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PHYSICO-CHEMICAL CHARACTERIZATION OF ALPHA AMYLASE FROM *BACILLUS SUBTILIS* IMD34 USING FOOD WASTE AS SUBSTRATE

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ABSTRACT

Amylase are the most important group of industrial enzymes that hydrolyze starch into amylopectin. The industrial demand of amylase predominantly from the microbial origin has been recently increased and enhances the research for amylase with high stability at extreme industrial conditions. Thus, this study is aimed to characterize amylase from *Bacillus subtilis* IMD34 obtained from previous using agro waste. Extracellular amylase production was carried out in basal medium by submerged fermentation technique. The production medium with 10% potato peels at pH 6.8 and 35 °C enhanced amylase activity (720 U/mL) than other wastes such as banana peels and yam peels. This optimized media increased the bacterial growth rate and activity of amylase compared to the non-supplemented basal medium. Further, amylase was purified and assessed their molecular weight (~39 k Da) in 12 % SDS -PAGE. The enzyme activity was observed at pH 6.8 and 35°C and stable over a wide range of pH (4-10), and temperatures (20-50°C). In addition, enzyme activity was stimulated in the presence of Mg^{2+}, Ca^{2+} and Mn^{2+} and was unpretentious after treating with surfactants (SDS, Triton X-100, Tween 80), organic solvents (ethanol, methanol, chloroform and acetone) and amylase inhibitors (EDTA and β-mercaptoethanol). These enzymic characteristics makes the amylase obtained in this study a promising candidate for various industrial sector application.

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1. Introduction

Amylase has been one of the leading hydrolases to the enzyme market in the last decade as their demand continue to increase with an expected market value estimated at US$14,506million in the year 2027[1]. Amylases are important in the following industries including food, detergent, pharmaceuticals and biotechnology [2]. Living things including plants, animals and microbes produces amylase [2-4], but microbial amylase production is preferred as it has an edge over other sources due to microbe's amenability and bulk recovery in fermentation [5-7]. Amongst these bacteria, *Bacillus* sp. is the most prominent source of soluble extracellular enzymes especially amylases with potential to secrete more than 20g/L [8-10]. Also, *Bacillus* sp. is ubiquitous to soil environment and aids in the recycling of nutrients through the secretion of biomolecule degrading hydrolases like amylase, cellulase, protease, etc. *Bacillus* strains have been reported to tolerate hash conditions, depletion in nutrient and abiotic stress in fermentation process [9]. Researchers have made used of microbial production of α-amylase in a separate fermentation medium by bacteria [8,9]. One of the greatest hurdles in the large-scale production of enzymes is the substrates' cost, predicted at 30-40% of the entire process [10]. Food wastes products, such as those obtained from bananas, potatoes, yam, and plantain, could be a source of low-cost carbon substrate in submerged fermentation [10-12]. These food wastes comprise carbon and nitrogen nutrients essential for the growth and metabolism of microorganisms. Diverse form of bacteria colonizes food waste and can be considered a source of primary and secondary metabolites [13, 14]. These wastes could be employed in the production of high value products with great commercial and economic values and this also enable the remediation of the waste from the environment as it could serve as a nuisance to the public. Organic wastes have been utilized for producing garbage enzymes which are used as antimicrobial agents to treat domestic, municipal and industrial drainage system and to clean air and remove the bad odour [15]. To enhance the cost-effective production of various industrial important enzymes, the massive agro waste have been used as an alternative and sustainable substrate [16]. With the rising in the demand for amylase, there's a drive in the search for low cost and sustainable substrate for large scale production of this valuable biomolecules. *Bacillus subtilis* IMD34 have been studied previously [24-25] using placket Burman design and optimization of process parameters for α-amylase production using submerged fermentation methods to be an excellent amylase producer, but the purification and characterization of this enzyme using physical and chemical properties were not carried out. In view of the above, the study was aimed at the effect of various food wastes on amylase production, purification and characterization of extracellular α-amylase produced by *Bacillus subtilis* IMD34 and to design a low-cost production media with high yield capacity.

Key words: Amylase, *Bacillus subtilis* IMD34, Agro-waste and physico-chemical characteristics

2.MATERIAL AND METHODS

Amylolytic bacterial isolates

Amylase producing bacterial isolate *Bacillus subtilis* IMD34 was obtained from previous studies [24-25]. All chemicals used in the experiment were of analytical grade. The organism was maintained on nutrient agar (Oxoid, Difco, USA) slant and preserved at 4^0C.

Submerged Fermentation

All experiments were carried out in triplicate using 250ml Erlenmeyer flasks containing 30ml basal media (soluble starch, 6g; peptone, 6g; Mg SO4.7H2O, 0.5g; K2HPO4,2g;

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Na2HPO4, 5g; NaCl, 2g; (NH4)2SO4, 4g; Fecl3.6H2O, 0.05g; CaCl2.2H2O, 0.05g; distilled water 1000ml, pH adjusted to 7.0) pH adjusted to 7.0. Flasks were autoclaved at 15psi and 121^{0}C for 15 minutes, after which they were inoculated with 18h old culture with cell concentration of 2x10^8 using aseptic procedure and incubated on an orbital incubator shaker (Model 10X400.XX2.C, Sanyo Gallenkamp PLC, UK) at 37^{0}C and 100rpm for 24h [3,24].

Optimization of production media

Effect of food waste as substrate in α-amylase production

The food waste used in this research which were locally collected, comprise potato peels, yam peels and banana peels. The wastes were cleaned, and dried at 65°C in an oven. The products were milled using a blender before use to obtain fine powder. These wastes were assessed for their effect on bacterial growth and α-amylase production. The amylase production liquid medium was supplemented individually with different concentration of (0, 5, 10, 15 %) of food wastes and inoculated with 2 % of broth culture containing 2×10^{8}CFU/mL. It was incubated at 37^{0}C and 100 rpm in orbital shaker. Amylase activity was measured at interval of 4 h for up to 72h of fermentation period. The proximate compositions of food waste were determined by following association of official analytical chemists' protocol [16].

Effect of pH and temperature

The bacterial isolate was grown in amylase production media adjusted with pH ranging from 4-9 (at the interval of 1 unit) and incubated at 37^{0}C. The enzyme activity was assayed after 48 h incubation. Similarly, the amylase production was carried out at different temperature by incubating the culture broth at various temperatures (25, 37, 45, and 55^{0}C) and the enzyme activity was measured after 48h incubation.

Cell growth and amylase production kinetics

The optimized production media was inoculated (pH 7) with 2 % of 24 h old *Bacillus subtilis* IMD34 broth culture (2×10^8 CFU/mL) and incubated for 48 h at 37^{0}C. Cell growth was measured by recording the OD_{520nm} value of broth culture for every 24 h. Similarly, the enzyme activity was checked using cell free supernatant (CFS) aliquots collected aseptically at every 24 h.

Extraction and purification of α-amylase

The culture fluid was centrifuge using a refrigerated high speed ultra-centrifuge at 10,000xg (4^{0}C) for 5minutes, after which the cell free supernatant (CFS) was assayed for extracellular α-amylase using DNSA reagent method [25]. A standard curve was prepared with glucose and the calibration curve so established was used to convert the spectrophotometer values to glucose equivalent and activities calculated [24-25]. In order to achieve maximum precipitation of enzyme, several (NH4)2SO4 concentrations (30, 40, 50, 60 and 70%) were used. Ammonium sulphate was used for salting out preferentially because it is soluble in water and high ionic strength can be attainable. At high ionic strength, salt may remove water of hydration from proteins and reduce solubility, hence proteins are coagulated. Crude enzyme extract was saturated to different concentrations of $(NH_4)_2SO_4$ by adding calculated amount of $(NH4)_2SO_4$ in 10 mL crude extract under constant day. Then it was kept overnight at 4°C and centrifuged as 15000 rpm at 4°C by the centrifuge. The pellets were collected, dissolved in minimum quantity of buffer and dialyzed against distilled water while the supernatant was discarded [17-19]. Total protein content was determined by the Lowry's method with bovine serum albumin as a standard [20].

Gel filtration chromatography

A pooled fraction from dialysis loaded on Sephadex G-50 gel filtration column (16×2 cm) was equilibrated with 50 mM malonate buffer of pH 4.5. The 200 µL/run of sample applied and 100 mM phosphate buffer (pH 6.0) having 0.15 M NaCl was used as elution buffer and 20 major positive fractions collected with the flow rate of 0.5mLmin^{-1}.

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After each purification step, the total protein content and enzyme activity were determined to calculate specific activity and purification factor (Irshad et al., 2011).

Characterization of α-amylase

Molecular weight determination of purified αamylase

Sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE) analysis

The molecular weight of purified α-amylase was determined by SDS-PAGE with 12 % gel. The gel was stained with Coomassie Brilliant Blue R-250 and the enzyme molecular mass was compared with standard protein ladder of 11-250 k Da. (New England Biolabs, USA).

Effect of physical and chemical factors on α-amylase activity

pH effect

Alpha amylase activity was assayed at different pH levels ranging from 3 to 9. α-Amylase was analyzed at pH 3 to 3.5 in 100 mM succinate buffer, pH 4 to 5 in 100 mM citrate buffer, pH 6 to 7 in 100 mM phosphate ($Na_2H_5O_4$ and NAH_2SO_4) buffer, and pH 8 to 9 in 100 mM sodium phosphate buffer.

Temperature effect

Purified α-amylase was evaluated at different temperature ranging from 10 to 100^0C at optimum pH 5.5; the enzyme was incubated at varying temperatures for 15 min.

Substrate concentration effect: determination of Km and Vmax

The Michalis-Menten kinetic constants (*K*m, *V*max) were determined by using varying concentrations of starch ranging from 0.5 to 3 mg/mL following the method described by Metin *et al.* (2010).

Metal ions effect

Purified amylase was treated with various metal ions such as magnesium, calcium, ferrous, manganese, copper, zinc and mercury (1 and 5 mM) incubated at 15minutes

Surfactant Effect

Surfactants such as SDS, Triton X-100, Tween 80 (1, 5 and 10 %) incubated at 15minutes

Organic Solvent Effect

Organic solvents such as ethanol, methanol, chloroform, acetone, hexane (10 and 20 %) incubated at 15 minutes

Inhibitors Effect

Inhibitors such as ethylenediaminetetraacetic acid (EDTA), phenylmethylsulfonylfluoride (PMSF), tetramethyl ethylenediamine (TEMED) and β–mercatptoethanol (1 and 5 mM) for 1 h and then their residual activity was assessed.

The enzyme was incubated at 35°C (assay temperature) in the presence of varying concentration solutions (pH 5.5) of the respective compounds for 10 to 15 min [17].

3.RESULTS AND DISCUSSION

Table 1: Proximate composition of food waste used to produce α-amylase from *B. subtilis* IMD34

% composition	Potato peels	Banana peels	Yam peels
Carbohydrate	67.41	60.21	63.14
Protein	12.42	10.42	11.62
Fat	0.95	0.82	0.88
Moisture	0.84	1.29	1.22
Other	18.38	27.26	23.14

Table 2: Protein concentration and specific activity of α-amylase from *B. subtilis* IMD34 employing different purification procedures

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Purification Procedures	Enzyme volume	Total protein(mg/ml)	Amylase activity(U/ml)	Specific activity(U/ml)	Purification fold
Crude amylase from basal media	480	920	3766.8	6.0	1.0
Crude amylase from optimized media	485	915	3776.2	6.2	1.2
Ammonium sulphate precipitate	15	48.20	2014.6	58.0	12.8
Dialysis sample	10	34.10	1974.0	76.10	18.4
Gel filtration	6	8.20	924.10	124.0	36.2

Table 3: Metal ions, Inhibitors, surfactant and organic solvent effect on purified α-amylase activity carried out in triplicate

Ions	**Concentration**	**% residual activity**
Mg^{2+}	1mM	85±3.5
	3mM	95±2.6
Ca^{2+}	1mM	115±3.8
	3mM	154±2.4
Fe^{2+}	1mM	74±2.2
	3mM	80±4.0
Mn^{2+}	1mM	92±2.2
	3mM	82±3.5
Inhibitors		
EDTA	1mM	100±0
	3mM	96±3.6
PMSA	1mM	72±2.8
	3mM	42±3.2
Mercaptoethanol	1mM	92±4.2
	3mM	80±3.8
Surfactant		
SDS	1mM	92±2.6
	3mM	64±3.6
Triton X-100	1mM	98±4.0
	3mM	84±3.8
Tween 80	1mM	96±2.2
	3mM	80±4.5
Organic solvents		
Ethanol	5%	98±4.0
	10%	95±3.5
	15%	84±2.8
Methanol	5%	90±3.0
	10%	86±2.5
	15%	78±3.4
Chloroform	5%	98±4.2
	10%	90±3.5
	15%	88±2.2
Acetone	5%	86±4.6

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	10%	78±4.0
	15%	68±3.8

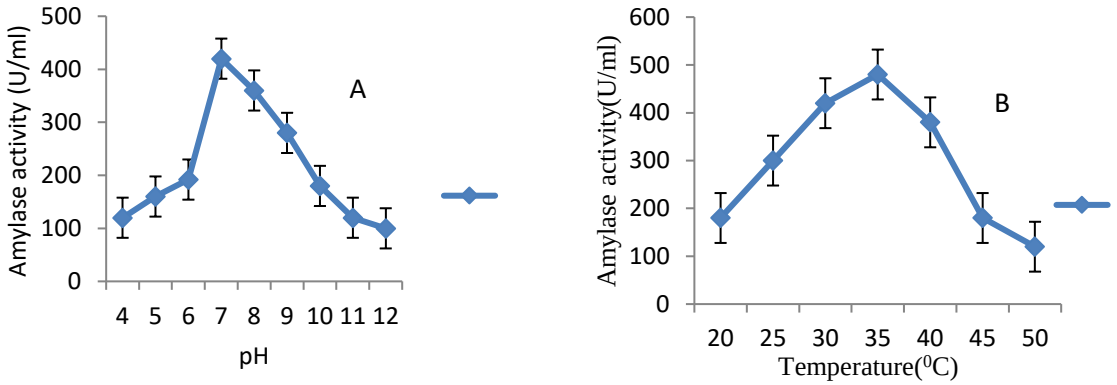
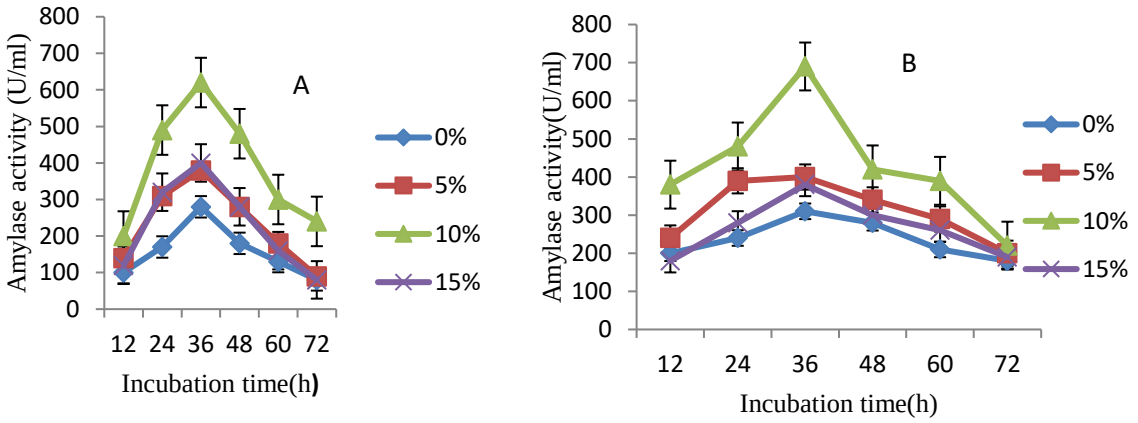


Figure 1: Kinetic study of cell growth and α-amylase production (activity) (a) basal media (b) media with food waste as carbon source incubated at 35^{0}C for 48h performed in triplicate.



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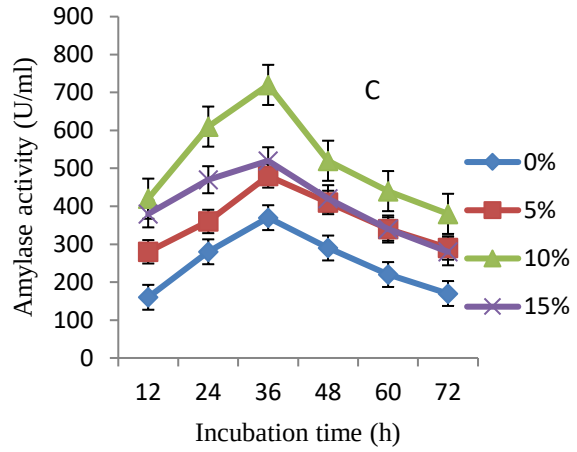

Figure 2: α-amylase production by *B. subtilis* IMD34 in submerged fermentation using optimized media of food waste (a) banana peels (b) yam peels (c) potato peels.

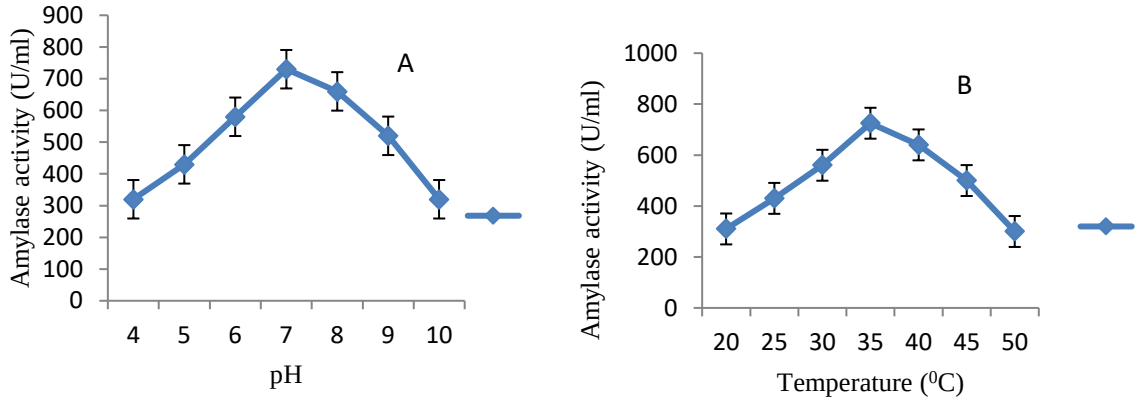

Figure 3: Effect of (a) pH (b) Temperature on α-amylase production using optimized substrate (10% potato peels) after 48h of incubation.

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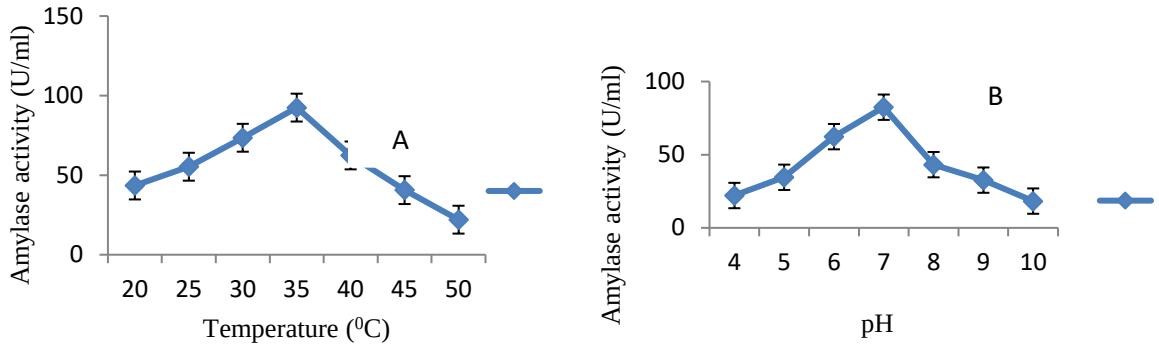

Figure 4: Purified α-amylase activity treated at various (a) temperature (b) pH under 2h.

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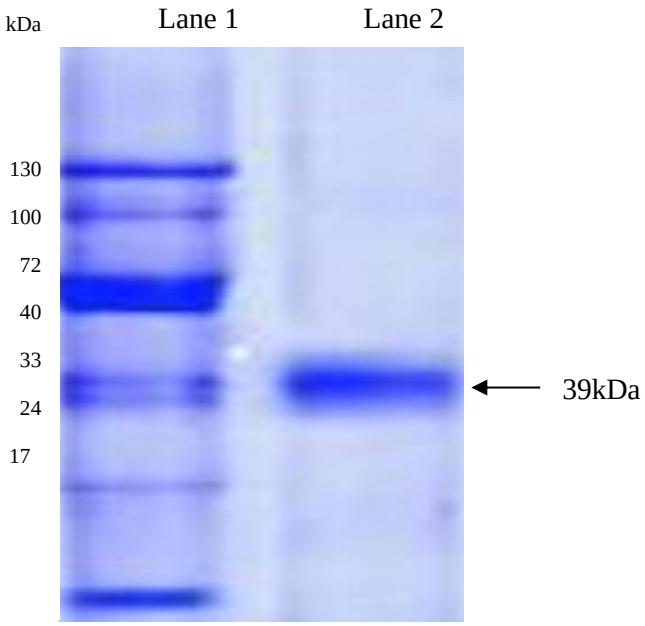

Figure 5: SDS PAGE analysis of purified amylase lane 1: marker protein, lane 2: purified fraction 7.

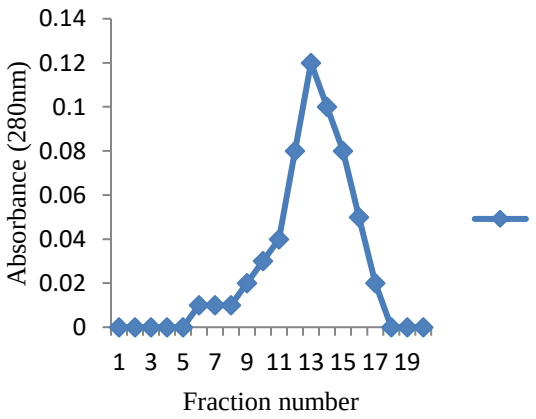

Figure 6: Gel filtration chromatography of α-amylase produced by *Bacillus subtilis* IMD34.

Nigeria, the most populated country in African continent agricultural sector produces huge quantity of solid waste that may be global health threat if they are allowed to accumulate extensively for long time. Thus, such waste must be remediated/recycled to produce added value products which is useful for her teaming population and reduces the risk of health crises. Agro wastes have been used to

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produce the industrial important enzymes such as L-asparaginase [18], alpha amylase [18,20], xyalanase [19], inulinase [21], protease [22], etc. This study is aimed at the utilization of agro waste products such as banana peels, yam peels and potato peels as substrate for amylase enzyme production. *Bacillus* specie is well known as one of the most important genera that produces biocatalyst of industrial value currently harnessed in various sectors, including food, beverage, pharmaceutical, medical, leather and detergent [21]. This special industrial interest is not only due to their generally recognized as safe (GRAS) status and also to their fastest growth and their potential for secreting extracellular enzymes.

Amylase activity of *B. subtilis* IMD34

The ability of *B. subtilis* IMD34 to produce alpha amylase under submerged fermentation condition was evaluated using basal medium at pH 6.6 reported earlier [24]. The figure 1a shows that after 36 h incubation the growth was continuously increased up to 48 h and followed by a steep decline. Nevertheless, the enzyme production increases from 24 h continuously up to 36h along with the growth pattern. Our findings are in concurrent with the report of [23], who stated growth of *Bacillus subtilis* Y25 peaked at 36 h of incubation period beyond which a steady decline occurred. A similar pattern of bacterium growth and α-amylase production, in relation to incubation period, was reported by [19]. Our observations were in contrary to the growth pattern of *Bacillus* sp. ST that reached a stationary phase after 26 h only [7] indicating the variability in growth pattern of strains due to their source, growth conditions and other parameters. The maximum enzyme production in this study was at 36 h with highest amylase activity (420 U/mL) (figure 1a) then the production rate tends to decrease gradually up to 48 h and a steep decrease up to 60 h. The enzyme secretion pattern of the current strain is quite similar to that reported [17]. Maximum enzyme production in this study was observed at stationary phase. All these findings clearly revealed the key role of extracellular amylase in metabolism and organism's survival [17].

Effect of pH and temperature on amylase production of *B. subtilis* IMD34.

Bacillus species is commonly preferred for industrial production of enzymes due to their acidophilic, alkalophilic and thermophilic potential and this varied from strain to strains based on their source and environmental conditions. Furthermore, the growth temperature and media pH are the most critical factors influencing enzyme production. Thus, in this study, the current *B.subtilis* IMD34 strain was assessed for efficiency to produce the extracellular amylase under various pH and temperatures in broth supplemented with potato peels (10%). The *B. substilis* IMD34 strain could produce amylase at wide range of pH from 4-10 and maximum production (780 U/mL) was observed at pH 6.8 (Figure 3a). At pH 4-5, the lesser enzyme production was recorded which increases gradually to peaked at pH 6.8 indicating the importance of growth pH in metabolic functioning of bacteria especially during biomolecules secretion. The optimum pH for *B. subtilis* Y25 α-amylase production was reported to be 8.0 [23]. [10] had a similar pH for α-amylase

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production from *B. aquimaris* VITP4. Our findings had a similar pH, as reported for α-amylase production from strains of several *Bacillus* species to be pH 7.0 [13]. Yang et al. [12] had reported that αamylase production from Bacillus species mostly occurs in pH range of 6 to 10.0. The optimum pH has an effect on the conformation of the active to the substrate. Any change in this optimal pH translate to an active site conformational change which affects the biomolecules rate of reaction [13]. It has been clearly stated that a bacterial strain growing under certain pH condition require a similar condition for their better metabolic process. With respect to temperature, the isolate highest amylase activities (680U/ml) were observed at 35^0C (figure 3b). This result is in contrary to the report of Alajadena et al., [23] who observed maximum production of α-amylase to occur at temperature of 450C. There have been similar reports on incubation temperature for amylase production from *B. licheniformis* RD24 [16] and *B. cereus* Ms6 [18]. At high temperatures, enzyme production decreased which might be due to growth inhibition and hence enzyme inactivation [14]

Optimization of amylase production media under submerged fermentation

The figure 2a, b & c depicted that amylase production was greatest in submerged fermentation with agro- waste as substrate. Among the three substrates (banana peels, yam peels and potato peels), 10 % potato peels supplemented fermentation media showed highest enzyme activity (720 U/mL) (figure 2C) at 36 h incubation at 35°C. Further incubation decreased the enzyme production because the substrate became limiting to the bacterial growth. This is followed by banana peels with 690 U/mL of enzyme activity at 36 h incubation (Figure 2B). The current result agreed with the report that potato peels is rich in lignin (6–12%), pectin (10–21%), cellulose (7–10%), and hemicelluloses (6–9.4%%), hence has been used as s substrate to produce various enzymes [16]. Alpha-amylase by *Bacillus subtilis* is one of the studies that have been designed to accomplish industrially important enzymes using potato peels. Similarly, De et al., [12] used Bnanana peels to produce microbial amylase by bacterial isolate. The proximate composition of three waste products, banana peels, yam peels and potato peels were assessed and showed in table 1. High amount of carbohydrate and protein was present in potato peels followed by banana peels and these two waste products only produced amylase with high enzyme activity indicating nutritional composition especially carbohydrate and protein content majorly influencing the amylase production during submerged fermentation. This result agrees with the report of Ozdemir et al., [18] who stated that substrate rich in carbon and nitrogen content do enhance bacterial growth and improved fermentation process.

Kinetics of cell growth and amylase production

Process parameters were optimized with pH 6.9 and temperature 35^0C for amylase production with media supplemented with 10% of potato peels (based on the optimization study). The figure 3A showed that the amylase activity and growth increase steadily from pH 4 to 5 and in a steep pattern to peaked at pH 6.8 at 36h of incubation

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thereafter enzyme activities decrease steadily. Amylase secretion goes hand-in - hand with increase growth pattern. The maximum amylase activity (720 U/ml) was attained at 36 h. After that enzyme production was stayed nearly constant up to 48 h. Yang et al. [21] reported that maximum production of amylase (573 U/mL) was at 48h of incubation. The present results clearly indicate that *B. subtilis* IMD34 could completely utilized the substrate and other nutrients within lesser period (36 h). Thus, the present strain, IMD34 could be effective in utilizing agro-waste which are a cheaper source of substrate and convert them into valuable amylase enzyme. The amylase produced from submerged fermentation was pelleted by ammonium sulphate precipitation and purified further by dialysis against sodium phosphate buffer. The dialysate was further checked for their protein concentration and enzyme activity. The protein concentration and specific activity of the amylase obtained from basal broth, optimized broth, ammonium sulphate precipitated sample and dialysate were listed in Table 2. The enzyme activity was comparatively higher than that of the previously reported amylase.

The molecular mass of purified amylase was analyzed by SDS-PAGE.

The existence of one band revealed the amylase purity and its readiness for industrial application. The molecular weight was about 39 kDa (Figure 5). The molecular weight of amylase from *Bacillus* sp. was reported to vary between 40.0 and 60.0 KDa with only a few exceptions [22]. Yang et al. [21] found that the molecular weight of *B. licheniformis* amylase was 53.13 KDa. Annamalai et al. (2014) reported a similar kind of result (21 kDa) for amylase produced by *Bacillus* sp.

Effect of pH, temperature, metal ions, surfactants, organic solvents and inhibitors on enzyme activity

The amylase activity was checked after treating at various physical and chemical conditions to assess their optimum reactive condition and stability. The purified amylase of B. subtilis IMD34 was active over a wide range of pH from 4 to 10 and maximum activity (100 %) was observed at pH 6.8 and minimal activity (45 %) was observed at pH 10. Further, the amylase was stable over low pH 5 and retained 50 % of its activity whereas at pH 8.5, 70 % activity was retained (Figure 4B). The results show a wide range of pH stability of amylase from *B. subtilis* IMD34 and this characteristic makes the enzyme a good candidate for use in various industrial applications including food, detergent, tanning, etc. Hadder et al. (2009) reported that pH 9-12 is optimum for anticipated industrial applications and thus the current amylase is found to be a good fit for such industrial applications. Similarly, the amylase from *B. subtilis* IMD34 was active over a wide range of temperature ranging from 20 to 50°C revealing their thermostability nature. The maximum enzyme activity (100 %) was recorded at 35°C and the enzyme retained its activity up to 50°C with 30 % (Figure 4A) of its activity. Thus, the optimal temperature for amylase activity was 35°C which is concurrent with previous report for *B. subtilis* B22 [8] and *B. firmus* CAS 7 [10]. 473U/mL from *B. firmus*

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[13], 432 U/mL from *B. subtilis* B22 [15], 380 U/mL from *Bacillus* sp. [20].

Table 3 show the activity of amylase in the presence of various metal ions, surfactants, organic solvents and inhibitors. The enzyme activity was stimulated when treated with Mg^{2+}, Ca^{2+} and Mn^{2+} and significantly reduced by Fe^{2+} treatment. A similar kind of stimulatory effect of Mg^{2+}, Ca^{2+}, Mn^{2+} was reported [11] and Deb et al., [12]. These metals (Mg^{2+}, Ca^{2+}, Mn^{2+}) may prevent the unfolding of the amylase by which they preserve the native form and activity of the enzyme [15]. In contrast, metal ions, Fe^{2+} affect the active site of the enzyme and leads to reduction in enzyme activity [8].

Various surfactants treatment was also carried out on purified amylase, the enzyme showed 98% activity at 1mM of non-ionic surfactants (Triton X-100 and Tween 80) and the activity were decreased to 84% and 80% after treated with 3mM of Triton X-100 and Tween 80 respectively. But the anionic detergent like SDS reduced the amylase activity from 92 % and 64 % at 1 and 3mM concentration. The amylase exhibited 98 % of activity after treated with 5% ethanol but drops with increase in concentration to 15% of the solvent, this indicates the interference of the solvent with enzyme activity. More than 80% of amylase activities was recorded for methanol, chloroform and acetone. Generally, solvents are toxic in nature and can disturb the structural and hydrophobic interactions leading to lose of enzyme activity [6]. Our findings show that amylase from *B. subtilis* IMD34 was solvent tolerant. This is concurrent to the reports from *B. subtilis* DR8806 [4], *B. subtilis* B22 [5], *B. cereus* [3], *B. licheniformis* K7A [2]. The tolerance of amylase against the solvents is due to the presence of large amount of acidic (negative charged) amino acids than basic (positively charged) amino acids [15] and the negative charges makes more stable by forming a hydrated ion network with cations [22]. Similarly, the amylase inhibitors like EDTA and β-mercaptoetahnol did not show significant effect on enzyme activity at their 1 mM concentration and 5mM concentration with activities at the range of 80-100%. It was observed that the least concentration of PMSF (1 mM) minimized the activity to 72% and at 5 mM concentration enzyme activity was not noticeable. The amylase was not affected by EDTA revealing it as a metalloamylase class of enzymes as the catalytic site remains intact, on the contrary PMSF completely inhibited the enzyme activity. This result is in consistent with previous reports on *B. licheniformis* [11] and *B. subtilis* B22 [22]

4.CONCLUSION

The production of microbial amylase by exploiting agro wastes not only gives insight on the burden of environmental pollution but also increases the economic value of these wastes. In addition, these wastes could also improve the yield of enzyme at cost effectively. In this present study, an amylase producing bacterial isolate, *B. subtilis* IMD34 obtained from previous studies was used. The amylase production was significantly improved (720 U/mL) in optimized media containing 10 % potato peels at pH 6.8 and 35°C. The active site of enzyme was stimulated by metal ions such as Mg^{2+}, Ca^{2+} and Mn^{2+}. In addition, the amylase exhibited

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higher tolerance to wide ranges of physical conditions (pH and temperatures), surfactants (SDS, Triton X-100 and Tween 80), various solvents (ethanol, methanol, chloroform, and acetone) and inhibitors (EDTA and β-mercaptoetahnol). These potential characteristics make this enzyme as an efficient candidate for commercial production of amylase at cost effective manner and their application in various industrial sectors like detergents, leather, food, etc.

5.REFERENCES

[1] Abdel-Fattah, Y. R., Soliman, N. A., El-Toukhy, N. M., El-Gendi, H., & Ahmed, R. S. (2012). Production, purification and characterization of thermostable α-amylase produced by *Bacillus licheniformis* isolate AI20. Journal of Chemistry, 20(13),1-11. https://doi.org/10.1155/2013/673173.

[2] Anupama, A., & Jayarama, G. (2011). Detergent stable, halotolerant α-amylase from *Bacillus Aquimaris* VITP4 exibits reversible unfolding. International Journal of Applied Biology and Pharmaceutical Technology, 2(2), 366-376.

[3] Asgher, M., Asad, M. J., Rahman, S. U., & Legge, R. L. (2007). A thermostable α-amylase from a moderately thermophilic *Bacillus subtilis* strain for starch processing. Journal of Food Engineering, 79,950-955. https://doi.org/10.1016/j.jfoodeng.2005.12.053

[4] Ayansina, A. D. V., Adelaja, A. O., & Mohammed, S. S. (2017). Characterization of amylase from some *Aspergillus* and *Bacillus* species associated with cassava waste peels. Advances in Microbiology, 7, 280-292.

[5] Azad, M. A., Bae, J. H., Kim, J. S., Lim, J. K., Song, K. S., Shin, B. S., & Kim, H. R. (2009). Isolation and characterization of a novel thermostable alpha-amylase from Korean pine seeds. Nature Biotechnology, 26, 143-149. https://dx.doi.org/10.1016/j.nbt.2009.09.006

[6] Bakare, M. K., Omoboye, O. O., Adewale, I. O., Awojobi, K. O., & Oyedeji, O. (2014). Purification and characterization of thermostable alpha-amylase by *Bacillus licheniformis* RD 24 isolated from decayed refuse at Obafemi Awolowo University Campus, Ile-Ife, Nigeria. Frontiers of Biological and Life Sciences, 2(4), 74-84. http://dx.doi.10.12966/fbls.12.03.2014

[7] Benjamin, S., Smitha, R. B., Jisha, V. N., Pradeep, S., Sajith, S., Sreedevi, S., Priji, P., Unni, K. N., Sarath, Josh, M. K. (2013). A monograph on amylases from *Bacillus* spp. Advances in Biosciences and Biotechnology, 4, 227-241. doi:10.4236/abb.2013.42032.

[8] Bijttebier, A., & Goesaert, H., & Delcour, J. A. (2008). Amylase action pattern on starch polymers. Biologia, 63(6), 989-999.

[9] Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical Biochemistry, 72, 248-254 https://doi.org/10.1016/0003-2697(76)90527-3.

[10] Castro, A. M., Carvalho, D. F., Freire, D. M. G., & Castilho, L. R. (2010). Economic analysis of the production of amylases and

[DOI: 10.22034/FSCT.21.155.121]

other hydrolases by *Aspergillus awamori* in solid-state fermentation of babassu cake. Enzyme Research, 1-9. http://dx.doi.org/10.4061/2010/576872

[11] Das, S., Singh, S., Sharma, V., & Soni, M. L. (2011). Biotechnological applications of industrially important amylase enzyme. International Journal of Pharma and BioSciences, 2(1), 486-496.

[12] Deb, P., Talukdar, S. A., Mohsina, K., Sarker, P. K., & Sayem, S. A. (2013). Production and partial characterization of extracellular amylase enzyme from *Bacillus amyloliquefaciens* P-001. Springerplus, 2, 154. http://dx.doi.org/10.1186/2193- 1801-2-154.

[13] Demirkan, E., Sevgi, T., & Baskurt. M. (2017). Optimization of physical factors affecting the production of the α-amylase from a newly isolated Bacillus sp. M10 strain. Karaelmas Fen ve Muhendislik Dergisi, 7(1), 23-30. http://dx.doi.org/10.7212%2Fzkufbd.v1i1.458

[14] El-Helow, E. R. (2001). Identification and molecular characterization of a novel *Bacillus* strain capable of degrading tween-80. FEMS Microbiology Letters, 196(2), 119-122. https://doi.org/10.1111/j.1574-6968.2001.tb10551.x

[15] El-Kady, E. M., Asker, M. S., Hassanein, M. S., Elmansy, E. A., & El-Beih, F. M. (2017). Optimization, production and partial purification of thermostable α-amylase produced by marine bacterium *Bacillus* sp. NRC12017. International Journal of Pharmaceutical and Clinical Research, 9(8), 558-570. https://dx.doi.org/10.25258/ijpcr.v9i08.9581

[16] Elmansy, E. A., Asker, M. S., El-Kady, E. M., Hassanein, S. M., & El-Beih, F. M. (2018). Production and optimization of α-amylase from thermo-halophilic bacteria isolated from different local marine environments. Bulletin of the National Research Centre, 42, 31-37. http://dx.doi.org/101186/s42269-018-0033-2

[17] Femi-Ola, T. O., & Olowe, B. M. (2011). Characterization of alpha amylase from *Bacillus subtilis* BS5 isolated from Amitermes evuncifer Silvestri. Research Journal of Microbiology, 6, 140-146. http://dx.doi.org/10.3923/jm.2011.140.146

[18] Ozdemir, S., Fincan, S. A., Karakaya, A., & Enez, B. (2018). A novel raw starch hydrolysing thermostable α-amylase produced by newly isolated *Bacillus mojavensis* SO-10: purification, characterization and usage in starch industries. Brazilian Archives of Biology and Technology 61, 103-107. http://dx.doi.org/10.1590/1678-4324-2018160399.

[19] Prakash, B., Vidyasagar, M., Madhukumar, M. S., Muralikrishna, G., & Sreeramulu, K. (2009). Production, purification and characterization of the extremely halotolerant, thermostable and alkali-stable α-amylases from *Chromohalobacter* sp. TVSP 101. Process Biochemistry, 44, 210-215. https://doi.org/10.1016/j.procbio.2008.10.013.

[DOI: 10.22034/FSCT.21.155.121]

[20] Tamura, K., Stecher, G., Peterson, D., Filipski, A., & Kumar, S. (2013). MEGA 6: molecular evolutionary genetics analysis version 6.0. Molecular Biology and Evolution, 30(12), 2725-2729. http://dx.doi.10.1093/molbev/mst197.

[21] Yang, H., Liu, L., Li, J., Du, G., & Chen, J. (2011). Heterologous expression, biochemical characterization, and overproduction of alkaline α-amylase from *Bacillus subtilis*. Microbial Cell Factories, 10, 77. http://dx.doi:10.1186/1475-2859-10-77.

[22] Zeng, J., Gao, X., Dai, Z., Tang, B., & Tang, X. F. (2014). Effects of metal ions on stability and activity of hyperthermophilic pyrolysin and further stabilization of this enzyme by modification of a Ca^{2+}-binding site. Applied and Environmental Microbiology, 80(9), 2763-2772. http://dx.doi:10.1128/AEM.00006-14.

[23] Aladejana, O. M., Oyedeji, O., Omoboye, O. O., & Bakare, M. K. (2020). Production, purification and characterization of thermostable alpha amylase from *Bacillus subtilis* Y25 isolated from decaying yam (*Dioscorea rotundata*) tuber. Notulae Scientia Biologicae, 12(1), 154-171. http://dx.doi:10.15835/nsb12110521.

[24] Henshaw, E. E., & Sherifat, W. (2019). Effect of Agitation Speed and Incubation Time on Amylase Production by *Bacillus* Species Isolated from Malted and Fermented Maize (*Zea mays*). Microbiology Research Journal International, 27 (3), 1-7.

[25] Henshaw, E. E., & Lennox, J. A. (2022). Optimization of Independent Variables for the Production of Extracellular Alpha Amylase by *Bacillus subtilis* IMD34 Using Plackett-Burman Design. Sultan Qaboos University Journal for Science, 27 (2), 77-83.

[DOI: 10.22034/FSCT.21.155.121]