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Optimization of Carbon and Nitrogen Sources and Effect of Metal Ions on Amylase Production by Lactic Acid Bacteria Strains Isolated From Fermented Starchy Foods

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ABSTRACT

α-Amylases (EC 3.2.1.1; 1,4-α-D-glucanglucanohydrolase) are enzymes that are widely studied and used in industries. Amylase production is affected by substrate and heavy metals. The effects of various carbon and nitrogen sources and heavy metals concentrations on α-amylase production by submerged fermentation (smF) from lactic acid bacteria strains were investigated. Amylase producing bacteria were isolated from fermented starchy samples collected from local producers in Aba, Abia state thereafter screened for amylase activity on starch agar medium. Effect of carbon and nitrogen sources on amylase production was carried out. The effects of nutrient conditions on the amylase production were found to be significant ($P< 0.05$). Cassava flour (1%w/v) was found to be favorable carbon source for α-amylase production by all the strain with the amylase activity of 1.16 U/ml for *Lactobacillus plantarum*FMO2, 0.80 U/ml for *Lactobacillus plantarum*Z2 and 0.92 U/ml for *Lactobacillus pentosus* BSR3 compared to the control (starch) 0.47U/ml. Of the nitrogen sources tested, peptone (1.5w/v) led to the production of maximum amount of α-amylase of 1.11U/ml, 0.84U/ml and 0.96 U/ml for *Lactobacillus plantarum*FMO2, *Lactobacillus plantarum*Z2 and *Lactobacillus pentosus* BSR3 respectively compared to the control (yeast) 0.42U/m. The effects of different heavy metals on the production of α-amylase from *Lactobacillus plantarum*FMO2 as the was activated by Ca^{2+}, strongly inhibited by Cu^{2+} but less affected by Mg^{2+} and Fe^{2+}at 0.1w/v concentrations. The application of cheaper and readily available agro-industrial substrates such as cassava flour as carbon source and peptone as nitrogen source with Ca^{2+} may be considered a better nutrient additive for optimum growth of the bacteria and amylase production.

Keywords: Amylase activity, submerged fermentation, lactic acid bacteria, metal ions.

Introduction

Enzymes play a pivotal role in various industries, ranging from food and beverage to pharmaceuticals and biofuels. Microbial enzymes are widely used in industrial processes and α-amylase holds significant importance due to its ability to catalyze the hydrolysis of starch, a complex polysaccharide, into simpler sugars. Among the wide array of enzymes, α-Amylase is considered one of the most important industrial enzymes, having applications in industrial processes such as brewing, baking, textiles, pharmaceuticals, starch processing, and detergents. The demand for amylase has surged in recent years, leading to extensive research on the identification and optimization of microbial sources for its production (Adrio and Demain, 2014).

Microorganisms have become increasingly important as producers of industrial enzymes due to their biochemical diversity and ease of improving the enzyme productivity. α-Amylases are some of the most versatile enzymes in the industrial enzyme sector and account for approximately 25% of the enzyme market (Sidhu *et al*., 1997).

Amylases are among the most important enzymes and are of great significance for biotechnology, constituting a class of industrial enzymes having approximately 25% of the world enzyme market (Rajagopalan and Krishnan, 2008; Reddy *et al.*, 2003). They can be obtained from several sources, such as plants, animals and microorganisms.

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The amylases of microorganisms have a broad spectrum of industrial applications as they are more stable than when prepared with plant and animal α-amylases (Tanyildizi *et al*., 2005). The major advantage of using microorganisms for the production of amylases is the economical bulk production capacity and the fact that microbes are easy to manipulate to obtain enzymes of desired characteristics. α-Amylase has been derived from several fungi, yeasts and bacteria. The use of amylases produced from *Lactobacillus* is considered as safe because they are non-pathogenic and the end product of fermentation is lactate which is used as flavoring agent in the food industry. Amylolytic strains of *Lactobacillus plantarum* have been isolated from fermented cassava, maize and sorghum (Guyot, 2010; Haydersah *et al*., 2012). Amylolytic strains of *Lb. fermentum* were isolated from ogi and mawe, a Benin maize sourdough product (Tchekassi *et al*., 2014), from traditionally fermented Nigerian Foods (Sanni *et al*., 2002). Optimization of culture conditions is very important for maximum microbial growth and enzyme production by microorganisms (Kathiresan and Manivannan, 2006). Among these physical and chemical factors as; optimum temperature, pH value, carbon and nitrogen sources are the most important for enzyme production by microorganisms (Gupta *et al*. 2003). Different carbon and nitrogen sources like organic and inorganic nitrogen sources are basic need for the enzyme production. Heavy metals also affect the production of α-amylase. It was strongly inhibited by Co^{2+}, Cu^{2+}, and Hg^{2+} but less affected by Mg^{2+}, Zn^{2+}, Ni^{2+}, Fe^{2+}, and Mn^{2+} (Asgher *et al*., 2007). With these nutrients, its proper amount is also important for better enzyme production (Anto *et al*., 2006; Deb *et al*., 2013). In this study, medium supplements and metal ion effect at different concentrations were checked and optimized for the large and better amylase production obtained from fermented starchy foods such as retted cassava and ogi.

Material and Methods

Sample Collection

Samples of freshly fermented maize gruel (ogi) and retted cassava (fufu) were obtained from local producers located at Umungasi in Aba North Local Government Area of Abia State. The fermented products were transferred into universal sterile bottles. These samples were packaged inside a cooler containing ice cubes and quickly brought to the laboratory in sterile containers for immediate analysis.

Lactic acid bacteria (LAB) previously isolated from fermented starchy foods, and screened to have potential for amylase production and amylase activity by the authors (Ejike and Onyeanula, 2024) and molecularly identified as *Lactobacillus plantarum* FMO2, *Lactobacillus plantarum*Z2 and *Lactobacillus pentosus* BRS3 by the authors (Ejike and Onyeanula, 2024) were employed for the present study.

Optimization of Parameters for Amylase Production

Effect of carbon sources on amylase production

The effect of carbohydrate sources was studied as described by Demissie (2014) by replacing soluble starch in basal medium with different sugars, gelatinized and raw natural crude starch sources (glucose, fructose, maltose, cassava, maize flour, rice, and sorghum flours at final concentration of 1% (w/v).

A hundred milliliter (100ml) of each media was dispensed and sterilized at 121^0C for 15mins while for the medium containing raw cassava; starch powder was sterilized at 120°C for 2 h in a hot oven and used as raw cassava starch for enzyme production. After which 5ml of the broth culture was used to inoculate the various media. Fermentation was carried out in an orbital shaker at 120rpm at 37^0C for 48hours. The culture broth was filtered through Whatman no 1 filter paper at room temperature and filterate recovered. The cell free filterate was used for amylase assay.

Effect of nitrogen sources on amylase production

Nitrogen sources were tested by replacing yeast extract with various nitrogen sources (peptone, tryptone, beef extract, soyabean meal, and urea at final concentration of 1.5% (w/v)).Each of the nitrogen sources was used as a sole source of nitrogen in place of yeast extract employed in the basal medium.

Fermentation was carried out in an orbital shaker at 120rpm at 37^0C for 48hours. The culture broth was filtered through Whatman no 1 filter paper at room temperature and filterate recovered. The cell free filterate was used for amylase assay (Demissie, 2014).

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Effect of metal ions on amylase activity

The effect of metal salts on amylase activity was determined by adding 0.1% (w/v) of metal salts ($CaCl_2.2H_2O$, $MgSO_4.7H_2O$, $FeSO_4.7H_2O$, $CuSO_4.5H_2O$) to the standard assay. The effect of metal salt on amylase activity was evaluated by pre-incubating the enzyme in the presence of the above effectors for 30 min at 70°C. The remaining activity was determined as described above Adegoke and Odibo (2019).

Statistical analysis

All the experiments were done in triplicates and data were represented as means ± standard deviation of mean. The study was subjected to one–way analysis of variance (ANOVA) using SPSS 23.0 to determine the significant variations between the tests. Mean analysis was carried out using Duncan Multiple Range test at 95% confidence level.

Results

The effect of different carbon sources (Table 1) on the production of α-amylase by *Lactobacillus plantarum*FMO2, *Lactobacillus plantarum*Z2 and *Lactobacillus pentosus* BSR3, were tested. Cassava flour was found to be favorable carbon source for the production of α-amylase by all the strain and amylase activity of 1.16U/mL for *Lactobacillus plantarum*FMO2, 0.80U/ml for *Lactobacillus plantarum*Z2 and 0.92U/ml for *Lactobacillus pentosus* BSR3 was observed.

The result of the five nitrogen sources (peptone, tryptone, beef extract, soyabean meal, and urea) at final concentration of 1.5% (w/v) substituted for yeast for the production of α-amylase from each strain is presented in Table 2. The maximum α-amylase production was obtained with peptone with 1.11U/ml, 0.84Uml and 0.96Uml for *Lactobacillus plantarum* FMO2**,** *Lactobacillus plantarum*Z2 and *Lactobacillus pentosus* BRS3 respectively.

Table 1: Effect of carbon sources on amylase activity

Carbon source (1% w/v)	Amylase activity (U/ml)		
	***Lactobacillus plantarum*FMO2**	***Lactobacillus plantarum*Z2**	***Lactobacillus pentosus* BRS3**
Starch (control)	0.47 ± 0.16^{a}	0.47 ± 0.20^{a}	0.47 ± 0.31^{bc}
Glucose	0.44 ± 0.19^{ab}	0.34 ± 0.22^{bc}	0.36 ± 0.23^{ab}
Fructose	0.36 ± 0.11^{ab}	0.23 ± 0.31^{ab}	0.22 ± 0.07^{cd}
Maltose	0.22 ± 0.10^{b}	0.30 ± 0.24^{c}	0.40 ± 0.26^{ab}
Cassava flour	1.16 ± 0.10^{c}	0.80 ± 0.17^{bc}	0.92 ± 0.06^{cd}
Rice flour	0.28 ± 0.13^{bc}	0.27 ± 0.11^{abc}	0.24 ± 0.04^{d}
Sorghum	0.45 ± 0.07^{bc}	0.37 ± 0.20^{bc}	0.30 ± 0.19^{ab}

Values are means of triplicates ± standard deviation. Means with different superscript across a row are significantly diff ($p < 0.05$).

Table 2: Effect of nitrogen sources on amylase activity

Nitroge source (1.5% w/v)	Amylase activity (U/ml)		
	***Lactobacillus plantarum*FMO2**	***Lactobacillus plantarum*Z2**	***Lactobacillus pentosus* BRS3**
Yeast (control)	0.42 ± 0.17^{a}	0.42 ± 0.17^{a}	0.42 ± 0.17^{bc}
Peptone	1.11 ± 0.25^{b}	0.84 ± 0.22^{bc}	0.96 ± 0.23^{ab}
Tryptone	0.33 ± 0.70^{a}	0.23 ± 0.31^{ab}	0.22 ± 0.07^{cd}
Beef extract	0.35 ± 0.67^{a}	0.18 ± 0.24^{c}	0.40 ± 0.26^{ab}

Values represented are means of triplicates ± standard deviation. Means with different superscript across a row are significantly different ($p < 0.05$).

Table 3 shows the effect of various metal ions Mg^{2+}, Ca^{2+}, Cu^{2+} and Fe^{2+} on α-amylase at different concentrations, when compared with the control. The relative activities in the presence of Mg^{2+}, Ca^{2+}, Cu^{2+}, and Fe^{2+} were determined as 83.6%, 117.8%, 52.1%, and 95.9% respectively. The purified enzyme was activated by Ca^{2+}, but slightly inhibited by Fe^{2+} and Mg^{2+}.

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Table 3 Effect of metal ions on amylase activity

Metal ions	Amylase activity (U/ml)	Relative activity (%)
Control (without metal)	0.71 ± 0.02^{b}	100
$CaCl_2$	0.85 ± 0.05^{c}	117.8
$MgSO_4$	0.62 ± 0.03^{b}	83.6
$FeSO_4$	0.70 ± 0.10^{b}	95.9
$CuSO_4$	0.33 ± 0.06^{a}	52.1

Values are mean ±standard deviation of triplicates analysis. Values in the same column with different superscripts are significantly different at P≤ 0.05

Discussion

In this study, different fermentation parameters (carbon and nitrogen) were optimized for α-amylase production by conducting a series of experiments. The presence of specific metallic ion along with essential nutrient source can inhibit or enhance amylase activity (Shanti *et al*., 2013). The study revealed that the levels of the α-amylase production varied greatly with the type and concentration of carbon and nitrogen sources used.

The addition of carbon sources, either in the form of monosaccharides or polysaccharides could affect the enyme production (Grata *et al*., 2010). However, effects of carbon source differ widely, depending on bacterial strains (Grata *et al.*, 2008). Therefore, amylase production was investigated by replacing starch in the basal medium with different sugars at 1% (w/v) concentration under optimal cultural conditions and nitrogen source [i.e. pH 9.0, 40˚C, 2% (v/v) inoculum and 1.5% (w/v) yeast extract]. Starch is a commonly accepted nutritional component for induction of amylolytic enzymes. This material was considered as a reference (Suman and Ramesh, 2010). Other carbon sources reduced α-amylase production compared to starch (0.47U/ml) as seen in table 1. Of the starches used, the cassava flour has given maximum yield of α-amylase (1.16, 0.80 and 0.92U/ml) at $P < 0.05$with the *Lactobacillus plantarum* FMO2, *Lactobacillus plantarum*Z2 *and Lactobacillus pentosus*BSR3 strains respectively. This finding suggests that although amylolytic lactic acid bacteria species may be isolated from different carbohydrate sources, starchy flours from cassava proved to be the best inducer of amylase production. Gelatinized starchy sources of corn and sorghum flour were reported most suitable for α-amylase and lactic acid production by *L. fermentum* 04BBA19 (Fossi *et al*., 2011).

The nitrogen sources are of secondary energy source to organisms, which play an important role in the growth and enzyme production. Various nitrogen sources were tested and screened at the concentration of 1.5%. Results in Table 2 showed that peptone significantly ($P < 0.05$) supported higher amylase production (1.11U/ml, 0.84 U/ml and 0.94 U/ml) followed by the control (yeast extract) 0.42±0.17U/ml, an organic nitrogen source. The results obtained are in concordance with those obtained by previous investigators. Shaista *et al*. (2003) observed maximum amylase activity upon supplement with 0.2% peptone as an inorganic nitrogen source for *Bacillus* species.

Owing to the high activity observed in all the lactic acid bacteria strains, *Lactobacillus plantarum*FMO2 showed maximal activity, it was selected for heavy metal optimization. Metals have long been known to stabilize and activate enzymes by directly involving in catalysis or structural modifications. Table 3 showed that various metal ions Mg^{2+}, Ca^{2+}, Cu^{2+} and Fe^{2+} affected α-amylase activity at different concentrations, when compared with the control. The relative activities in presence of Mg^{2+}, Ca^{2+}, Cu^{2+}, and Fe^{2+} were determined as 83.6%, 117.8%, 52.1%, and 95.9% respectively. It was observed that only Ca^{2+}enhanced amylase activity. Fe^{2+} ions retarded amylase activity to greater extent on increasing concentration, whereas, Mg^{2+} ions showed same behavior but its retarding effect was lesser. The enhancement in amylase activity with Ca^{2+} ions could be based on its ability to interact with negatively charged amino acid residues such as aspartic and glutamic acid (Hossain and Uddin 2011). Most of the α-amylases are considered as metalloenzymes which require calcium ions (Ca^{2+}) for their activity, structural integrity and thermal stability (Kadziola *et al*., 1994).Although in most of the cases it was observed that calcium increased amylase activity but it depended on the source of amylase.

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Magnesium ion might have altered enzyme structure after binding to its sites resulting in modification in activity (Leveque *et al*. 2000). However certain Ca^{2+} independent α-amylase have been reported from *Bacillus thermooleovorans* NP54 which produced thermostable enzyme without Ca^{2+} (Malhotra *et al*., 2000). In the case of *B. licheniformis,* previous studies suggested that α-amylase production was enhanced due to the increasing availability of the calcium ion in the fermentation medium (Souza, 2010).

In conclusion, the results of this study has indicated that *Lactobacillus plantarum* FMO2 among other strains isolated from fermented Ogi possesses starch degradation capability through the production of extracellular amylase. Supplement of nutrients and metal ions and its optimum concentration is important for the higher alpha amylase enzyme production. After this investigation it clearly showed that, optimization of production medium such as cassava flour and peptone as carbon and nitrogen sources have proven to be better nutrient supplements in the production of amylase. Metal ions also play a very important role in the growth and production of amylases and the action of metallic ions on amylase vary from one species to other.

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