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Amylase Production by Solid State Fermentation of Agro-industrial Wastes Using *Bacillus* species

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Authors' contributions

This work was carried out in collaboration among all authors. Author CNO designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Authors ANE and CNO managed the analyses of the study. Author OO the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

This study evaluated amylase production by *Bacillus* species employing the solid state fermentation (SSF) method using five agro-industrial wastes namely corn cobs, potato peel and maize straw, groundnut husk and corn chaff. Five *Bacillus* species were tested for amylase production abilities and *Bacillus subtilis* showed the highest amylase production ability after incubation. Corn chaff gave maximum enzyme production (3.25 U/ml) while the least enzyme was recorded on groundnut husk (2.35 U/ml) at 25. Potato peel had maximum enzyme production by *Bacillus subtilis* (3.05 U/ml) at pH 7.0 while the least enzyme production was from groundnut husk (2.84 U/ml) at pH 4.0.Thus there was an increase in enzyme production with corresponding increase in substrate concentration. The results obtained in this study support the suitability of using agro-industrial wastes as solid state fermentation substrates for high production of amylase. It's also a means of solving pollution problems thus making solid state fermentation an attractive method.

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Keywords: Agro-industrial wastes; amylase; Bacillus species; fermentation; solid state.

1. INTRODUCTION

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 [1]. Amylases are of great significance in biotechnological applications ranging from food, fermentation, detergent, pharmaceutical, brewing and textile to paper industries [2]. To meet the higher demands of these industries, low cost production of amylase is required.

The amylases can be derived from several sources, such as plants, animals and micro-organisms. Because of their short growth period, the enzymes from microbial sources generally meet industrial demands [3]. The first enzyme produced industrially was an amylase from a fungal source in 1994, which was used for the treatment of digestive disorders [4].

Amylase is produced in bacteria, fungi, plants and animals. The major bacteria belong to *Bacillus* species and fungi such as *Aspergillus niger, Penicillium* sp*., Cephalosporium and Rhizopus* are the major α-amylase producing microorganisms [5]. However, due to efficient production strategies, microorganisms have substantial potential to contribute to a number of industrial applications [6]. Such industrially important microorganisms are found within the *Bacillus* species because of their rapid growth rates that lead to short fermentation cycles, their capacity to secrete proteins into extra cellular medium and general handling safety [7].

Production of these α amylases has been investigated through submerged (SmF) and solid-state fermentation (SSF) [8]. 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 [9]. Therefore this study focused on the production of amylase enzyme by solid state fermentation of different agro-industrial wastes (corn cobs, potato peel and maize straw, groundnut husk and corn chaff) using *Bacillus* species.

2. MATERIALS AND METHODS

2.1 Collection of Substrate

Five Agro industrial wastes namely corn cobs, potato peel, maize straw, groundnut husk and corn chaff were collected from different locations in Umuahia. They were washed with distilled water 2-3 times and then treated with 1% NaOH for 30 min. The substrates were autoclaved and dried in oven at 80ºC for two days. Dried substrates were ground using a grinder to fine particles [10].

2.2 Test Bacterium

Stock culture plate of *Bacillus* species sourced from National Roots Crops Research Institute, Umudike maintained on Nutrient Agar slant was used as starter culture for the fermentation.

2.3 Screening of Test Bacterial

Primary screening of test bacteria for production of alpha amylase was done by the Starch Agar Plate Method described by Joanne et al. [11]. Species that showed the widest zone of clearance in starch hydrolysis were selected for use in Solid State Fermentation.

2.4 Solid State Fermentation Technique

Solid state fermentation experiments as described by Rajshree and Rajni [12] were conducted in 100 ml Erlenmeyer flasks containing 1 g of the substrate impregnated with 10 ml of sterile liquid nutrient broth (beef extract 3 g, peptone 5 g). The flasks were autoclaved at 121ºC for 15 min, and inoculated with 1 ml of the prepared inoculum, thoroughly mixed and incubated at 37ºC for 5 days.

2.4.1 Enzyme extraction

The amylase enzyme was extracted from Solid State Fermentation medium by a simple contact method described by Jamieson et al. [10]. After incubation, 100 mL sodium phosphate buffer of pH 6.9 was added into each experimental flask. The flaks were shaken (150 rpm) for half an hour and the material was filtered through a filter paper. The filtrate was centrifuged at 1000 (r) for 10 min at -10ºC. The supernatant was carefully collected and used as crude enzyme extract.

2.5 Amylase Enzyme Assay

For assay, previously inoculated nutrient starch broth was centrifuged at 8000 g for 12 minutes and the supernatant was used as crude enzyme source. The assay of amylase was conducted following the method of [10].

2.6 Optimization of Fermentation Parameters for Amylase Production and Activity

Optimization of agro industrial wastes samples fermentation was for the following parameters for amylase production: incubation period, temperature, medium pH, and substrate concentration [13].

2.7 Statistical Analysis

One-Sample T-Test was used to investigate the significant difference in the effects fermentation parameters of the substrates for amylase activity at 95% confidence interval. The data were analyzed using the program IBM SPSS Version 16.

3. RESULTS

Table 1 shows the shows the identification and characterization of *Bacillus* spp

Table 2 shows the effect of incubation period on amylase enzyme. The isolate showed highest production of amylase after 35 hours of incubation at 2.11 U/ml, 2.33 U/ml and 2.39 U/ml respectively.

Table 3 shows the effect of Temperature on amylase production. The maximum enzyme production was detected at 40ºC (2.52 U/ml, 2.35 U/ml, 2.45 U/ml, 2.30 U/ml and 2.44 U/ml) for each of the substrates respectively.

Table 4 shows the effect of pH of the medium on amylase production. Maximum enzyme activity was at pH 7.0, enzyme was produced maximally (2.55 U/ml), (2.54 U/ml), (2.34 U/ml), (2.43 U/ml) and (2.49 U/ml) respectively. It was recorded at pH8 that the activity of enzyme were slightly declined (2.35 U/ml), (2.30 U/ml) and (2.25 U/ml) for each substrate at 24 hours of incubation.

Table 5 shows the effect of substrate concentration on amylase production. There was increase in enzyme production with increase in substrate concentration up to 5 g.

4. DISCUSSION

This study evaluated amylase production by solid state fermentation of agro-industrial wastes using *Bacillus* spp. The amylase production by *Bacillus subtilis* is influenced by number of fermentation parameters. *The Bacillus subtilis s*howed the highest amylase production at 24 hours of incubation with Potatoes Peel having the highest production of amylase (2.36 U/ml) at 35ºC, followed by Corn Cobs which also recorded high amylase production (2.34 U/ml) at 35ºC. Hence Potatoes Peel is the best substrate for enzyme activity when compared to other agro-industrial wastes in this study. Similar result was reported by Ikram-ul-Haq et al. [14], who found out that wheat bran was a better substrate for α-amylase production by *Bacillus licheniformis.* Gangadharan et al. [15] have reported that maximum amylase production was achieved at 24-48 h incubation period. *Bacillus subtilis* has shorter period of incubation for the production of α-amylase when compared to earlier reports. Chandrashekhar et al. [16] evaluated the production of amylase at 12, 24, 36, 48 and 60 hours using *B. subtilis* cultured on banana waste and found more production at 24 hours, which corroborate with the present study. Above this incubation period, the amylase enzyme activity started to decrease. This may be due to the decrease in growth of the isolate. Most of the studies reported the highest enzyme production between 35 hours and 48 hours [17] on the contrary, (B5) showed optimum production after 25 hours, thus proving early harvesting time for industrial use.

Temperature is one of the important physical factors influencing the enzyme production [18]. Corn chaff produced the maximum enzyme production at 30ºC (2.75 U/ml). This could be due to the mesophilic nature of the organism. The finding of this present study supports the finding of Asgher et al. [19] who found that amylase produced by *Bacillus subtilis* JS2004 gave the best activity at 40ºC.

The result of the effect of temperature on enzyme production by *Bacillus subtilis* was almost identical to that reported for *B. lichemiformis* growing on wheat bran [20], for *Bacillus subtilis* growing on banana stalk [21], for *Bacillus megaterium* isolated from cassava waste [22]. Whereas, Vipul et al. [23] reported that the optimum temperature of enzyme activity was 40ºC. These results indicate the independent nature of the temperature effect irrespective of

Table 1. Identification and characterization of *Bacillus* species

Colonial features	Gram Reaction	Cell Arrangement	Spore stain	Catalase	Oxidase	Coagulase	Indole	Citrate	Motility	Methyl Red	Voges-P	Suspected bacteria
White Moisture	+	Short Rod	+	+	-	-	-	+	+	+	+	*Bacillus* spp

Key: - = Absent, + = Present

Table 2. Effect of incubation period on amylase activity (U/ml)

	Sample Substrate and Optical Density Reading (540nm)					
Incubation Period (hr)	**Corn Cobs**	**Potato Peels**	**Maize Straws**	**Groundnut Husks**	**Corn chaffs**	**Standard Values**
25	$1.46^{a} \pm 0.71$	1.45a± 0.71	$1.44^{a} \pm 0.71$	$1.32^{c} \pm 0.71$	$1.48^{a} \pm 0.71$	0.00
30	$1.75^{b} \pm 0.71$	$1.79^{b} \pm 0.71$	$1.75^{c} \pm 0.71$	$1.68^{d} \pm 0.71$	$1.81^{b} \pm 0.71$	0.00
35	$2.84^{c} \pm 0.71$	$2.86^{d} \pm 0.71$	$2.83^{c} \pm 0.71$	$2.75^{b} \pm 0.71$	$2.89^{c} \pm 0.71$	0.00
40	$2.61^{d} \pm 0.71$	$2.59^{d} \pm 0.71$	$2.60^{d} \pm 0.71$	$2.55^{b} \pm 0.71$	$2.65^{d} \pm 0.71$	0.00
45	$2.52^{e} \pm 0.71$	$2.55^{e} \pm 0.71$	$2.56^{e} \pm 0.71$	$2.50^{c} \pm 0.71$	$2.02^{c} \pm 0.71^{a}$	0.00

Same superscripts down the columns are not significantly different (P≤0.05)
Values are mean ± standard deviations from two replicates

Table 3. Effect of temperature on amylase activity (U/ml)

Substrate	Temperature (ºC)				
	25	**30**	**35**	**40**	**45**
Corn Cobs	$1.75^{b} \pm 0.71$	$2.45^{a} \pm 0.71$	$2.65^{a} \pm 0.71$	$2.94^{a} \pm 0.71$	$2.80^{c} \pm 0.71$
Potatoes Peel	$1.72^{c} \pm 0.71$	$2.35^{a} \pm 0.71$	$2.70^{b} \pm 0.71$	$2.95^{b} \pm 0.71$	$2.65^{d} \pm 0.71$
Maize Straw	$1.85^{c} \pm 0.71$	$2.55^{a} \pm 0.71$	$2.80^{c} \pm 0.71$	$3.02^{c} \pm 0.71$	$2.72^{e} \pm 0.71$
Groundnut Husk	$1.71^{a} \pm 0.71$	$2.35^{d} \pm 0.71$	$2.76^{d} \pm 0.71$	$2.32^{d} \pm 0.71$	$2.62^{c} \pm 0.71$
Corn chaff	$1.70^{a} \pm 0.71$	$3.25^{e} \pm 0.71$	$2.55^{e} \pm 0.71$	$2.75^{e} \pm 0.71$	$2.57^{b} \pm 0.71$

Same superscripts down the columns are not significantly different (P≤0.05)
Values are mean ± standard deviations from two replicates

Table 4: Effect of pH amylase activity (U/ml)

Substrate	pH				
	4.0	5.0	6.0	7.0	8.0
Corn Cobs	1.90^{a}± 0.71	2.02^{b}± 0.71	2.50^{d}± 0.71	3.04^{a}± 0.71	2.85^{a}± 0.71
Potatoes Peel	1.95^{a}± 0.71	2.05^{c}± 0.71	2.55^{d}± 0.71	3.05^{a}± 0.71	2.80^{a}± 0.71
Maize Straw	1.92^{a}± 0.71	2.00^{d}± 0.71	2.55^{d}± 0.71	2.99^{c}± 0.71	2.75^{b}± 0.71
Groundnut Hust	1.81^{c}± 0.71	2.45^{e}± 0.71	2.64^{a}± 0.71	2.84^{c}± 0.71	2.65^{c}± 0.71
Corn chaff	1.85^{c}± 0.71	1.92^{e}± 0.71	2.62^{a}± 0.71	2.93^{d}± 0.71	2.65^{c}± 0.71

Same superscripts down the columns are not significantly different (P≤0.05)
Values are mean ± standard deviations from two replicates

Table 5. Effect of substrate concentration on amylase activity (U/ml)

Substrate	Substrate concentration (g)				
	1	2	3	4	5
Corn Cobs	1.46^{b}± 0.71	1.94^{a}± 0.71	2.39^{a}± 0.71	2.75^{a}± 0.71	3.32^{a}± 0.71
Potato Peels	1.55^{a}± 0.71	1.76^{b}± 0.71	2.07^{b}± 0.71	2.89^{b}± 0.71	3.49^{b}± 0.71
Maize Straws	1.02^{c}± 0.71	1.34^{c}± 0.71	2.70^{c}± 0.71	3.06^{c}± 0.71	3.21^{c}± 0.71
Groundnut Husks	1.52^{d}± 0.71	1.71^{b}± 0.71	1.94^{d}± 0.71	2.82^{b}± 0.71	3.05^{d}± 0.71
Corn chaffs	1.34^{e}± 0.71	1.86^{e}± 0.71	2.15^{e}± 0.71	2.69^{a}± 0.71	2.94^{e}± 0.71

Same superscripts down the columns are not significantly different (P≤0.05)
Values are mean ± standard deviations from two replicates

the type of solid substrate used. It was also observed in this study that the enzyme production declined below and above 40ºC temperature and this was due to lesser growth of the bacteria [24]. Vasantha and Hemashenpagam [25] also evaluated the influence of temperature on amylase production.

Among the physicochemical parameters, pH of the growth medium plays an important role by inducing morphological changes in the organism and in enzyme secretion. Variation of pH results due to substrate consumption (eg: protein hydrolysis) and metabolite production like organic acids. Increase in pH from 4 to 6 increases enzyme activity, further increase in pH up to 9 decreases activity. *Bacillus subtilis* could grow and produce α-amylase over a wide range of pH (4-11). Potatoes peel had maximum enzyme production (2.55 U/ml) at pH 7.0 Similarly, [26] observed pH 7 as optimum for amylase production by *Bacillus amyloliquefaciens*. For the amylase production, most of the *Bacillus* sp. reported to have optimum pH between 7-10 [27]. [21] reported production of α-amylase by *Bacillus subtilis* on banana fruit stalk and got optimum activity at pH 7.0. [28] reported production of α-amylase by *Bacillus subtilis* utilizing banana peel and got optimum activity at pH 7.0. It was recorded that at pH 8 the activity of enzyme slightly declined to (2.35 U/ml), (2.30 U/ml) and (2.25 U/ml) for each substrate at 24 hours of incubation. When pH is altered below or above the optimum the activity it appears to be decreased or becomes denatured [29]. Different organisms have different pH optima and decrease or increase in pH on either side of the optimum value results in poor microbial growth [30] went on to report 6.8 as an optimum pH for the production of amylase by *B. subtilis.*

It has been suggested that the metabolic activity of bacteria is very sensitive to pH level of media. [31] reported that the initial pH of solid substrate was found to have an impact on α-amylase production by *Bacillus subtilis* grown on Poat Moss (PM). Further, the type of buffer used in nutrient solution is a key factor in governing α-amylase production by the *Bacillus subtilis*.

It was observed in this study that after 24 hours of incubation at 35ºC, broth slightly increased from 1g to 5g, having maximum enzyme production at 2.99 U/ml, 2.82 U/ml and 2.71 U/ml from the various substrates. Thus, the ability of enzyme production means the more substrate concentration the more the enzyme production. This could be attributed to the fact that bacteria might have utilized medium faster and has undergone decline phase due to nutrient depletion. The difference in enzyme production could be attributed to certain factors which are associated either with the structure of the substrate or with the composition of individual substrates. These results support the suitability of using agro-industrial wastes as solid substrate for high production of α-amylase [32].

The contents of synthetic media are very expensive and these contents might be replaced with more economically available agricultural by-products to reduce the cost of the media [14]. Therefore, agro-industrial wastes and by-products such as starchy materials had been used for Biosynthesis of amylases to solve the pollution problems and obtain a low cost media [33]. The use of agricultural wastes makes solid--state fermentation (SSF) an attractive alternative method [34].

5. CONCLUSION

Among the cheap sources tested, potatoes peel was best for maximum amylase production at 35ºC. The optimum activity of enzyme was obtained at 40ºC incubation temperature and 35 hours incubation period.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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