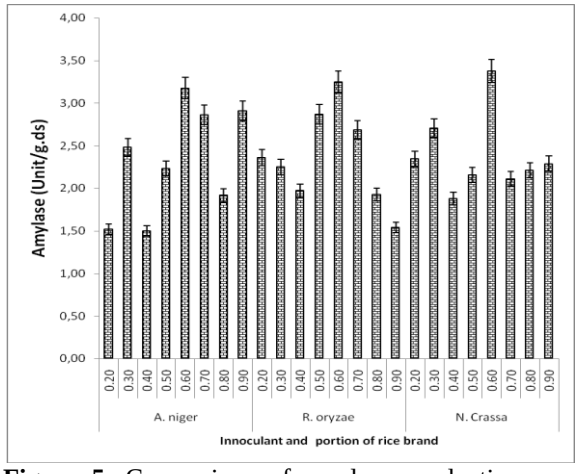

Figure 5. Comparison of amylase production as affected by media composition and inoculant type. The enzyme activity was determined at 4 days incubation period, the SSF was at 30 ºC.

The effect of moisture content

Initial moisture content affects enzyme production. Maximum enzyme production was achieved at 60 %. Substrate moisture is one of the important abiotic factor that affect growth of fungi in various media for enzyme production (Latifian *et al*., 2007). Maximum cellulase activities by *Trichoderma reesei* QM9414 and *T. reesei* MCG77 in solid-state fermentation using rice bran as substrate

obtained at 70 % humidity. Not only initial moisture content but also temperature are important factor that affect maximum enzyme activity on *T. reesei* MCG77 (Latifian *et al*., 2007). In the case of *A. niger*, temperature and moisture are the most important factor that affect the myclial growth. The increase of water content to more than 55 % at the temperatures 35 and 40 °C decreases microorganism growth (Hamidi-Esfahani *et al*., 2004). Therefore adjusting the initial moisture and water content are crucial to obtain highest enzyme activity.

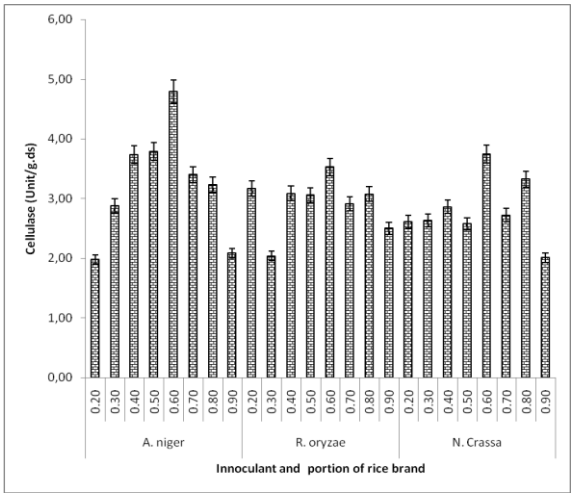

Figure 6. Comparison of cellulase production as affected by media composition and inoculant type. The enzyme activity was determined at 4 days incubation period, the SSF was performed at 30 ºC.

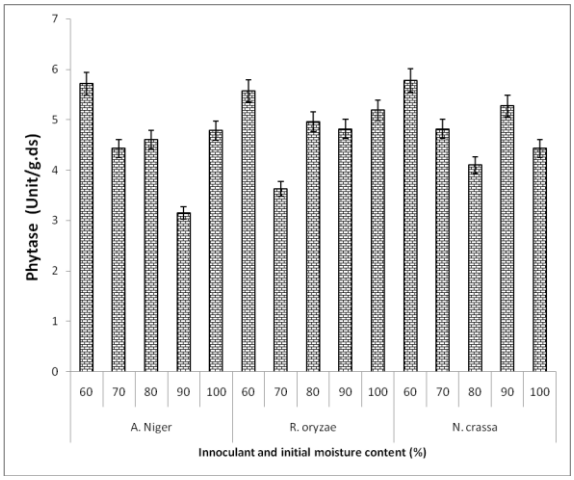

Figure 7. Comparison of phytase production as affected by initial moisture content and inoculant type. The enzyme activity was determined at 4 days incubation period, the SSF was at 30 ºC.

Slightly higher moisture content (64 %) was needed for production of phytase by *Aspergillus ficuum* NRRL 3135 on canola meal in solid state fermentation (Ebune *et al*., 1995). They also observed that phytase production increased with an increase in inoculum age between 2 and 5 days.

Our finding is similar to that of El-Batal and Karem (2001), who obtained similar phytase activity at moisture content of 60% on rapeseed meal by *A. niger* A-98. They observed not only moisture content but also addition of surfactant Tween 20, Tween 40, Tween 60, Tween 80 as well as oliec acid increased phytase production, but Triton X-100 inhibit phytase activity (El-Batal & Karem, 2001) (Figure 7).

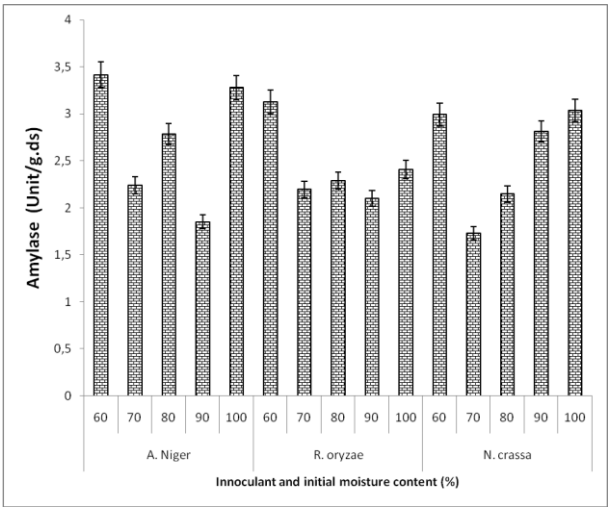

Figure 8. Comparison of amylase production as affected by initial moisture content and inoculant type. The enzyme activity was determined at 4 days incubation period, the SSF was at 30ºC.

Production of amylase by fungi was also affected by initial moisture content (Figure 8). Not only sargassum and rice bran, other substrate such as oil cakes such as coconut oil cake sesame oil cake , groundnut oil cake, palm kernel cake and olive oil cake can be used to produce amylase in solid state fermentation (Ramachandran *et al*., 2004). They carried out for the production of α-amylase using *Aspergillus oryzae* and they found maximum amylase production was obtained at moisture content of 64 % moisture. They also found, except Mn, all other metal ions such as Ca, K, Na, Mg were inhibitory for the enzyme activity.

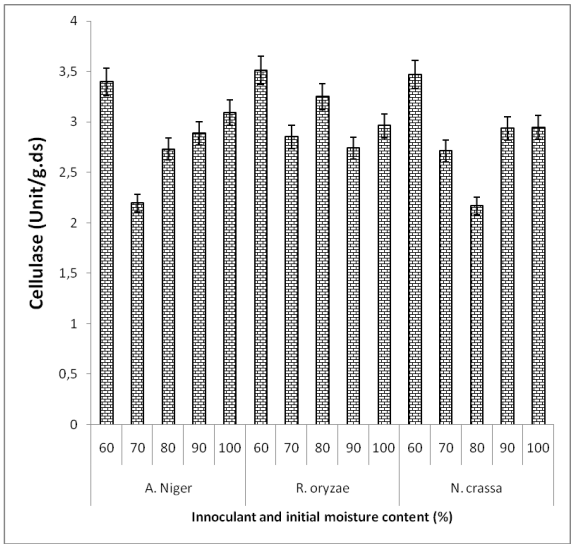

Figure 9. Comparison of cellulase production as affected by initial moisture content and inoculant type. The enzyme activity was determined at 4 days incubation period, the SSF was at 30 ºC.

Cellulase production was also affected by moisture content (Figure 9). We found 60 % initial moisture content was optimal for cellulase production. Higher moisture content (70 %) was needed to produce highest cellulase by *Trichoderma* sp using apple pomace as substrate (Sun *et al*., 2010). They found not only moisture content but also incubation temperature and inoculum size influenced the cellulase production. Lactose and corn-steep solid supplement to the apple pomace increase enzyme production.

The effect of temperature.

Temperature affected enzyme production. Maximum enzyme production was achieved at 30 ºC (Figure 10). Temperature affect biomass growth and enzymes activities in solid state fermentation is well documented (Bellon-Maurel *et al*., 2003). We obtained better biomass growth enzymes activities at 30 °C. When temperature increased the enzymes activities was lower (Figure 10). Higher temperature than 40 °C for phytase production in solid state fermentation is also possible (Bhargav *et al*., 2008). The most important is how to get good biomass growth and produce higher hydrolytic enzymes (Hamidi-Esfahani *et al*., 2004).

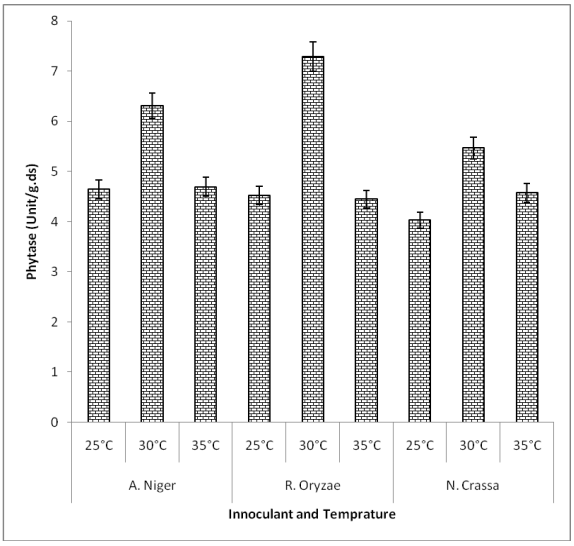

Figure 10. Comparison of Phytase production as affected by incubation temperature content and inoculant type. The enzyme activity was determined at 4 days incubation period, the SSF was at 25 to 35 ºC, with initial moisture content of 60 %.

Higher amylase production was also observed at 30°C by all cultures (Figure 11). Depend on isolate for fermentation, amylase production is optimal at 30 to 40 °C (Francis *et al*., 2002; Anto *et al*., 2006 ; Das *et al*., 2011).

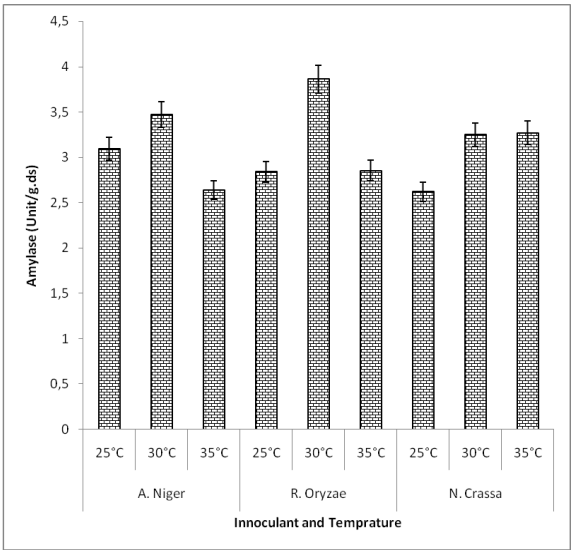

Figure 11. Comparison of amylase production as affected by incubation temperature and inoculant type. The enzyme activity was determined at 4 days incubation period, the SSF was at 25 to 35 ºC, with initial moisture content of 60 %.

Cellulase production was also affected by temperature (Figure 12). Optimum temperature for cellulase production depend on isolate and culture condition (Mekala *et al*., 2008). For instance, high cellulase production

by *Trichoderma reesei* RUT C30 was obtained at 33 °C. While *Aspergillus niger* produced highest celluase *on Jatropha curcas* seed-cake at 40 °C (Ncube *et al*., 2012).

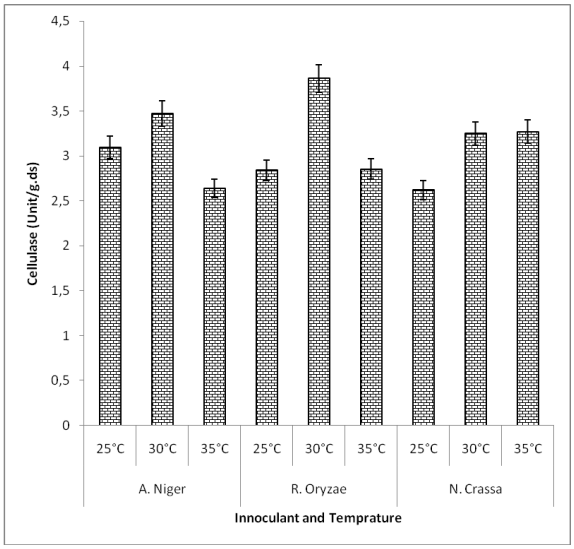

Figure 12. Comparison of cellulase production as affected by incubation temperature content and inoculant type. The enzyme activity was determined at 4 days incubation period, the SSF was at 25 to 35 ºC, with initial moisture content of 60 %.

Effect of nitrogen sources

Nitrogen sources affect enzyme production. Sodium nitrate was the best nitrogen source for amylase, phytase, and cellulase production. However the effect of other N-sources yeast extract, urea and peptone was quite variable. Nitrogen supplement affect phytase production. For instance, addition of ammonium sulphate together with casein, and glucose increased Phytase production (>85%) by *Mucor* and eight *Rhizopus* strains on canola meal, coconut oil cake, wheat brand in solid state fermentation. They use the crude enzymes directly in animal feed rations with enhanced cost efficiency (Bogar *et al*., 2003).

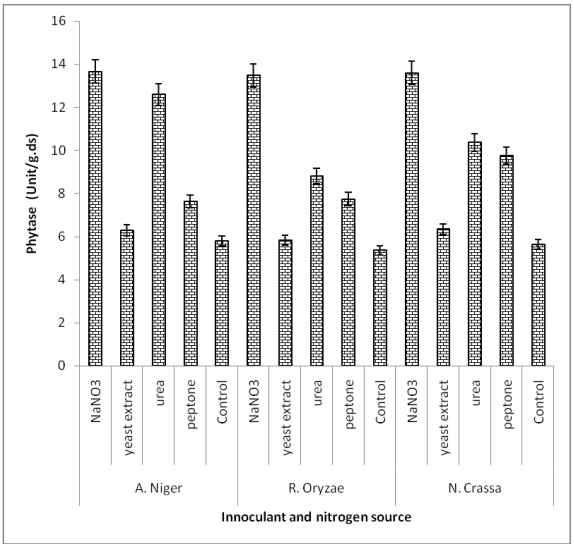

Figure 13. Comparison of phytase production as affected by nitrogen sources and inoculant type. The enzyme activity was determined at 4 days incubation period, the SSF was at 30ºC, with initial moisture content of 60%.

In the case of amylase production, *A. niger* was the best isolate for amylase production. We observed sodium nitrate supplement was good for *A. niger* and *R. oryzae* (Figure 14). *A. niger* produce higher α-amylase on mixed substrate of wheat brand and potato peel + banana when nitrogen source and the Carbon source were supplemented to the media (Shailima Vardhini *et al*., 2013). In case of *R. oligosporus*-ML-10, increased of amylase production by 70 % obtained when NH_4NO_3 (0.25 %) and yeast extract (0.25 %) supplement together with Tween soluble starch (0.001 %) and asparginine (0.0001 %) added to enzyme production medium (Gautam *et al*., 2013). High amylase activity was obtained using sodium nitrate as N-sources by *A. oryzae* in solid-state fermentation using 14 agro-industrial wastes as substrate. Enzyme production was growth associated and maximum titers (15,095 U/gds) were obtained after 72 hours when incubated at 30°C on wheat bran with initial moisture content, of 60 %; initial medium pH 5.0 (Sivaramakrishnan *et al*., 2007).

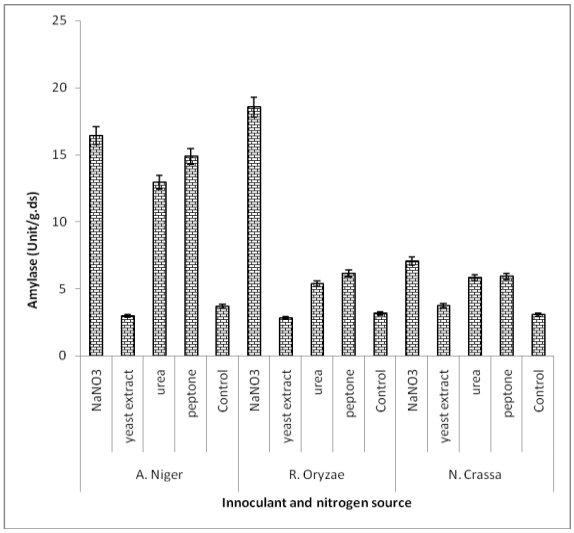

Figure 14. Comparison of amylase production as affected by nitrogen sources and inoculant type. The enzyme activity was determined at 4 days incubation period, the SSF was at 30 ºC, with initial moisture content of 60 %.

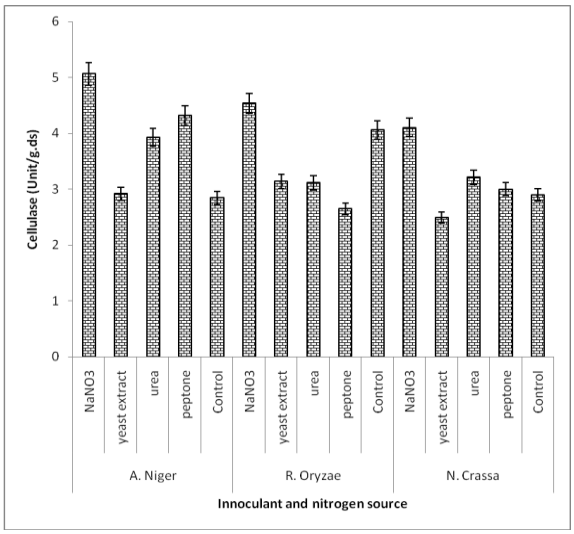

Figure 15. Comparison of cellulase production as affected by nitrogen sources and inoculant type. The enzyme activity was determined at 4 days incubation period, the SSF was at 30 ºC, with initial moisture content of 60 %.

Cellulase production by *Trichoderma koningii* AS3.4262 on vinegar waste not only affected by carbon but also nitrogen sources (Liu & Yang, 2007). *Aspergillus niger* grown on Jatropha curcas seed-cake supplemented with ammonium chloride supplementation did not increase production of cellulase, but xylanase was increased by 13 %. In case of *Cellulomonas cellulans* grown on waste materials such as cassava bagasse, pine leaves, wheat bran and rice bran in solid state fermentation, addition of nitrogen sources such as yeast extract, beef extract, peptone, malt extract were taken.

Among them, yeast extract was selected the best nitrogen source for cellulase production. Maximum production of cellulase was obtained at an initial moisture content of 80% with an initial pH of 6 of which implies that N-species affect hydrolytic enzyme activities differently (Ncube *et al*., 2012; Sugumaran *et al*., 2013).

Conclusion

Formulated media containing sargassum and rice bran can be used to produce amylase, cellulase and phytase. The enzyme production was affected by particle size, iniatial moisture content, temperature, and nitrogen sources. Hence optimum enzyme production in solid state fermentation could be achieved by manipulating those factors.

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