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Studies on screening and optimization of amylase enzyme production using bacteria isolated from soil

Abstract

Various inevitable parameters have been studied, searching for prospect enhancement of amylase enzyme production using limited energy, time and resources. These parameters include microbes, culture conditions and carbon sources. The present study aimed at screening and optimization of soil bacteria for their ability to degrade starch and produce amylase enzyme. The starch agar plate method was used to screen bacteria and submerged fermentation was for enzyme production. The mean bacterial counts of samples from Kasarawa (KS), Runjin sambo (RS) and Kalambaina (KL) area were 4.5×10^6, 6.6×10^6 and 4.1×10^6 CFU/g respectively. The mean hydrolysis zone of RS 23.3mm was maximal followed by KL 16.8mm and KS 16.3mm in diameter. Morphological and biochemical characteristics of the screened isolate were identified as KS-1 (*Bacillus subtilis*), RS-1 (*Bacillus licheniformis*) and KL-2 (*Bacillus cereus*). Production of amylase enzyme depends on growth parameters and energy sources which enzyme activity was optimal at 48hrs and 72hrs for *Bacillus licheniformis* and *Bacillus subtilis* respectively. The increased in temperature showed increased in enzyme activity by three (3) of the bacterial species between 25 and 55°C. The enzyme activity ranged from *B. subtilis* 1.78 to 4.55, *B. licheniformis* 2.97 to 6.52 and *B. cereus* 1.31 to 3.03Uml^{-1}. Meanwhile, all the isolates differed significantly. The enzyme activity of *B. licheniformis* was optimal at pH 7 (5.02Uml^{-1}) and pH 8 (4.44Uml^{-1}). The *B. subtilis* and *B. cereus* enzyme activity ranged from 2.04 to 4.85Uml^{-1} and 1.51 to 3.85Uml^{-1} respectively followed the same decreased trend as *B. licheniformis*. The best-observed carbon source was starch 5.67Uml^{-1} used by *B. licheniformis*, 3.89Uml^{-1} *B. cereus* and 3.55Uml^{-1} *B. subtilis.* In contrast, the nitrogen source was yeast extract utilized best by *B. licheniformis* 4.55Uml^{-1}, *B. subtilis* 3.61Uml^{-1} and *B. cereus* 3.13Uml^{-1}. The study presented *Bacillus* sp. 48hrs, 50°C, neutral pH, starch and yeast extract as the best parameters observed for amylase enzyme production.

Keywords: amylase activity, *Bacillus* species, production and soil

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Introduction

Amylases are glycoside hydrolases, enzymes that catalyze the hydrolysis of complex sugar such as starch (food stored in plants) and glycogen (food stored in animals) into subsequent simpler sugar molecules.[1] They separate the connecting bonds between subunits in polysaccharides, producing monosaccharides (glucose) and disaccharides (maltose). The polysaccharide (starch) contains glucose sub-units joined by glycosidic bonds made from amylose and amylopectin polymer.[2] Amylases are present in living plants, animals and microbes like fungi and bacteria.[1,3,4] The varied kinds of amylases include α (alpha) amylases which are presents in animals and microbes. The β (beta) and γ (gamma)-amylase presents in plants, differ in how they separate bonds in complex sugar molecules.[4,5] Alpha-amylase separate long-chain sugar molecules at random positions and tend to be quicker acting than other enzyme kinds as it can take action wherever on the substrate.[6,7] They also contain preserved amino acid like glutamate and aspartate in the site of catalysis.[4]

Microbial/bacterial amylases are suitably employed for biotechnological and industrial bases. They are significantly for wastewater treatment, Starch saccharification and liquefaction[8] and other varied industrial applications ranging from food, chemicals and fuel for transport.[1,4] The research study aimed to identify soil bacteria, screened and optimized for growth conditions and energy sources potentially for amylase enzyme production.

Materials and methods

Sample collection

Samples of soil were collected in sterile polythene bags, at three (3) different and three (3) within each locations including Kasarawa, Kalambaina and Runjin Sambo area within Sokoto metropolis. The samples transported to the laboratory were labelled with place, date and time of collection.

Isolation and enumeration of bacteria

Soil samples (1g) of each were serially diluted into 9ml distilled water containing test tubes up to 10^{-6}. The dilution 10^{-6} of each soil sample were spread plated (0.1ml) on sterilized solidified starch agar (SA) plates and incubated at 37°C for 24hours after which bacterial colonies were counted and expressed as colony-forming units per gram of soil (CFU/g). The bacteria were isolated, purified and identified based on conventional methods (cultural, morphological and biochemical characteristics) using the methods described.[9,10] Finally, Pure colonies were maintained in slant tubes at 4°C until use.[11]

Screening of amylase producing bacteria

The pure colony of each isolated bacteria was streaked on sterile individual starch agar plates, incubated at 37°C for one day. After a day, Lugol's iodine solution 1% was streamed on each plate for 20minutes and clear zones were recorded for positive amylase bacterial species using meter rule (mm) according to the method described.[12,13]

Inoculum preparation and fermentation

Mineral salt medium g/l (0.2 $MgSO_4.7H_2O$, 0.5 KNO_3, 6.0 peptone, 10.0 starch, 1 K_2HPO_4, 0.1 $CaCl_2$, and pH 7.0) as described[13] were put together in an Erlenmeyer flask 250ml capacity, sterilized at standard temperature and time. Test tubes containing nutrients broth (NB) were inoculated with a loop full of each test isolate, incubated at 37°C for overnight. About 1ml (1.4×10^6CFU/ml) of NB-culture mixture was added to the 99ml mineral salt medium in an Erlenmeyer flask and incubated in a rotary shaker at 150 rpm for 24hours.[13] The mixtures were centrifuged at 10,000rpm for 10 minutes. The supernatants were collected and used for enzyme amylase assay.[14,15] All experiments were carried out in duplicate.

Amylase enzyme assay

Amylase was assayed using the method.[13,16] The reaction mixture containing 1ml crude enzyme, 1ml soluble starch (1%), soaked in 1ml 0.2ml M phosphate buffer (at neutral pH) was added together in test tubes, covered and incubated at 60°C for 30mins. About 1 ml of 1M HCl was added to each tube for the reaction to stop. After then, about 1ml of iodine reagent (0.5% iodine and 1% KI) was added to each tube. The absorbance was examined at 620nm by spectrophotometer.

Optimization of parameters for amylase production

Varying parameters for enzyme optimization were observed such as temperature, pH, period of fermentation, carbon and nitrogen sources.

Effect of incubation period, temperature and pH

The best incubation period for enzyme amylase production was evaluated by inoculating a loop full of chosen bacterial isolate (after screening) into 100ml production media and incubated at 37±2°C. Effectivity was observed at 24, 48, 72 and 96hrs for 24hour intervals. In addition, temperatures were observed at intervals of 10°C as 25, 35, 45 and 55°C, and for optimum pH value determined include 5, 6, 7 and 8 using the same conditions of enzyme amylase production.[17,18]

Effect of nutrients for amylase production

The methods described[19] was adopted while determining the effects of carbon sources, including fructose, sucrose and starch, each was individually (at 1%) supplemented in a mixture containing production media and culture isolate/inoculum at neutral pH, incubated at 50°C for 2days.[20] Likewise, nitrogen sources as yeast extract, ammonium nitrate ($NH_4NO_{2)}$ and sodium nitrate ($NaNO_3$) was supplemented in a mixture and observed optimum amylase enzyme production.

Statistical analysis of data

For analysis, statistical software GenStat (17th edition) was used.[11] Means were compared between enzyme activities of each isolate concerning each parameter effect; ANOVA was used where the level of significance was at $P\leq0.05$. Results were presented in tables, graphical lines and bar charts.

Results and discussion

Bacterial cell count

The bacterial cell count was determined by calculating the colony forming units on agar plates. The results indicated an increase in bacterial cell count (Table 1). The RS soil sample showed maximal mean bacterial counts of 6.6×10^6 CFU/g, while KL expressed minimal mean bacterial counts, 4.1×10^6 CFU/g. The decrease in the number of bacterial cell counts in different samples could be attributed to the nature or topography of the soil.

Amylase producing bacteria

In this study, about 28 bacteria were isolated and characterized based on morphological and biochemical characteristics. The bacteria were classified as gram-negative rod bacilli with the ability to form spores. Of these isolates, 18 were identified as amylase producers (Table 2). The result demonstrated that RS-1 had the highest hydrolysis capability with a diameter zone of inhibition of 28.7mm, followed by RS-2 (27.2mm), RS-3 (24.5mm), RS-5 (22.7mm) and KS-1 (20.6mm). The isolates with the most miniature hydrolysis diameter zone of inhibition were from KL samples (Table 2). The possible reason could be the soil samples from KL had much athropogenic environmental interruption since KL is a cement industrialized area producing acidic substances that suppresses most bacterial growth.[21] Therefore, three isolates were determined as the best potential amylase producers according to their highest hydrolysis zones of inhibition. It has been reported that multiple species of bacteria are known to produce extracellular enzymes for several industrial processes.[22]

Morphology and biochemical characterization

To know the identity of selected isolates, Bergy's manual of the determinative bacteriology was used[23] to interpret the biochemical reactions. The results showed that all the isolates belonged to the genus *Bacillus* and share some common features; gram-positive rods, arranged in smaller chains, indole negative, are motile and can hydrolyze starch (Table 3). About three starch decomposers were identified as KS-1 (*Bacillus subtilis*), RS-1 (*Bacillus licheniformis*) and KL-2 (*Bacillus cereus*). The details of the result are presented in table 3. Soil is rich in microbes; hence, varied information and studies reported *B. subtilis*,[24-26] *B. licheniformis*[27] and *B. cereus*[28] as a source from soil and amylase enzyme producers.

Optimization studies

For the optimization study, the incubation period showed significant optimal amylase activity 6.51Uml^{-1} *B. licheniformis* at 48hours, 5.23Uml^{-1} *B. subtilis* at 72 hours and no increased enzyme activity observed above this period (Figure 1). Hence, at 48 and 72hrs, the amylase enzyme activity was not significantly different ($P\geq0.05$) between *B. subtilis* and *B. licheniformis*.[15,19,29,30] reported 48hrs as maximal enzyme activity.

At the temperature growth condition study, the amylase enzyme activity of each bacterial isolate was increased when the temperature rises. The activity of enzyme amylase of *B. subtilis*, *B. licheniformis* and *B. cereus* between 25 and 55°C ranged 1.78-4.55, 2.97-6.52 and 1.31-3.03Uml^{-1} respectively (figure 2). At 55°C, *B. licheniformis* produce greater enzyme activity 6.52 Uml^{-1} than *B. cereus* 3.03Uml^{-1} minimal. This may be due to the ability of bacteria *B. licheniformis* to have tolerated such temperature which may have given them the chance to produce more amylase enzymes. Some taxa in the genus *Bacillus* were classified as thermophilic bacteria[13] used to degrade starch and they include *B. licheniformis*. and *B. subtilis* are also mesophiles since they actively produced enzyme actively at 40°C (Figure 2).[31] The three (3) isolates were significantly differentiated ($P\geq0.05$) at 55°C. A Previous study showed that *B. subtilis* from soil produces maximal amylase activity at 40°C.[29,32,33] isolated three *Bacillus* species from temperate waste and produces more excellent amylase activity at higher temperature 90°C.

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Table 1 Total bacterial count of soil samples

Samples	Mean values (CFU/g)
KS	4.5×10^6
RS	6.6×10^6
KL	4.1×10^6

KS, Kasarawa; RS, Runjin Sambo; KL, Kalambaina; CFU, colony forming units

Table 2 Hydrolysis zones of soil isolates

Samples	Hydrolysis zones (mm)	Samples	Hydrolysis zones (mm)	Samples	Hydrolysis zones (mm)
KS-1	20.6	RS-1	28.7	KL-1	17.8
KS-2	12.4	RS-2	27.2	KL-2	21.1
KS-3	18	RS-3	24.5	KL-3	13.1
KS-4	17.6	RS-4	19.8	KL-4	15.3
KS-5	13.2	RS-5	22.7	KL-5	14.6
KS-6	16	RS-6	17.3	KL-6	19
Mean hydrolysis zones	16.3		23.3		16.8

Table 3 Conventional characteristics of bacteria isolated from soil samples

Isolate	Gram Reaction	shape	Arrang.	Cat	Citr	Indole	Motility	MR	VP	Starch
KS-1	+ve	Rods	Singly in chains	+ve	+ve	-ve	+ve	-ve	+ve	+ve
RS-1	+ve	Rods	Short chains in pairs	+ve	+ve	-ve	+ve	+ve	-ve	+ve
KL-2	+ve	Rods	Singly in chains	+ve	+ve	-ve	+ve	-ve	+ve	+ve

KS-1, *Bacillus subtilis;* RS-1, *Bacillus licheniformis;* KL-2, *Bacillus cereus;* Arrang, arrangement; Cat, catalase; Citr, citrate; MR, methyl red; VP, voges proskauer; +ve, positive

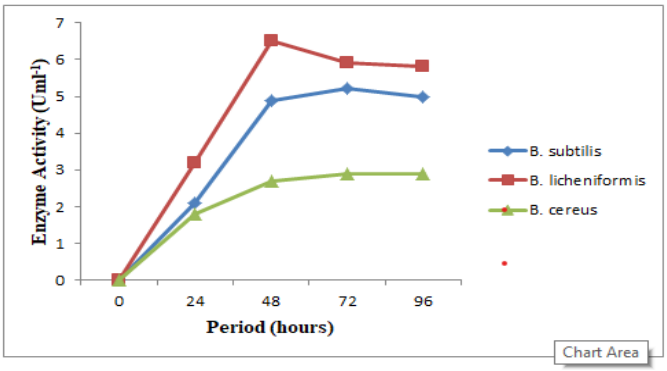

Figure 1 Effects of incubation period on enzyme amylase.

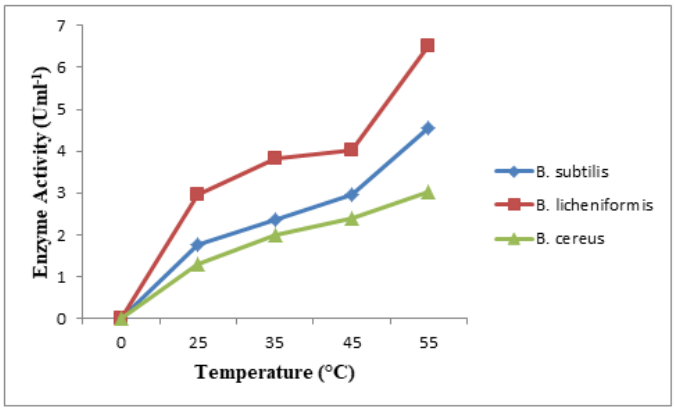

Figure 2 Effects of temperature on enzyme amylase.

The enzyme activity for different pH of the production medium was presented in Figure 3. Based on the pH values, the bacterial species produces enzyme activity ranged from 2.04-4.85, 2.65-5.02 and 1.51-3.85 for *B. subtilis*, *B. licheniformis* and *B. cereus* respectively. The bacterial species are alkalophilic as they present better enzyme activity between pH 7 and 9.[31] The optimal enzyme activity across all the bacterial species was at pH 7. A gradual reverse trend of the enzyme activity was observed at pH 8 (figure 3). The trends may because, the cells may not perform at basic nature of the medium; they may remain at rest. At pH eight alkalophilic flagellated *Bacillus* species becomes less active.[31] This agrees with the report[30] that there was decreased trend of 5.00-2.14 Uml^{-1} enzyme activity at pH 5 to11 and the optimum enzyme activity was pH 7. Similar results were reported.[15, 23,34]

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The results of biosynthesis of enzyme amylase on sugars (Figure 4) showed that starch was the best carbon source across all the isolate 3.55-5.67Uml^{-1} followed by 2.33-3.87Uml^{-1} sucrose and 1.78-3.25Uml^{-1} fructose the lowest. On nitrogen sources, the enzyme activity range indicated yeast extract 3.13-4.55Uml^{-1} as the best, followed by $NaNO_3$ 2.55-3.01Uml^{-1} and NH_4NO_2 2.25-2.79Uml^{-1} the lowest. Thus, each of the three (3) *Bacillus* species could produce greater enzyme at starch and yeast extract nutrients.

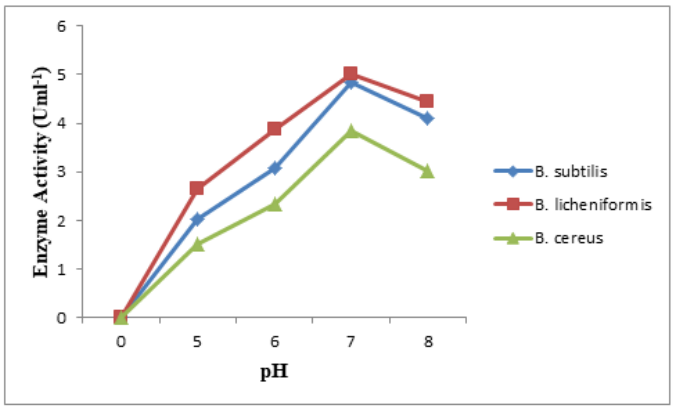

Figure 3 Effects of pH on enzyme amylase production.

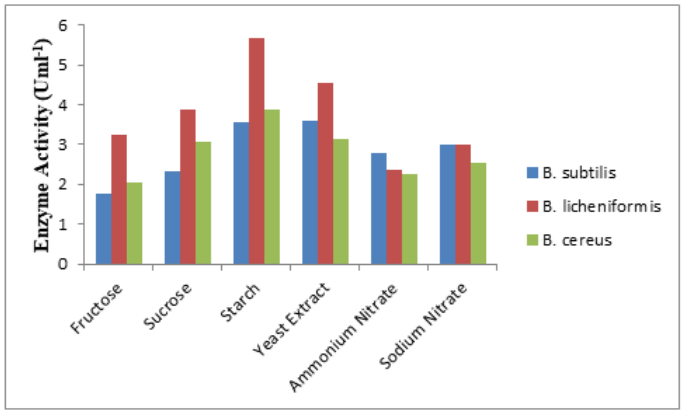

Figure 4 Effects of nutrients on enzyme amylase production.

Conclusion

Soil bacteria were successfully screened and best selected *B. subtilis*, *B. licheniformis* and *B. cereus* as starch degraders. The Present study shows 48hrs, 50°C, pH 7 as optimal or best parameters for amylase enzyme production by each isolate. Each of the three (3) *Bacillus* species could produce higher enzyme activity at higher temperatures. The best energy/carbon and nitrogen sources were starch and yeast extract.

Acknowledgments

None.

Conflicts of interest

The authors declare no conflict of interest.

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