

Effects of Optimization Condition on Solid State Fermentation of Various Agro-Allied Waste for Production of Amylase Enzyme

Anaegbu Chinonso Joel, Orukotan Abimbola Ayodeji, Mohammed Sani Sambo Datsugwai

Department of Microbiology, Faculty of Science, Kaduna State University, Kaduna, Nigeria

Email address:

crownbah@yahoo.com (A. C. Joel)

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Abstract: The effects of optimization condition on solid state fermentation of agro-allied waste for production of amylase using *Bacillus* sp was investigated. The amylase producing specie, was isolated from soil samples, rotten potatoe and spoiled fruit waste. Optimization of the amylase production using various agro-allied waste such as wheat bran, potatoe peel, Banana peel and rice bran were carried out. All the *Bacillus* sp isolates were screened by activity zone techniques with iodine solution. Out of 6 bacterial specie, only four (4) of the isolates showed positive results for amylase production. Bacterial strain-3 was found maximum at pH 6. The result showed that potatoe peel produced more enzyme activity under optimization conditions using Tap water, maltose, at pH 6, enzyme activity in the range of 0.555 iu/ml, 0.365 iu/ml, and 0.364 iu/ml respectively. Rice bran produced more enzyme activity of 0.342 iu/ml at 30°C while wheat bran showed an increased enzyme activity using peptone as source of nitrogen at 0.371 iu/ml, than any of the other agro-allied waste used for the amylase production. Owing to the prolific use of thermostable alpha amylase in various industries like paper, food, detergent, brewing and starch liquefaction process, the production of alpha amylase is still going on.

Keywords: Alpha Amylase, Optimization, Fermentation, Solid State Fermentation, Substrate Activity

1. Introduction

Microorganisms have become increasingly important as producers of industrial enzymes due to their biochemical diversity and the ease of improving the enzyme productivity through environmental optimization and genetic manipulation [1]. There are various reports on starch degrading microorganisms from different sources and respective amylase activity [2]. Among bacteria, *Bacillus* sp. is widely used for amylase production to meet the industrial needs. Amylase production in different *Bacillus* sp has been reported by several workers [3]. Amylases can be obtained from several sources such as plants, animals and microbes [4]. Many microorganisms especially several species belonging *Bacillus* sp are known to produce a variety of extracellular enzymes with a wide range of industrial application [5]. The microbial source of amylase is preferred to other sources because of its plasticity and vast availability [6].

Amylase enzymes play an important role in

biotechnological industries and has several potential applications in food, fermentation, textile and paper industries. The spectrum of applications of amylases has widened in many sectors such as clinical, medicinal and analytical chemistry [7].

All amylases are glycoside hydrolases and act on α -1, 4-glycosidic bonds. Amylases are significant enzymes for their specific use in the industrial starch conversion process. Amylolytic enzymes act on starch and related oligo-and polysaccharides. In the food industry amylolytic enzymes have a large scale of applications, such as the production of glucose syrups, high fructose corn syrups, maltose syrup, and reduction of viscosity of sugar syrups, reduction of turbidity to produce clarified fruit juice for longer shelf-life, solubilisation and saccharification of starch in the brewing industry [8].

[9] found the maximum amount of amylase production in

B. subtilis followed by *B. megaterium*. The maximum amylase producing *Bacillus* sp was taken for optimization studies through submerged fermentation by varying the temperature, pH, moistening condition, carbon and nitrogen source, since the production of amylase enzymes are influenced by diverse physico-chemical and biological factors [10].

However nowadays the new potential of using microorganism as biotechnological sources of industrially relevant enzymes has stimulated interest in exploration of extra cellular enzymatic activities in several microorganisms [11]. It is important to determine the production yield of *Bacillus* species in the production of Amylase enzyme from some agro-allied wastes. This study is therefore aimed at evaluating the effects of optimization condition on solid state fermentation of agro-allied waste for production of amylase enzyme using species of *Bacillus*.

2. Materials and Methods

2.1. Collection of Samples

Agro-allied waste such as wheat bran was produced from local market within kaduna metropolis, while Rice bran was obtained from institute for agricultural research zaria, and banana peel and potatoe peel were gotten from dumpsites within kaduna metropolis. Soil samples were collected from agro-allied dumpsites in central market and kawo market using sterilized spatula into sterile polythene bags separately. The samples were transfered to microbiology laboratory kaduna state university.

2.2. Isolation of Bacteria for Amylase Production

One gram (1g) of each soil sample was mixed with 9 mL of sterile saline (9 g/L NaCl) to obtain 10^1 . The samples was then serially diluted to 10^{-6} with saline. One millilitre (1ml) of 10^4 and 10^6 were inoculated on nutrient agar plate and skimmed milk agar plate using pour plate method. After 24 hours of incubation at 37°C, single colonies of different sizes were selected and the diameters of colonies were measured. Single colonies showed different morphological characteristics such as size, shape, colour, elevation and margin were identified from different plates streaked with diluted samples. Single colonies which formed clear halos with Gram's iodine were identified as starch utilizing organisms. The halo diameters of selected single colonies was measured after 24 hours of incubation to determine the halo diameter to colony diameter ratio. Selected single colonies were purified by repeated streaking and were transferred to starch-nutrient agar slant as described by [12].

The developed isolates were observed microscopically for morphological features, and biochemical tests such as grams staining, catalase, oxidase, lecithenase production, citrate utilization, vorges proskeur reaction, indole production, nitrate reduction, urease activity, and starch hydrolysis were carried out on each of the isolates respectively.

2.3. Screening of Bacterial Isolates for Alpha Amylase Production

Primary screening of bacterial isolates for production of alpha amylase was done by the starch agar plate method. Out of 6 specie, the 4 specie that showed the biggest zone of clearance in starch hydrolysis which were selected for production in solid state fermentation (SSF).

Amylase activity was assayed by measuring the reducing sugar formed by the enzymatic hydrolysis of soluble starch. The reaction mixture containing 1 ml of 1% (w/v) soluble starch in citrate phosphate buffer (pH 6.5) and 1 ml culture extract enzyme was incubated at 40°C for 30mins. The reaction was stopped by addition of 2 ml of dinitrosalicylic acid (DNS) reagents. The reaction mixture was heated for 5 minutes in boiling water bath and the absorbance was taken at 540 nm to estimate the reducing sugars released. The activity of amylase enzyme was determined as IU/ ml. Enzyme activity was calculated from the amount of reduced sugar produced in 30 minutes.

2.4. Inoculum Preparation for Alpha Amylase Production

The selected bacterial specie were inoculated in nutrient broth consisting of (g/ L⁻¹): peptone, 5; Beef extract, 3; NaCl as described by [13] and incubated at 37°C for 24 hours to get a standardized inoculum.

2.5. Substrate Preparation for Alpha Amylase Production

Four (4) different types of agro-industrial wastes were used as substrate viz., Wheat bran, Rice Bran, potatoes peel and banana peels were dried and powdered to obtain a particle size of 1.0 to 2.0 mm for each respectively. they were autoclaved and inoculated, this was optimized using different conditions such as moistening condition, sugar fermentation, temperature condition, source of nitrogen and pH. Solid state fermentation was performed with all the four (4) substrates and their enzyme production was checked by assay [13].

2.6. Solid State Fermentation Technique for Production of Alpha Amylase

Five grams (5g) of the substrate impregnated with 10 ml of sterile liquid nutrient medium containing (%): [KH₂PO₄-0.1, NaCl-0.25, MgSO₄.7H₂O-0.01, CaCl₂-0.01] were transferred to 100 ml Erlenmeyer flasks. The flasks were autoclaved and inoculated with 1ml of the prepared inoculum. These was thoroughly mixed and followed by incubation at 37°C for 5 days. One milliliter 1ml of the samples were aseptically taken at interval and assayed for amylase activity [13].

2.7. Enzyme Assay of Alpha Amylase

Estimation of amylase activity was carried out according to the DNS (3, 5 dinitro salicylic acid) method. One ml of 1% starch was incubated with different dilutions of the enzyme extract and 1ml of citrate-phosphate buffer (pH 6.0). The reaction mixture was incubated at 50°C for 30 minutes. The

reaction was stopped by adding 2 ml of DNS and kept in boiling water bath for 10min. The absorbance was determined at 540nm using a Spectrophotometer (Shimadzu, Thermoelectric cell holder, S-1700) against glucose as the standard. One unit of enzyme activity is defined as the amount of enzyme, which releases 1 μ mole of reducing sugar as glucose per minute, under the assay conditions (U/ml/min). The assay was carried out in triplicates and standard error was calculated [13].

2.8. Optimization Studies for Enzyme Production

In a sequential order, the various physicochemical factors as moistening condition, source of carbon or sugar fermentation, source of nitrogen, temperature condition, pH condition optimization affecting the enzyme production were optimized for maximum yield [13].

2.8.1. Effect of Temperature

To study the effects of temperature on amylase production using solid state fermentation was carried out at different temperatures (20, 30, 40, and 50).

2.8.2. Effect of pH

The fermentation medium were prepared with varying pH values (2.0, 4.0, 6.0, 8.0, 10.0, and 12.0) and the production of amylase enzyme [13].

2.8.3. Effect of Moistening Condition

The fermentation medium was checked for the production of amylase using different moistening conditions such as tap water, mineral salt and distilled water [13].

2.8.4. Effect of Carbon Source

The fermentation medium was prepared with different carbon sources such as sucrose, fructose, mannitol and maltose (0.5% level) and assessed for amylase production [13].

2.8.5. Effect of Organic Nitrogen Source

Different organic and inorganic nitrogen sources such as peptone, yeast extract, NH₄CL AND NH₄FESO₄ (2.0% level) were incorporated to the fermentation medium and assessed for their effect on amylase production for each respectively [13].

3. Results and Discussion

Table 1. Spectroscopic Determination of Amylase Activities Of Bacterial Isolates.

Bacterial isolates	Absorbance (540nm)	enzyme activity (iu/ml)
S1	1.322	0.244
S3	2.933	0.542
S5	1.009	0.187
S6	0.870	0.161

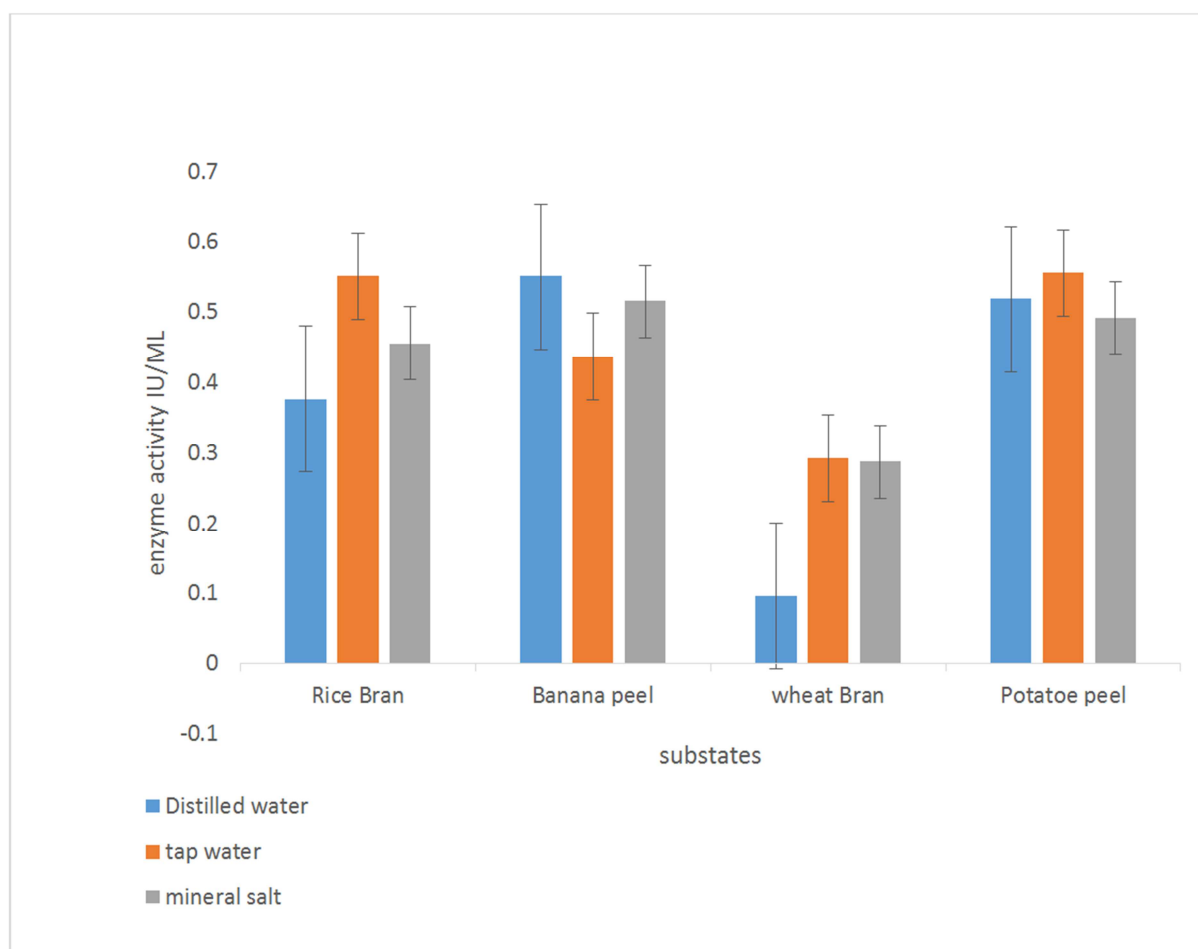


Figure 1. Enzyme Assay for Optimization of Moistening Condition on Various Agro-wastes.

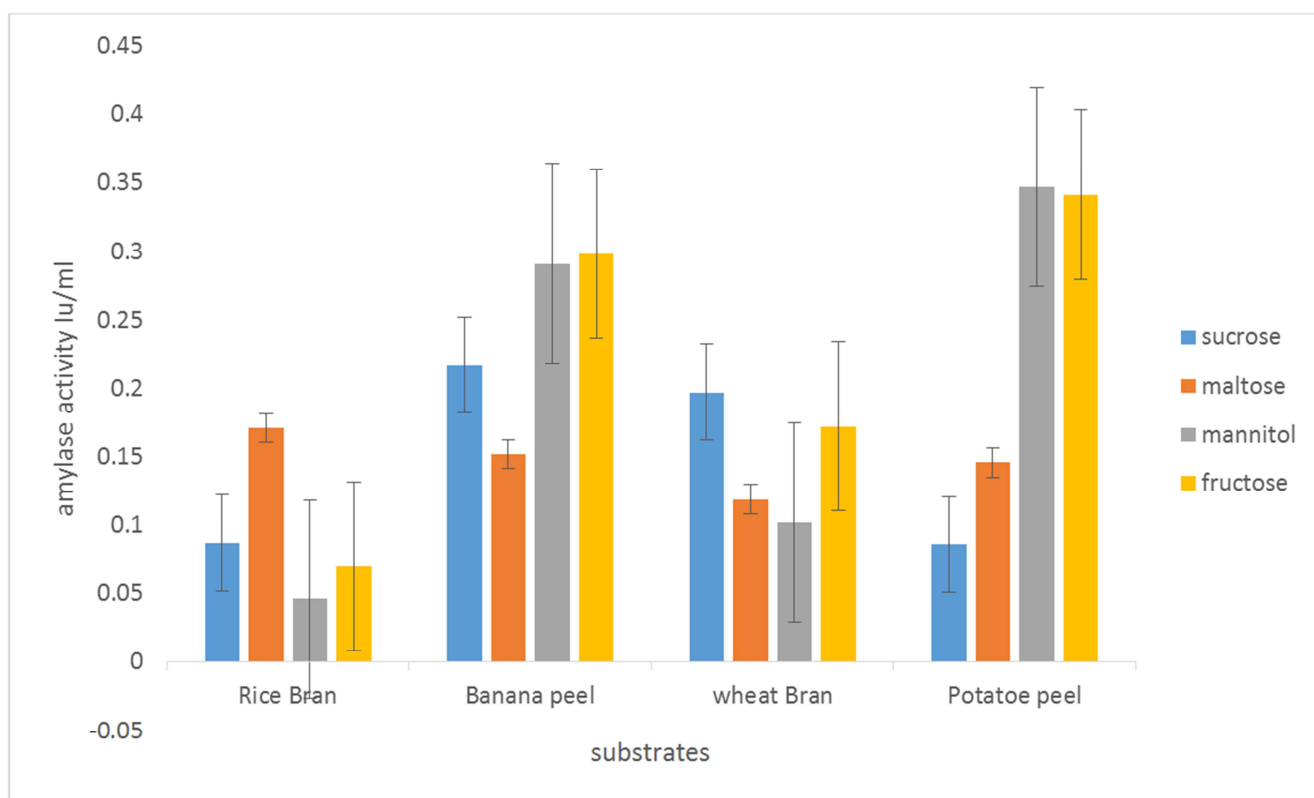


Figure 2. Enzyme Assay For Sugar Fermentation On Various Agro-wastes.

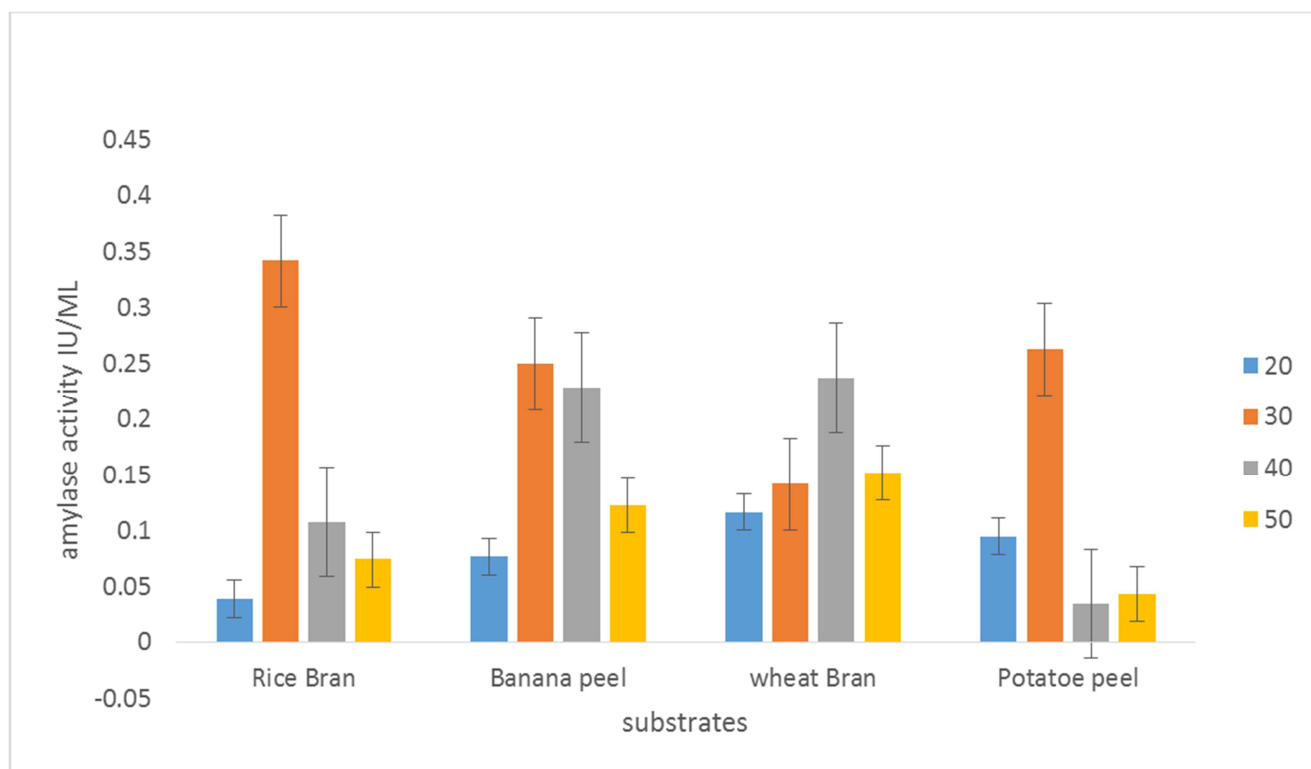


Figure 3. Enzyme Assay for Temperature Optimization on various Agro-wastes.

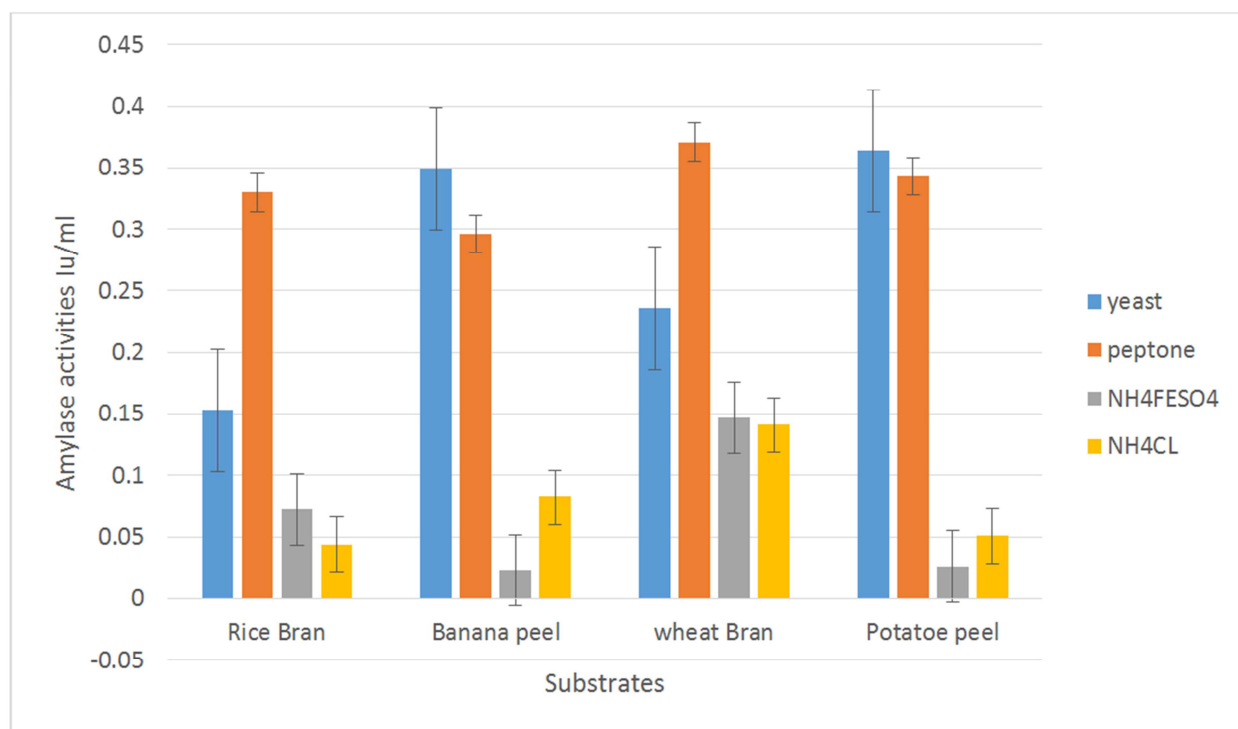


Figure 4. Enzyme Assay For Nitrogen Optimization on various Agro-wastes.

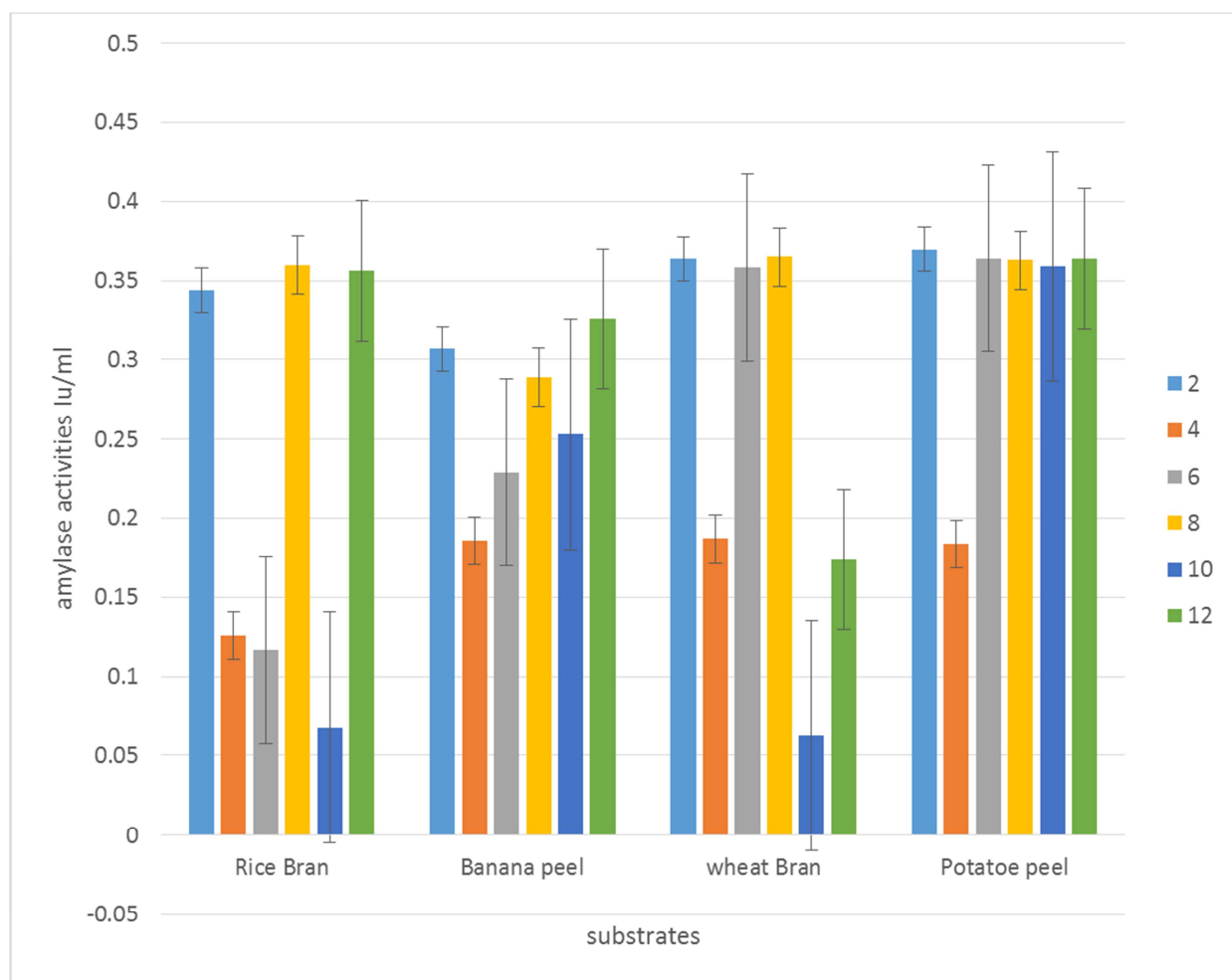


Figure 5. Enzyme Assay For pH Optimization on various Agro-wastes.

Isolation and Characterization of *Bacillus* sp

The *Bacillus* sp was characterized as aerobic, Gram positive and rod shaped bacteria. physiological tests showed that the cells could survive and grow in the medium pH ranging from 5.0 to 11.0 and under saline conditions of 10.0% NaCl₂. The biochemical tests, revealed the isolate belong to a member of *Bacillus* this was according to Bergey's Manual of Systematic Bacteriology [14]. Bacterial specie produced amylase enzyme, in only 72 hour in solid state fermentation [15].

enzyme assay for moistening condition from this study according to figure one (1) showed that for *Bacillus* sp; tap water recorded the highest enzyme activity with Rice bran, wheat bran and potatoe peel at 0.550Iu/ml, 0.292Iu/ml and 0.555Iu/ml respectively (figure 1). This could also be as a result of ions and metals contained in tap water which makes it condusive for growth of bacterial. For Banana peel mineral salt also showed high enzyme activity at 0.515 Iu/ml (figure 1). this agrees with [16] who stated that all media with different moistening agents supported enzyme production but maximum α -amylase production (810 U/g) was recorded when phosphate buffer (0.1 M; pH 7.0) was used as the moistening agent. Results of this study were also in agreement with the observations made by [17], who stated that the best moistening agents were tap water (pH 6.5) and distilled water (pH 6.8).

Enzyme assay for sugar fermentation from this study according to figure two (2), revealed highest enzyme activity using different sugars which consist of sucrose, maltose, mannitol and fructose and with different substrates (figure 2). The best substrate and highest enzyme activity was with potatoe peel with mannitol at 0.348 Iu/ml followed by banana peel with Mannitol at 0.344 Iu/ml, then wheat bran with mannitol at 0.319 Iu/ml and then rice bran with sucrose at 0.148 Iu/ml (figure 2). Growth and enzyme production of any organisms are greatly influenced by the nutrients available in the growth medium. α -amylase is an inducible enzyme [18]. The carbon sources in the medium are found to exert a profound effect on the enzyme production behaviour. Some carbon sources supported good growth with low enzyme production while others supported good growth as well as enzyme secretion [18]. The results is not in agreement with the reports of [18] for *B. subtilis*. [17] For *Bacillus* sp. PS-7 who reported maximum amylase production when starch was used as carbon supplement. Glucose and fructose supplementation resulted in the repression of enzyme production. This might be due to the feedback inhibition caused by the presence of glucose and fructose as reported by [19]. The repression is higher with glucose than fructose. Glucose acted as a catabolic repressor for the enzyme production.

Temperature Optimization showed that the optimum incubation temperature for production of amylase enzyme was at 30°C (figure 3). The highest enzyme activity was seen with rice Bran at 30°C at 0.342 Iu/ml followed by potatoe peel at 30°C at 0.263 Iu/ml, then banana peel at 0.250 Iu/ml

finally with wheat bran at 20°C at 0.237 Iu/ml (figure 3). [20] reported the optimum temperature for the growth of lysinibacillus as 30°C to 40°C. Also according to [21] Amylases are known to be active in a wide range of temperature between (40-90°C) and pH between (4-11). The optimum temperature of this amylase was 50°C, which is similar to other *Bacillus* amylases described by [18]. This identifies the unique characteristic of this *Bacillus* sp that grows at 37°C as a mesophile but produces enzymes that are active and stable at high temperatures between (50-70°C). This is also similar to findings reported by [22]. The stability of the enzyme was found to be independent of divalent calcium ions. The enzyme stability trend, as reported in the present study, agrees with the behavior of amylases from *Bacillus* sp as reported by [22].

Added nitrogen sources have been reported to have an inducing effect on the production of various enzymes in SSF system. Among the various organic and inorganic nitrogen sources tested nitrogen optimization in this study showed highest enzyme activity with peptone water with 0.371 Iu/ml and 0.330 Iu/ml with wheat bran and rice bran respectively, followed by yeast extracts with 0.364 Iu/ml and 0.349 Iu/ml for potatoe and banana peels respectively (figure 4). Effectiveness of organic nitrogen compounds on amylase production has been reported by [23]. The inhibitory effect of inorganic nitrogen has also been well demonstrated by [24]. It is observed that organic nitrogen compounds increased α -amylase production than when compared to inorganic compounds. The result obtained is in concurrence with the work reported earlier by [25] for *B. subtilis* who reported yeast extract as the best nitrogen supplement for amylase production.

The pH Optimization revealed that, amylase enzyme activity was 0.370 Iu/ml with potatoe peel at pH 2, while subsequent optimum pH for rice bran and wheat bran was at 0.360 Iu/ml and 0.365 Iu/ml respectively at pH 8 while banana peel gave an optimum enzyme activity of 0.326 Iu/ml at pH 12. This is in agreement with [26] who reported that amylases are active and stable over a wide range of pH between (3.5-12), though some are only stable within a narrow pH range. This is not in agreement with [21] who reported that the pH optima of the enzyme was found to be 6 with stability in the range of pH 5-7. The enzyme activity was found to be enhanced by lower concentrations of calcium, cobalt, magnesium and sodium ions. Similar findings have been reported by [27], However their reports of NaCl being less effective for enzyme activity contradicts this study where NaCl has been found to enhance the activity equally.

4. Conclusion

The findings of the current study, revealed that *Bacillus* sp could be used as a candidate strain for the production of amylase by solid state fermentation. Exposure of the *Bacillus* sp to other environmental parameters actually influenced the

growth and production of economically valuable metabolites through cultural techniques and specie improvement process. This might aid in providing excellent information for exploiting its biological potential. Moreover the studies also reveals the values as well as the microbial wealth of amylase producing bacteria which can be a boon for the development of biotechnological processes most especially in the industries.

Recommendations

Alpha amylase produced after solid state fermentation from *Bacillus subtilis* appears to have potential in industries due to its thermal, pH and detergent stability. It is therefore recommended that further studies be conducted to continue with the goal of performing its production in pilot scale. Data originated from this study will help to design experimental set up for large scale production of amylase. Evaluation of other biochemical and biophysical parameters like sensitivity towards ions, inhibitors, reaction kinetics and structural studies are to be performed to validate its use in industries. Divalent cations like calcium and magnesium ions increase the thermal stability of amylase so it will be relevant to check the effects of these ions in the fermentation media for the improvement of thermal stability of amylase. This may pave the pathway to justify its commercial utility. Alpha amylase production using microbial source and solid state fermentation has been conducted for past few years in search of thermostable enzyme. Owing to the prolific use of thermostable alpha amylase in various industries like paper, food, detergent, brewing and starch liquefaction process, the production of alpha amylase is still going on.

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