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Exploring the Production of Amylase Using Rice Grains as Substrate via Solid State Fermentation

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Abstract
One of the most widely used enzymes in industries is amylase. It is used in the hydrolyses of starch to produce sugar syrup, which contains glucose and maltose. This study was aimed at exploring the production of amylase using solid state as means of fermentation. Rice grains were used for the fermentation process, which lead to the production of enzyme. The rice grains were soaked in distilled water and washed and it was then dried at 45 - 50 ° C for pre-treatment. Nutrient broth was prepared and inoculated with Bacillus species. The inoculated broth was centrifugedto get the supernatant at 3000 rpm for 10 mins. 5 ml of sterile distilled water was used to re –suspend the cell pellet and added to 250 ml Erlenmeyer flask containing 4 g of pre-treated rice straw. The fermentation medium was evenly mixed with the rice grain and microbial cell. The flask containing the mixture of fermentation media, microbial cells and solid substrate was incubated at 37 ^{0}C. The a-amylase enzyme was extracted at different intervals for 7 days. Sample 1 had an enzyme activity of 12.66 ± 0.126 U/g on day 0 and an activity of 55.55±1.154 on day 7. Sample 3 had the highest activity of 101.48 ± 1.28 U/mg on day 3. The rice grain proved to be effective for amylase production, showing the feasibility of using agricultural product like rice grain as substrate for the production of amylase with optimal production conditions.

Keywords: Hydrolyses, Pallet, Fermentation, Pre-treatment, Extracted

INTRODUCTION
The enzyme amylase, is one of the most widely used enzymes in the industry. It hydrolyses starch and is used commercially for the production of sugar syrups from starch which consist of glucose, maltose, and higher oligosaccharides (Hagihara *et al*., 2001). This enzyme is of great importance in biotechnological application ranging from food, fermentation, pharmaceutical, brewing and textile to paper factories (Kunamneni *et al*., 2005). Very low cost production process, is pertinent to meet the high demand of this enzyme.

This enzyme is mainly produced by bacteria, fungi, plants and animals. Microoganisms have sustainable potential in enzyme production; hence new strategies are derived to speed up its production process (Sodhi *et al*., 2005). Industrially important microorganisms are found within the *Bacillus* species because of their rapid growth rates that lead to short fermentation

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cycles, their capacity to secrete proteins into extra cellular medium and general handling safety (Pandey *et al.,* 2000).

Microorganisms such as *Bacillus*, *Aeromonas, Lactobacillus, Streptococcus* and *Micrococcus* have been tested for α amylase production using submerged or solid state production method for α- amylase (Jeon *et al*., 2010). Solid state fermentation (SSF) even though conventional but is still extensively employed due to less energy requirements, high product yield, less catabolic repression and end product inhibition, low capital investment and better product recovery (Regulapati *et al*., 2007). Raw materials such as banana peel, rice starch, dried potato peel, coconut oil cake and mustard oil cake have been used as substrates for SSF (Swain and Ray, 2007).

Solid state fermentation process has gained more awareness, and it's now been used extensively. Moreover, it has more merits over the submerged fermentation process because it has simple technique, low capital investment, cheaper production of enzyme having better physiochemical properties and better product recovery (Baysal *et al.,* 2003). Parameters that affects microbial synthesis of enzyme in solid state production process are; inoculum concentration, substrate particle size and substrate moisture level. Thus, it involves the screening of a number of agro –industrial materials for microbial growth and product formation (Sodhi *et al.,* 2005). Temperature condition and the state of pH are known to be very pertinent parameters in the production of enzymes from bacteria; hence, the thermal and the pH stability of the enzyme, which is a function of the exposure time, must also be taken into account.

Production of these α-amylases has been investigated through submerged (SmF) and solid-state fermentation (SSF) (Perez-Guarre *et al*., 2003). However, the contents of a synthetic medium are very expensive and uneconomical, so they need to be replaced with more economically available agricultural and industrial by-products, as they are considered to be good substrates for SSF to produce enzymes (Kunamneni *et al*., 2005).

Materials and Methods

Sample Collection

The materials used in the study were PDA (Potato Dextrose Agar), $(NH_4)_2SO_4$, KH_2PO_4, K_2HPO_4, $MgSO_4.7H_2O$, lactose, maltose, phosphate buffer, DNS (Dinitrosalicylic acid), starch and distilled water. Rice strew was used as the substrate.

Preparation of Substrate and Pre – treatment.

This study used solid-state fermentation (SSF) process with rice grain as solid substrate support. Rice grain was bought from Uselu market in Benin City. The grains were soaked in distilled water and washed. It was then dried at 50 °C. The dried rice grain was grinded and soaked with 2.0 % (w/v) NaOH and heated at 86 °C for 3 h. The treated rice straw was then filtered and washed with distilled water until no traces of acid or alkali could be detected and dried in an oven at 60 °C for 2 days.

Microorganism

Bacillus subtilis used in this study was obtained from the Microbiology Departmental laboratory of University of Benin. Pure culture were obtained by streak plate method (Dimowo and Omonigho 2017; Asikhia and Dimowo, 2023). Using nutrient agar slant, the culture were sub cultured and stored at 4 ^{0}C.

Inoculum Preparation and Amylase production.
250 ml flask was used in preparing nutrient broth. The organism was inoculated into the broth medium for 8 hrs for optimum productivity/growth. 10 ml of the inoculated broth was centrifuged at 3000 rpm for 10 mins. Using 5 ml of sterile distilled water, the cell pallet was re-suspended and added to 250 ml flasks containing 4 g pre-treated rice straw. A control experiment was also prepared without pre-treated rice straw. 10 ml of fermentation media comprised of $MgS0_4.7H_20$ (0.2 g/l), (0.2 g/L), $CaCl_2$ (0.02 g/L), KH_2PO_4 (1.0 g/L), $NH_3H_2PO_4$ (1.0 g/L), NH_4NO_3 (1.0 g/L), $FeCl_3$ (0.05 g/L) and glucose, was evenly mixed with the rice grain and cells. The initial pH of fermentation media was maintained throughout the experiment at pH 7.0. Each experiment of solid state fermentation was carried out in triplicates. Incubation was carried out at 37 ^{0}C. The amylase enzyme was extracted at different intervals for 7 days.

Enzyme Extraction
Amylase enzyme was extracted by mixing 50 ml of 0.1M phosphate buffer (pH 7) with the whole solid substrate and shaken on a rotary shaker at 250 rpm for 30 mins. The buffer containing enzyme was separated from solid substrate using filter paper. The filtrate was centrifuged at 4000 rpm for 20 mins. The clear brown supernatant was used as the enzyme source for the enzyme assay analysis.

Enzyme Assay
Amylase activity was determined by the procedure of Bernfeld using soluble starch as a substrate. The reaction mixture containing 200 µL of 1% substrate (soluble starch) (w/v) in 300 µL 0.1 M phosphate buffer (pH 7) and 150 µL of enzyme solution was incubated at 3 7°C for 30 mins. The reaction was stopped by adding 400 µl of 3, 5- dinitrosalicylic (DNS) acid solution followed by heating in a boiling water bath for 5 mins and cooling at room temperature (Dimowo *et al.,* 2021). Then, distilled water was added until the solution volume was 12 ml. Absorbance of each solution was measured at 489 nm using a UV-Visible spectrophotometer. The initial reading was prepared by boiling the enzyme solution first in the hot water bath for 20 mins to denature the enzyme protein structure.

Enzyme activity (EA) calculation was based on the amount of glucose released from the degradation reaction of α –amylase enzyme on the substrate soluble starch as in the enzyme assay procedure. A Unit (U) of α-amylase activity was defined as the amount of enzyme that releases 1 µmol of reducing sugars as glucose per minute, under assay conditions of pH 7 and incubation temperature of 37 °C with phosphate buffer solution. The enzyme activity was expressed in U/mg of solid substrate.

Statistical analysis
All experiments were performed in triplicates (n = 3) and a one way ANOVA analysis was carried out using SPSS 16.0 software. Least significant differences were also evaluated by Duncan's new multiple range tests.

RESULTS
Figure 1; explains the optical density of the fermentation medium for the production of amylase using rice grains. From the result obtained, day 3 and day 5 had a better optical density for the production of amylase compared to day 0 and day 7

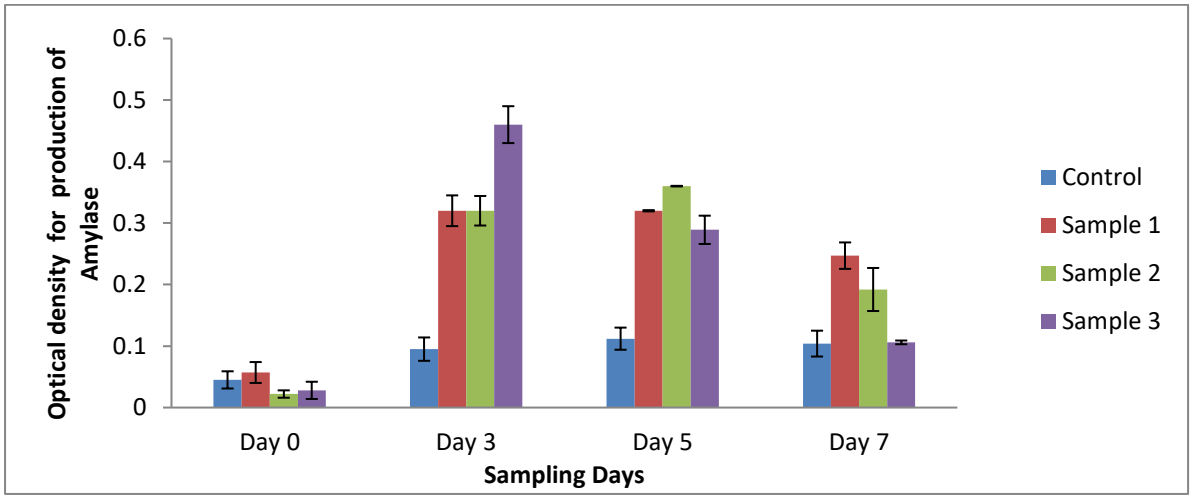

Figure 1: Optical Density for the Production of Amylase using Rice straw substrate

Figure 2: depicts the determination of amylase activity during production using solid state fermentation. In all, the samples tend to have more activity of amylase on day 3 and 5. The control had an optical density of 4.543±0.125 on day 0 and 32.098 ± 0.125 on day 7. Sample 3 had the highest amylase activity of 101.48 ±1.283 U/mg on day 3 and it reduced to 23.704 ± 0.128 on day 7.

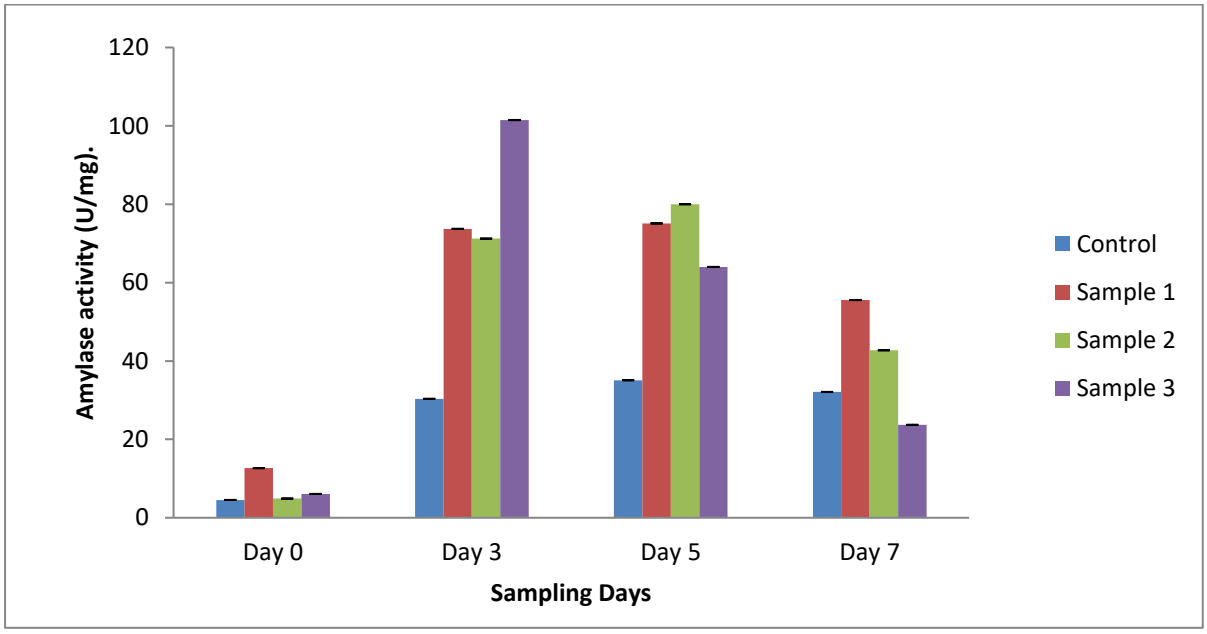

Figure 2: Amylase activity during production using Solid State Fermentation

DISCUSSION

Amylase is an inducible enzyme that is easily produced when there is a source of carbon like starch, its hydrolytic product, or maltose. Glucose has been known to induce only a minimal level of amylase in some fungal species and xylose has been reported to strongly repress amylase production (Yabuki *et al.,* 1977). In this study, amylase was produced by *Bacillus subtilis* in growth fermentation medium containing rice as carbon and growth source at 37 ^{0}C. Amylase activity was optimum on the third day with starch as carbon source and tryptone as nitrogen source. When ammonium chloride was used as nitrogen source and glucose as

carbon source, amylase activity was optimum on the fifth day. Conditions of growth of the organism seemed viable for amylase production. Enzymes occur in every living cell, hence in all microorganisms. Each single strain of the organism produces a large number of enzymes, hydrolysing, oxidizing or reducing and metabolic in nature (Prescott *et al.,* 2005)

However, Rehana and Nand in 1989, reported the occurrence of Amylase –producing organisms in soil. In this study, the production of amylase via solid state fermentation using rice grain as examined, table 1 shows the optical density of the rice grain in respect to the number of days or hrs. Comparing the results from the optical density of the rice grain from Day 0 to Day 7, it was evident that day 3 (0.46 ± 0.03) had a better optical density. Day 0 had the lowest optical density of 0.028 ± 0.014 when compared to the optical density of Day 3, Day 5 and Day 7 in respect to the number of days or hours taken for the fermentation of rice grain. However, values of optical density obtained in this research were lower compared to the values obtained by Dimowo *et al.,* (2021), in the production of L-asparaginase. Table 2 shows the enzyme activities during the fermentation of rice grain. There was a fast increase in the enzyme activity of rice grain with its peak on Day 3 (101.48 ± 1.283). There was a steady but slow decrease in the enzyme activity of rice grain on Day 7.

It is evident from this study, that rice grain serve as ideal fermentation bases for obtaining high yields of amylase from *Bacillus* sp. Rice grain contains a lower level of proteinaceous matter and a higher level of carbohydrate than other substrates (Krishnan and Chandra, 1982), thus is a suitable nutrient source by itself for amylase production. However, Ikram-ul-Haq *et al.* (2003) have reported wheat bran as the best substrate for amylase production by *Bacillus licheniformis* using different agricultural by-products. Amylases are present endogenously in wheat flour but their activities vary greatly depending on the type and variety of grain, the environmental conditions during cultivation, and the state of maturity at harvest (Dupont and Altenbach, 2003). A study was carried out on different agricultural grain substrate such as rice grain, millet and wheat and were tested for the production of alpha amylase by parental and its mutant derivatives. The production of alpha amylase was 10-folds better by the mutant strain *B. licheniformis* GCUCM-30 than the parental strain when pearl millet starch at 1.5 % level and nutrient broth concentrations at the level of 0.25 % was supplemented to the fermentation medium (Pratima and Umender, 1989; Mamo and Gessesse, 1999).

Alpha amylase, it's an extracellular; enzyme, that can degraded α,1-4 linkage of starch and extensively used in starch liquefaction, paper, food, pharmaceutical and sugar industries. Highly active alpha amylase is required to meet the demand of above-mentioned industries. Mutant strains of *Bacillus* have better ability for the production of alpha amylase. The mutant strains of *Bacillus* can be derived by mutagenesis and extensive screening. The fermentable carbon sources such as glucose, starch, lactose or fructose that are obtained after the processing of agricultural products can be used in the production of this enzyme. Therefore, they became very expensive for commercial production of alpha amylase. These expensive products can be replaced in the fermentation medium with the economically available agricultural by-products. The flours of different grains such as wheat, millet and rice can be used in the fermentation medium to increase the productivity of alpha amylase by the bacterium (Swain and Ray, 2007).

Recent studies have shown that the alpha amylase of *B. licheniformis* GCBU-8 shows great promise for the production of alpha amylase using an economical medium. Because of the immense usage of the enzyme for industrial applications, further studies are needed to optimize carbon and nitrogen sources for enhanced production of alpha amylase. A study has

been under taken to optimize agricultural starches as carbon source and nutrient broth as nitrogen source for the biosynthesis of alpha amylase. The results of comparative study of *Bacillus* strains for the selection of suitable low cost agricultural non-processed starch for the production of alpha amylase has been discussed. Different agricultural raw starches such as pearl millet, rice and wheat starches was tested for the production of alpha amylase (Swain and Ray 2007). The starches were added to the fermentation medium at 1 % level. The production of amylase by the parental strain was higher in the presence of soluble starch. However, its mutant derivatives gave optimum production of alpha amylase in the presence of pearl millet starch. Based on this study it showed that complex carbohydrate sources such as pearl millet starch can serve as basal and standardized medium for obtaining high yields of alpha amylase from *B. licheniformis*. With the addition of pear millet starch, the amount of nutrient broth was decreased from 1.0 % level to 0.25 % level by the mutant derivatives. As compared to soluble starch, the pearl millet starch showed significant benefit in enzyme production in consideration of the low cost of this starch.

CONCLUSION

The rice grain was proven to be effective for amylase production in the substrate studied, showing the feasibility of using agro-industrial by-product as substrate for the production of amylase with optimal production conditions.

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