PRINT ISSN 1119-8362
Electronic ISSN 2659-1502

Full-text Available Online at
https://www.ajol.info/index.php/jasem
http://www.bioline.org.br/ja

J. Appl. Sci. Environ. Manage.
Vol. 27 (4) 665-671 April 2023

Effect of Substrate Concentration and Temperature for Bio-Enzyme Production Using *Enterococcus faecalis* Isolated from Waste Dump Soil in Sokoto Metropolis, Nigeria

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ABSTRACT: The study evaluated the effect of substrate concentration and temperature on the production of amylase enzyme by *Enterococcus faecalis* from the wastes dump soil Sokoto Metropolis, Nigeria. Five bacteria species were isolated and biochemically characterized which include *E. coli, B. subtilis, S. aureus, E. faecalis* and *P. aeroginosa*. The screening of identified bacteria for amylase production was carried out and all bacteria produced a wide halo on a starch agar. *E. faecalis* produced highest zone of clearance with 70mm,*B.subtilis* 50mm,*S.aureus* 34mm, *E. coli* 29mm,then*B.cereus* with the least zone of clearance of 24mm. proximate analysis of the potato peels sample shows that the potatoes peels has high carbohydrate content with value of 75.737 recorded. The effect of substrate concentration was studied at 5g,10g,15g and 20g. The highest amylase production was recorded (20g) with value of 1.218 and 15g with the least absorbance value of 0.973.The effect of temperature were studied at 25˚C, 35˚C, 45˚C, and 55˚C. The temperature of 45˚C has the highest absorbance value of 1.691. While the least was recorded at55˚C with value of 0.970.These shows that temperature and substrate concentration has a vital role to play in the production of microbial enzymes. It's concluded in this study that waste dump site is a reservoir of important microorganisms and potato peels is a good substrate for enzyme production and therefore can be utilized instead of dumping it for natural degradation.

DOI: **https://dx.doi.org/10.4314/jasem.v27i4.4**

Cite this paper as: HUSSAIN, H; MAIKAJI, F. S; SALIHU, M (2023). Effect of Substrate Concentration and Temperature for Bio-Enzyme Production Using *Enterococcus faecalis i*solated from Waste Dump Soil in Sokoto Metropolis, Nigeria. *J. Appl. Sci. Environ. Manage.* 27 (4) 665-671

Dates: Received: 11 January 2023; Revised: 28 January 2023; Accepted: 10 April 2023
Published: 18 April 2023

Keywords: Enzymes; Amylase; Potato peels; Temperature; substrate

Recent shift and high cost of commodities worldwide has become a great challenge to the layman, this is attributed to the overall dependant on conventional and expensive raw materials for the production of commodities.Bio enzymes are the organic solution developed by fermenting vegetables or fruit wastes various other substances like water, sugar and salt are used along with the chosen microorganisms like bacteria and yeast (Thirumurugan, 2016).Amylase production from microbial sources can be produce in an abundant quantities because the great diversity of microbe producing amylase. Microbial enzymes have a wide range of features that make them quit useful in a variety of application (Ahmadi, 2012). Amylase is produced by bacterial species of bacillus (Asghar, *et al*; 2007), *Pseudomonas* (shi'u and Hung, 2003) and Clostridium (Kilic*et al*; 2005). Bacterial species such as *Bacillus subtilis* (Rajpat*et al*; 2013), *B. lichenifomis* and *Bacillus aeruginosa* are generally preferred for the production of α-amylase because they appear to be very productive (Reda, 2007; Niazi et al; 2010). For extreme thermophilic bacteria such as *Rhodothomus, marinus*and mesopholic bacteria such as *B. megaterium, B. macerans* and *B. coagulase* are generally selected and utilized (Gimbi and Kitabatake, 2002). High thermostable α-amylase are also obtained

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in hyperthermophilic and thermophilic Archea such as *pyrococcusfuriosus, Thermococcus hydrothermalis* (Arikan, 2008). Microbial enzymes derived from microorganisms include fungal and bacterial amylases, and diastases. Enzymes are sold primarily on activity basis that is a quoted cost for a specified activity, secondary feature such as degree of purity, extent of modifications and microbiological specifications can modify this cost of enzymes for analytical and medical purposes these are often in a state of medium to high purity, and are sold in terms of numbers of enzyme unit per lots, whilst those for industrial processing are quoted on a unit weight basis for a standardized products of guaranteed activity per unit weight this applies to most conventionally produced solid and liquid products (Maps enzyme, 2010).Agro waste are cheap available raw materials that can be exploited in the production of bio-enzymes when use as substrate. Bacteria can be isolated from environmental samples and utilized for better production of microbial enzymes at a cheaper cost.Peels of vegetables are the main bi-product of plant processing factories which has important organic compound. Potato waste contain valuable chemical component which are suitable to apply in food preservation and pharmaceutical industries (Grunert 2018).Low cost medium is required for the production of amylase, to meet the demand of industries (Aliyu *et al,* 2011). Both Solid State Fermentation (SSF) and submerged fermentation (SMF) could be used for the production of amylase, although traditionally these have been obtained from submerged cultures because of easy handling and greater control of environmental factors such as temperature and ph. Mostly synthetic media have been used for the production of bacterial amylase through SmF (Ajay *et al.,* 2010). This work was aimed at evaluating the effect of substrate concentration and temperature on the production of amylase enzymes by *enterococcus faecalis* from waste dump soil from Sokoto metropolis, Nigeria.

MATERIALS AND METHODS

*Sample collection:*The soil sample was collected at a depth of 15-30cm using soil auger from Sokotometropolis, Nigeria and was properly stored in a polythene bag. A fresh sweet potatoes were obtained from market, it was peeled, dried shed, and grinded using a sterile mortar and pestle sterilized and packed in a sterile container further analysis**.**

Preparation of potato peel: The potato sample was obtained and was dried over some period of time at room temperature. The dried peel was milled into fine powder by pounding it in a clean and sterile pestle and mortar and was kept in an airtight container for practical use.

Sterilization of materials: The glass wires (Test tube, pipettes, conical flasks, beakers, petri- dishes, and universal bottles) were washed with soapy water, and rinsed with distilled water, they were allowed to dry and wrapped with kraft paper and further sterilized in a hot air oven at 18^{o}C for 1 hour and stored at 4^{0}C.

Proximate Analysis: Proximate composition of the sweet potato [Ipomoea batatas (L.) Lam] were determined according to the Official Methods of Analysis recommended by the Association of Official and Analytical Chemists (AOAC, 2008).

Determination of Moisture Content: An empty container was weighed (W_0) and two (2g) of the sweet potato [Ipomoea batatas (L.) Lam]666was weighed (W_1) into a pre-heated, cooled and weighed silica dish which was dried in an oven for 24 hours at a regulated temperature of 105˚C to a constant weight (W_2). The moisture content was determined as percentage moisture given by:

$$MC\ (\%) = \frac{W_2 - W_3}{W_3 - W_1} x100$$

Where: MC = Moisture content; W1 = weight of empty crucible (g); W_2 = weight crucible + sample before drying (g); W_2-W1=weight of sample; W_3=weight of crucible + sample after heating (g); W2-W3= loss of weight (g)

Determination of Ash Content: Crucibles were thoroughly washed, cleaned and placed in a hot air-circulation oven for 2 hours and cooled at ambient laboratory temperature in a desiccator. The empty crucibles were transferred to the muffle furnace to burn off all organic matter and, also, to stabilize the weight of the crucibles at the temperature range of 550-600˚C dessicator to cool to room temperature. From, the defatted samples, 2g were accurately weighed into the labeled crucibles, placed in the muffle furnace and ashed (incinerated) at 600˚C for 3 hours. At the end of the ashing period, the ashed samples were removed into desiccator to cool to room temperature and reweighed:

$$Ash\ content\ (\%) = \frac{Wt.of\ ash}{Wt.of\ sample} x100$$

W_1=Initial weight of empty crucible; W_2=weight of sample and crucible before heating; W_3=weight of sample and crucible after heating

Determination of Crude Lipid: Dry extraction methods (AOAC, 2008) was used for this analysis.

Crude lipid was determined by ether extraction method using soxhlet apparatus. Two grams (2g) of moisture free sample was wrapped in filter paper, placed in fat-free thimble and then inserted into the extraction tube. The receiving beaker was washed and dried then weighed as W_1 and later transferred 250ml of n- hexane into it, which was fitted into the apparatus. The extractor was switched on at 60°C and allowing water to run from tap by the use of tube. This process was left for 6 hours after which there was sequentially eight siphoning to ensure clean colorless fat free solvent in the above receiving flask. The content of the flasks was then left only the fat extract in the flask and the flask was weighed as W_2. The percentage crude fat was calculated as follows:

$$\%\ crude\ lipid\ (wet) = \frac{W_2 - W_3}{W_3} x100$$

Where: W_1=Initial weight of empty crucible; W_2=weight of sample and crucible before heating; W_3=weight of sample and crucible after heating

Determination of Crude Fibre: Exactly 2g of the peels was weighed and labelled as W_0. It was then transferred to a porous crucible and placed into the fibre machine keeping the valve at off position. Thereafter, 150ml of H_2SO_4 solution and drops of acetone was added to the column. The cooler was opened to turn on the heating element (Power 90˚C - 100˚C). After boiling, the power was reduced to 30˚C and then left for 3 minutes. The valves were opened to drain the acid and distilled water was used to rinse the column three times to ensure complete removal of acid from the sample. The above procedure was repeated using 150 ml of NaOH and later the sample was dried in the oven for an hour at 1500 ˚C. After drying, the sample was cooled in the desiccators and weighed as (W_1). This weighed sample was placed in the furnace for oxidation of the organic matter for 3 hours at 600˚C. Which this was ash completely, then cooled and reweighed finally as (W_2) and the percentage was calculated as:

$$\%\ crude\ lipid\ (wet) = \frac{W_2 - W_2}{W_2} x100$$

Where: W0= Initial weight of empty crucible; W1= weight of mixture before heating; W2= weight of mixed sample after heating.

Determination of Crude Protein: Two gram (2g) sample was weighed and placed onto the bottom of a 500ml Kjeldahl flask. Then 10ml of concentrated $H_2SO_4$was added and mixed gently by swirling under a tap water. Thereafter, 10g of anhydrous Na_2SO_4 and 1g of $CuSO_4$ was mixed together, and 3g of this was introduced into the flask, Also, anti-bumping chips was added into the mixture. The Na_2SO_4 and $CuSO_4$ mixture were the catalyst, the entire mixture were boiled in the Kjeldahl flask in a fume cupboard until charred particles disappeared and a clear green solution was obtained. Then, the digested mixture was made up to 100ml with distilled water.

Protein N→$(NH4)_2\ SO_4$
$(NH4)_2\ SO_4 + CO2 \uparrow + H2O$ Catalyst
$(NH_4)2SO_4 + NaOH \rightarrow Na_2SO_4 + 2NH_4OH$
$NH_4OH \rightarrow NH_3 + H_2O$
$3\ NH_3 + H_3BO_3 \rightarrow NH_4 + BO_3$
$BO_3 + 3H+ \rightarrow H_3BO_3$

Where: 1000= the conversion of mgN/100g sample; 6.25= the protein- nitrogen conversion factor for milk and its by-products

Where: S=Sample of titration reading; B=bank titration reading; N=normality of HCl; D=dilution of sample after digestion; V=volume taken for distillation 0.014 = milli equivalent weight of nitrogen

Determination of Nitrogen Free Extract: Nitrogen Free-Extract (NFE) was calculated by subtracting the sum total of all the other items in the proximate analysis from 100.

Thus:NFE=100- % crude protein + % crude fat + %ash + %moisture + % crude lipid)

Isolation and Characterization Bacterial Species: The soil samples were homogeneously mixed and then sieved with the use of 2.0mm sieve to remove unwanted soil debris. One (1) gram of the soil was weighed into test tube containing 9ml of sterile distilled water, and agitated for a minute. Serial dilution of the soil was made up to 10^{-7} dilutions. Aliquot of 0.5ml of the prepared dilution was aseptically transferred onto the surface of solidified Nutrient agar. It was spread well with the use of a sterile syringe. Plates were prepared in duplicates and incubated at 37^0C for 18-48h and were observed for bacterial growth. Different colonies observed were then purify by repeat streaking for each distinct colony on nutrient agar until pure colony was obtained. The purified bacterial isolates was transferred on sterile nutrient agar slants bottles and stored for further identification. Isolates were identify using the identification scheme provided in Bergy's manual of determinative Bacteriology (2007), based on staining and biochemical reactions, such as Gram staining,

motility, oxidase, catalase, coagulase, indole, MR-VR etc.

Determination of Amylolitic activity of the organism: The screening for amylase producers was done by inoculating the bacterial isolate in a hole created using sterile chockboreron a sterile starch agar (containing 1% starch and 2% agar) plate method. The plates were incubated at 37ºC for 48 hrs.After incubation, the plates were flooded with 1% ofiodine solution for 5min (Bahadure et al., 2010).Base on high size of zone of clearance around the well the potential isolate was selected

Amylase enzymes production: Optimization of culture condition (Substrate Concentration and Temperature for the Production of Amylase): The optimization of fermentation condition was done as described by Bertrand *et al*. (2004).

The optimize parameters are;
1. Substrate concentration of 5g, 10g,15g and 20g.
2. Temperature of 25^0C, 35^0C, 45^0C, and 55^0C.

Amylase assay: Amylase activity was measured by the 3,5-dinitrsalicylic acid (DNS) method Miller.(19); Oyeleke and Oduwole.(2009), by monitoring the amount of reducing sugars liberated from starch. Amylase was assayed by adding 1ml of enzyme (crude extract/fermented broth supernatant) to 0.5 ml of 1% soluble starch and incubated for 30 min at 37^0C.The reaction was stopped by adding 1 ml of 3,5 dinitrosalicyclic acid followed by boiling for 10 min.The final volume was made to 5 ml with distilled water and the absorbance measured at 540 nm with a spectrophotometer (Jenway 6100). One amylase unit (U) was defined as the amount of enzyme per milliliter culture filtrate that released 1 microgram glucose per minute.

Preparation of stock solution: The media was prepared by dissolving 6g of $MgSO_4,7H_20$, 0.5g KCL, soluble starch 1.0 bacteriological peptone 6.0g and 50.0g of waste potato peel into 1 litre distilled water pH 6.3 was dissolved in 1000ml of distilled water and 50mls was dispensed into 100ml capacity conical flasks sterilized at 121^0C for 15min.The Solution were stored at room temperature for further use.

Effect of Substrate Concentration: The effects of substrate concentration determined assaying the activity of the enzymes of each with different substrate concentration of 5, 10, 15 and 20g.DNSA and TCA reagent was added and the absorbance was taken on the spectrophotometer at 540nm and 240nm as described by Betrand*et al*, 2004.

Effect of optimum Temperature: Optimal temperature for amylase activity was determined by assaying the activity of the enzymesat different temperatures ranges of 25, 35, 45, and 55^0C.Thermostability of the enzymes was done by maintaining the enzymes solution in water at these different temperatures for 30mins.DNSA and TCA was added and the absorbance was taken on the spectrophotometer at 540nm and 240nm. This was done according to the method of Bterand*et al*. (2004).

RESULTS AND DISCUSSION

The result of the proximate analysis of the sweet potato peels sample showed that the sample have a moisture content of 10%, this is in agreement with the finding of Banidele*et al.(*2015) and Adenuga (2010) for root-based foods. The ash content of 3.5%, crude protein content of 3.675, Lipid content of 1.0 and fiber content of 5.5 was recorded which is slightly lower than the value recommended for rich-protein food by WHO. This result agreed with the finding of O*laniran et al. (*2020) on his study of proximate composition of formulated cassava, cowpea and potato flour blends. This study does not agreed with the finding of Zulkifil*et al*., (2021) on his study of proximate composition of Malaysian local sweet potatoes. The results of isolated and identified amylase producing bacteria are showed in table 4. Starch rich residues may be a better potential source where amylase positive bacteria can be isolated (fossi*et al;* 2005). A total of ten (10) bacterial organisms were identified from the soil sample. The identified organisms include *E.coli* with highest frequency and percentage of 30%, *S.aureus and B.subtilis* with percentage of 20%, and *E.faecalis, B.cereus and P. aeruginosa* with least percentage of 10%.

Among the identified organisms *E.faecalis* and *Bacillus spp* are among the known amylase producing bacteria (Asad, 2011). This study is agreed with the finding of Asad, 2011.Who reported *Bacillus spp* as the best amylase producers. The results of bacteria isolated from the soil sample for amylase production has proved that amylase producers are abundantly present in soil sample collected from the municipal waste.

A total of five (5) bacterial isolates morphologically different were selected for determination of amylolytic activity of the organism. Based on the determination, all isolates were produced zone of clearance which indicates amylase production using well assay. Table 7 showed isolate with the highest zone of clearance was biochemically identified as *Enterococcus feacalis*. This isolate was used for further study.

Table 1 Physiochemical analysis of the soil sample.

Physicochemical parameters	Values
Power of hydrogen (pH)	6.98
Organic carbon (%)	6.409
Nitrogen(mg/kg^{1})	3.675
Phosphorous (mg/kg ^{1})	0.37
Cat ion exchange capacity (CEC)	5.17
Magnesium (mg/kg ^{1})	5.4
Calcium (mg/kg ^{1})	4.0
Sodium(mg/kg ^{1})	3.0
Potassium (mg/kg ^{1})	34
Electrical conductivity (cmol/kg)	5.84
Sand (%)	91.7
Clay (%)	0.98
Silt (%)	9.28

Table .2proximate analysis of the sweet potato peel

Parameters	Values
Moisture (%)	**10**
Ash (%)	**3.5**
Lipid (%)	**1.0**
Fibre (%)	**5.5**
Crude protein (%)	**3.675**
Nitrogen (%)	**0.588**
Carbohydrate (%)	**75.737**

Table 3. Clony count of the isolates sample

S/N	Colony Count
C1	160 × 10^{4} cfu/g
C2	120 x10 ^{4} cffu/g
C3	104 × 10^{3} cfu/g
C4	108 x 10 ^{4}cfu/g

Table 4. Shows the frequency and percentage of the bacterial organisms

Bacteria specie	Frequency of occurrences	Percentage occurrences
Escherichia. Coli	3	30(%)
Staphylococcus.aureus	2	20(%)
Bacillus.subtilis	2	20(%)
Enterococcus.Faecalis	1	10(%)
Bacillus.cereus	1	10(%)
Pseudomonas.aeruginosa	1	10(%)

Table 5. Amylolytic activity of the Bacteria species.

S/N	Organisms	Zone of clearance
1	*Bacillus subtilis*	50mm
2	*E.coli*	29mm
3	*E. faecalis*	74mm
4	*Bacillus cereus*	24mm
5	*Staph aureus*	34mm

Substrate concentration affects the production of amylase enzyme by *E.faecalis*. The concentration varied from 5g-20g .The maximum absorbance value of 1.218 was recorded from concentration of 20g, which reduce to 0.973 as the substrate concentration reduce to 15g.likewise, absorbance value of 1.196 was determined at 10g of substrate concentration which have reduced to 0.991 as the substrate concentration reduces to 5g. This indicates that with increase in substrate concentration absorbance increases and decrease with reduction of substrate. This result does not agreed with the finding of Hauwa H. *et al.* (2019) who recorded the increase of amylase activity as the increase of substrate concentration from 1% to 2% followed sharp decrease at 3% concentration of the substrate on her study in production of bio-enzyme with banana peels by *Aspergillus niger*.

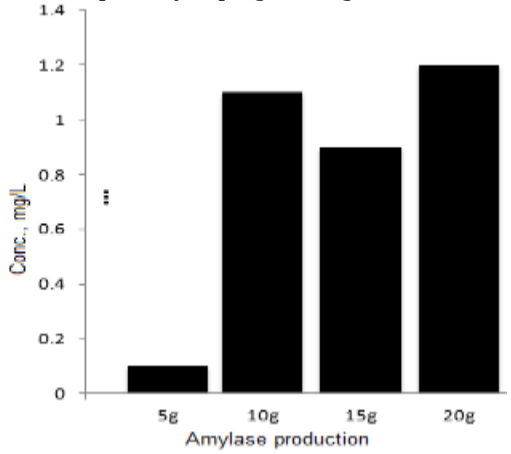

Fig 1; Effect of substrate concentration on bacterial species for amylase production by *E.faecalis)*

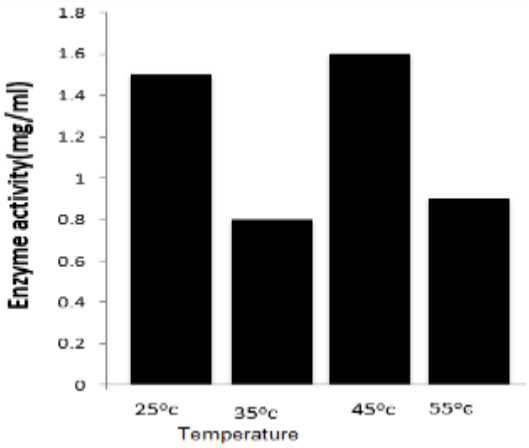

Fig 2. Effect of temperature on activity of amylase production by *E.*faecalis

This result agreed with the finding of Damir and Tari (2016) with heat bran by*Aspergillus* for polygalacturonase production. Figure 2 shows the effect of temperature on bacterial isolate. Highest temperature of 55 shows the lowest absorbance of 0.970 while temperature of 45 shows a highest absorbance of 1.691.Temperature is one of the important factorthat affect the metabolic activity of enzymes. Enzymes are more active at optimum temperature, and enzymatic reaction proceeds at maximum rate. The high temperature of 55^{0}C and 35^{0}C showed the absorbance value of 0.970 and 0.983 respectively. While the less incubation temperature of 25^{0}C and 45^{0}C shows the highest absorbance value of 1.673 and 1.691 respectively. This result confirmed the finding of Shibata *et al.* (2007) who reported that *Enterococcus specie* have the ability to produce

amylase at room temperature in a short period of time. There is a close-relationship between the growth of state culture and the acid production pattern of the medium as showed in figure 2. Increase incubation temperature from 25^0C to 35^0C decrease the rate of production. This result confirm with the finding of Panesar*et al. (*2010) who report a decrease in lactic acid production at increase in temperature from 30^0C to 37^0C.The result does not agree with the finding of Hauwa H. *et al*. (2019) who recorded the increase in amylase activity at temperature increase on study in production of bio-enzyme with banana peels by *Aspergillus niger*.

Conclusion: It is concluded from this study thatSubstrate concentration and temperature had significant effect on production of enzyme using *Enterococcus faecalis*. Agro wastes are important substrate to be utilized in the production of enzymes at low cost instead of dumping and allowed for natural degradation. It's also concluded that soil is an important reservoir for microbial exploitationfor production of important product.

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