Research Article

Optimization of culture parameters for production of raw starch-degrading amylase from isolated soil fungal species in Akure, Nigeria.

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ABSTRACT: The discovery, production and characterization of industrial enzymes from relatively cheap sources is at the center of biotechnological research with factors like carbon and nitrogen sources and their concentrations of great interest to researchers in the industry for low-cost media design. Fungi species isolated from the soil environment of a cassava-processing site in Akure metropolis, South-west Nigeria, were screened for amylolytic activity. Cultural parameters notably the carbon and nitrogen sources, incubation period, pH and temperature were optimized stepwise while maintaining the pre-optimized conditions to see their effects on the rate of production of raw starch degrading glucoamylase by three species of *Aspergillus* (*A. flavus, A. fumigatus* and *A. parasiticus*) with relatively high amylolytic index. Maximum enzyme activity was observed between 72 and 96 h of incubation while the pH and temperature optima for the enzyme production were observed to be between pH 5.0-5.5 and 30-35 °C, respectively. Highest enzyme production was observed with the combined use of yeast extract and ammonium sulphate as nitrogen sources. Enzyme production for industrial use has a high market value as it is known to be a lucrative business in the world. The local use and exportation of indigenously produced enzyme could serve as a means of foreign exchange in an emerging economy such as we have in Nigeria.

KEYWORDS: Raw-starch, Glucoamylase, Biotechnology, Optimization, Economy.
INTRODUCTION

Starch, a principal storage form of carbohydrate in plants and one of the most abundantly distributed polysaccharides in nature is known, together with cellulose, to be the most abundant carbohydrate polymers on earth (Anto et al., 2006). Commercially, the world production of starch amounted to approximately 58 million tons (roughly 69% from com, 10% from cassava, 9% from sweet potatoes, 6% from wheat, 6% from potatoes, and less than 1% from other sources) and serves as raw material for the industrial production of important products when hydrolyzed mechanically or by enzymatic means using amylases (Peters, 2007).

Amylases or amylolytic enzymes are a group of hydrolases capable of hydrolyzing starch and related saccharides by specifically cleaving glycosidic bonds in the molecules (Suganthi et al., 2011). They are of high industrial importance with an estimated value of about 25-33% of the global enzyme market next to proteases (Rao et al., 1998; Nwagu and Okolo, 2011), with applications in the food, pharmaceutical, paper, textile and biofuel industries (Ominyi, 2013).

At present, there are more than 30 known amylolytic and related enzymes (Janeček, 1997), which have been derived from plant, microbial or animal origin, but only a selected few are able to hydrolyze raw starch (Van Zyl et al., 2012). Raw starch digesting enzymes (RSDE) can act directly on raw starch granules below starch gelatinization temperature, a high energy demanding process that increases the overall cost of starch degradation in the industry, hence the continuous search for them.

The three best known amylases are α-amylase, β-amylase and glucoamylase belonging to glycoside hydrolase families 13, 14 and 15, respectively (Horváthová et al., 2000). However, glucoamylase is unique among the amylase enzyme family in that it can completely hydrolyze starch and other 1,4-linked glucose-oligosaccharides into glucose (Lam et al., 2013). It has received considerable usefulness for various industrial applications including starch liquefaction and saccharification (Deshmukh et al., 2011), production of glucose and fructose syrups (Riaz et al., 2007), production of biofuel (Pervez et al., 2014).

The use of microorganisms as potential sources of industrially viable enzymes has gained popularity amidst scientists in the field of biotechnology owing to the facts that these microorganisms reproduce fast when grown on the required media, produce enzymes of interest in economic bulk production capacity (Shah et al., 2014), and also, they are easy to manipulate to obtain enzymes of desired characteristics (Renge et al., 2012).

Despite the wide application of extracellular enzymes industrially, they are not produced in Nigeria, but are imported at very high importation cost leading to the high cost of finished industrial products (Oyeleke et al., 2012). The local production of glucoamylase from soil microorganism using readily available starch-based agro-residues in the country will certainly help in conserving foreign exchange thereby improving the nation’s economy. We seek to discuss such optimized production process in this work.

MATERIALS AND METHODS

Materials

Raw cassava starch and corn starch were obtained from a local market in Akure, Southwest Nigeria and authenticated at the Department of Crop, Soil and Pest Management, The Federal University of Technology, Akure, Nigeria (FUTA). Soluble starch, Bovine serum albumin (BSA), Sodium nitrate, Urea, Ammonium sulfate, Bradford assay kit, Dinitrosalicylic acid (DNSA) were products of Sigma-Aldrich Fine Chemicals, St. Louis, MO, USA. All other chemicals were commercial products of analytical grade. The water used was glass distilled.

Collection and Preparation of Soil Samples

Soil samples were collected randomly at a depth of 10 cm (approximate) from the soil environment of a cassava processing industry in Akure metropolis, South-West Nigeria (coordinates 7°10’N 5°05’E) in August, 2014 and were taken aseptically to Enzyme and Microbial Biochemistry Laboratory, Department of Biochemistry, Federal University of Technology, Akure, Nigeria for microbial analysis.

Isolation and Identification of Microorganisms

Isolation of microorganisms from the collected soil samples was done by serial dilution plate method on freshly prepared potato dextrose agar (PDA) plates fortified with 100 mg/L streptomycin. The fungal isolates obtained from the soil samples were identified taxonomically and characterized on the basis of morphological and microscopic features which included size, surface appearance, texture and colour of the colonies according to the method of Gilman (1971). Pure cultures of the soil fungi species were maintained on streptomycin-fortified PDA slants and stored at 4 °C with periodic sub-culturing on new agar slants to avoid contamination.

Plate Screening of Microorganism for amylolytic activity

For the plate screening, the organism was inoculated onto starch agar medium prepared by adding soluble starch (1% w/v) to prepared growth medium before sterilizing in an autoclave. A well of 6 mm diameter was cut at the middle of
the agar plates using a sterile cork borer and agar containing pure culture from freshly sub-cultured PDA plates were neatly cut using a sterile cork borer and placed in the wells. Plates were incubated at 37 °C for 72 h, after which they were stained with a solution containing potassium iodide, KI (0.1% w/v)/ I₂ (0.1% w/v) in 1.0 M HCl for 15 min and later de-stained with distilled water.

Screening for Glucoamylase Production in Liquid culture

The fermentation broth was formulated as described earlier according to Silva et al. (2005) with slight modifications for the growth of the microorganisms and consequently for the production of enzyme in Erlenmeyer flasks. The basal mineral medium contains yeast extract 0.67%, MgSO₄·7H₂O 0.05%, FeSO₄ 0.01%, CaCl₂·2H₂O 0.01%, KH₂PO₄ 0.02%, (NH₄)₂SO₄ 0.133% and soluble starch 1%. Enzyme was produced in a submerged fermentation process under a constant shaking condition at 150 rpm in a rotary shaker.

Enzyme Activity Assay and Determination of Protein Content

Glucoamylase activity was determined by a standard assay method earlier described by Cereia et al. (2000). Enzyme solution (1 mL) was added to a test tube containing 1 mL of 1% (w/v) soluble starch buffered with 0.05 M acetate buffer, pH 5.5 and incubated at 60 °C in a water bath for 10 min. The amount of reducing sugar (glucose) released was estimated by the DNS method (Miller, 1959). One unit of enzyme activity was defined as the amount of enzyme required to liberate one micromole of reducing sugar per minute under assay conditions. The protein concentration of the solutions was determined by the method of Bradford (1976), using bovine serum albumin (BSA) as the standard protein.

Optimization of Process Parameters for Enhanced Production of Glucoamylase

Effect of Carbon source on Enzyme Production

The effect of different starches such as soluble starch, potato starch, corn starch and cassava starch used as substrate on enzyme production was determined at the cultural conditions of pH 5.5, temperature of 30 °C and incubation time of 96 h. The activity of the glucoamylase produced was determined as described by Cereia et al. (2000).

Effect of Nitrogen source on Enzyme Production

Different nitrogen sources were used individually and in combination to determine their effects on the rate of production of glucoamylase under submerged fermentation conditions of pH 5.5 and 30 °C using soluble starch as the sole carbon source. Glucoamylase activity in liquid culture was determined under standard assay conditions after 96 h of incubation.

Effect of Incubation Period on Enzyme Production

The effect of incubation period on enzyme production was investigated by incubating the fermentation medium at pH 5.5 and at 30 °C for 7 days. The glucoamylase activity of the medium was determined at 12 h interval according to the assay method earlier described.

Effect of pH on Enzyme Production

The effect of pH on the rate of glucoamylase production under submerged fermentation was determined by adjusting the pH of the fermentation medium to different pH between 3.5 and 6.0. The fermentation process was allowed to go on for 72 h at 30 °C before it was terminated and the activity of glucoamylase in the liquid culture was determined.

Effect of Temperature on Enzyme Production

The effect of temperature on glucoamylase production was investigated by incubating the fermentation medium at temperature ranging from 25 - 45 °C. The pre-optimized conditions of pH and time of incubation were maintained at pH 5.0 and 72 h. The activity of the glucoamylase produced in liquid culture was determined according to the assay procedure earlier described.

RESULTS

Identification and Screening of Microorganisms for Amylolytic Activity

Plate (Qualitative) Screening

Eight species of fungi were isolated and identified from the soil sample. A clear halo zone around the colonies indicated amylolytic activity. The four isolated Aspergillus species; A. niger, A. flavus, A. fumigatus and A. parasiticus, were observed to be very good producers of amylases with zones of clearance of 3.40 cm, 3.31 cm, 3.34 cm and 3.18 cm respectively. The isolated Penicillium citrinum is a weak producer of amylase with a zone of clearance of 1.38 cm while Geotrichum albidium produced little amount of amylase and it is classified as a weak producer with just 0.67 cm halo-clear zone (Table 1).

Quantitative Screening for Glucoamylase Production in Liquid Culture

Six soil fungal species including four Aspergillus species (A. niger, A. flavus, A. fumigatus and A. parasiticus), one Penicillium species (P. notatum) and one Trichoderma species (T. viride) with high amylolytic activity index were selected from the plate screening result and further screened for the production of glucoamylase by submerged fermentation. Among the set studied, A. niger gave the
highest yield (activity of 32.5 U/mL) and cell mass of 2.3 g/L

Table 1: Plate (qualitative) Screening of isolated soil fungi species for amylolytic potential

<table>
<thead>
<tr>
<th>Isolate code</th>
<th>Halo-zone diameter (cm)</th>
<th>Amylolytic potential</th>
<th>Name of Isolate Identified</th>
</tr>
</thead>
<tbody>
<tr>
<td>CNP-1</td>
<td>2.66</td>
<td>+ ++</td>
<td>Penicillium notatum</td>
</tr>
<tr>
<td>CFA-1</td>
<td>3.31</td>
<td>+ ++</td>
<td>Aspergillus flavus</td>
</tr>
<tr>
<td>CCE-1</td>
<td>2.38</td>
<td>+ ++</td>
<td>Trichoderma viride</td>
</tr>
<tr>
<td>CFU-1</td>
<td>3.34</td>
<td>+ ++</td>
<td>Aspergillus fumigatus</td>
</tr>
<tr>
<td>CNP-2</td>
<td>1.38</td>
<td>+</td>
<td>Penicillium citrinum</td>
</tr>
<tr>
<td>CNA-1</td>
<td>3.40</td>
<td>+ ++</td>
<td>Aspergillus niger</td>
</tr>
<tr>
<td>CSP-1</td>
<td>3.18</td>
<td>+ ++</td>
<td>Aspergillus parasiticus</td>
</tr>
<tr>
<td>CGA-1</td>
<td>0.67</td>
<td>-</td>
<td>Geotrichum albicans</td>
</tr>
</tbody>
</table>

+ + +, very good producer; + +, good producer; +, weak producer; -, poor producer

Table 2: Quantitative Screening of glucoamylase producing organisms in liquid culture.

<table>
<thead>
<tr>
<th>Isolate code</th>
<th>Fungal Species</th>
<th>Enzyme Activity (U/mL)</th>
<th>Dry Cell mass (g/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CNP-1</td>
<td>Penicillium notatum</td>
<td>8.9</td>
<td>0.76</td>
</tr>
<tr>
<td>CFA-1</td>
<td>Aspergillus flavus</td>
<td>18.6</td>
<td>1.45</td>
</tr>
<tr>
<td>CNA-1</td>
<td>Aspergillus niger</td>
<td>32.5</td>
<td>2.3</td>
</tr>
<tr>
<td>CFU-1</td>
<td>Aspergillus fumigatus</td>
<td>25.7</td>
<td>1.92</td>
</tr>
<tr>
<td>CSP-1</td>
<td>Aspergillus parasiticus</td>
<td>16.2</td>
<td>1.34</td>
</tr>
<tr>
<td>CCE-1</td>
<td>Trichoderma viride</td>
<td>10.4</td>
<td>0.79</td>
</tr>
</tbody>
</table>

followed by A. fumigatus with an enzyme activity of 25.7 U/mL and 1.92 g/L cell mass while P. notatum showed the lowest enzyme activity with a value of 8.9 U/mL and dry cell mass of 0.76 g/L (Table 2).

Effect of Carbon (Starch) Source on Enzyme Production

The influence of the different carbon (starch) sources used as substrates on glucoamylase production by the selected fungal isolates is shown in Figure 1. At the end of 96 h incubation period at 30 °C, the liquid culture containing soluble starch gave the highest enzyme yield of 17.4 U/mL, followed by A. fumigatus with an enzyme activity of 24.4 U/mL and 14.3 U/mL respectively for A. flavus, A. fumigatus and A. parasiticus while the culture containing corn starch gave the lowest yield of glucoamylase 11.5 U/mL, 15.3 U/mL and 8.2 U/mL for the fungi species respectively.

Effect of Nitrogen source on Glucoamylase Production

As shown in Figure 2, when yeast extract alone was added to the fermentation media, the glucoamylase activity in culture
was 18.65, 15.50 and 12.31 U/mL for *A. fumigatus*, *A. flavus* and *A. parasiticus*, respectively. Ammonium sulphate gave 17.78, 15.93 and 14.87 U/mL for *A. fumigatus*, *A. flavus* and *A. parasiticus*, respectively, while sodium nitrate yielded 13.65, 12.22 and 11.09 U/mL for the respective organisms. The combination of yeast extract and ammonium sulphate as nitrogen source gave the highest yield of 29.325 U/mL for *A. fumigatus* while the combination of Ammonium sulphate and sodium nitrate gave the lowest yield (23.778 U/mL). Also for *A. flavus*, yeast extract and sodium nitrate when combined gave the highest enzyme activity of 25.03 U/mL while the smallest enzyme activity recorded was 21.350 U/mL for *A. fumigatus*, *A. parasiticus* and *A. parasiticus*, respectively. The combination of yeast extract and ammonium sulphate gave the highest yield of 23.979 U/mL for *A. parasiticus* while the combination of ammonium sulphate and sodium nitrate gave the lowest yield of 19.165 U/mL.

**Effect of Incubation Period on Glucoamylase Production**

It was observed that appreciable production of the amylolytic enzyme started after 24 h of incubation and continued steadily with increasing time of incubation, reaching a maximum value (24.4 U/mL, 26.3 U/mL and 17.7 U/mL respectively for *A. flavus*, *A. fumigatus* and *A. parasiticus*) at the 72nd hour of incubation. Thereafter, further increase in the incubation period resulted in decrease in the amount of glucoamylase produced (Figure 3).

**Effect of pH on Glucoamylase Production**

The dependence of the rate of glucoamylase production on the pH of the production basal media by the species of *Aspergillus* under study; *A. flavus*, *A. fumigatus* and *A. parasiticus* is shown in Figure 4. It was observed that the rate of enzyme production increased as the pH of the medium increased from pH 3.5 until enzyme production reached maximum at pH 5.0 and then declined. At pH 3.5, the production of the enzyme was low for all the species of *Aspergillus* (3.4 U/mL, 4.3 U/mL and 5.6 U/mL, respectively for *A. flavus*, *A. parasiticus* and *A. fumigatus*). The enzyme production was found maximum at pH 5.0 for the three species (20.4 U/mL for *A. flavus*, 22.8 U/mL for *A. fumigatus* and 14.5 U/mL for *A. parasiticus*).

**Effect of Temperature on Enzyme Production**

The effect of temperature on the production of glucoamylase is presented in Figure 5. The amount of the enzyme produced by the *Aspergillus* species was relatively low at 25 °C but increased to maximum values of 22.8 U/mL and 30 U/mL for *A. flavus* and *A. fumigatus* respectively at 30 °C while the optimum temperature for the production of glucoamylase by *A. parasiticus* was observed to be 35 °C with a value of 18.3 U/mL.

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**Figure 3:** Effect of incubation period on glucoamylase production by three *Aspergillus* species under pre-optimized conditions: pH 5.5, temperature (30) °C. CFA-1: *Aspergillus flavus*; CFU-1: *Aspergillus fumigatus*; CSP-1: *Aspergillus parasiticus*. Error bar indicates mean ± standard deviation of three replicates.

**Figure 4:** Effect of pH on glucoamylase production by Three *Aspergillus* species under optimum conditions: fermentation time 72 h, temperature (30) °C, substrate concentration (1% w/v). CFA-1: *Aspergillus flavus*; CFU-1: *Aspergillus fumigatus*; CSP-1: *Aspergillus parasiticus*. Error bar
For the purpose of industrial production, there is a preference towards the usage of filamentous fungi for the production of enzymes because they present a better capability to secret high protein levels in the fermentation media (Gouka et al., 1997), either solid or submerged (Koutinas et al., 2003; Suganthi et al., 2011).

Enzyme production by microorganisms is influenced by many factors such as temperature, pH, incubation time, and carbon sources (Jacob and Prema, 2006; Palaniyappan et al., 2009). The cost of growth medium is estimated to account for 30-40% of the production cost of industrial enzymes; therefore, it is of great significance to optimize the conditions for cost-efficient enzyme production (Palaniyappan et al., 2009). Starch has been shown to be a good inducer for glucoamylase synthesis by some Aspergillus species when used as substrate (Ganzlin and Rinas, 2008; Zambare, 2010). In this study, three different raw starches and soluble starch were utilized among which soluble starch gave the highest yield. Appreciable production of the amylolytic enzyme started after 24 hours of fermentation and was continuously produced up to the 72nd hour (day 3) beyond which amylolytic activity in the fermentation media decreased. According to Feroza et al. (1998), this might be due to the fact that, with the passage of time, the nutrients become depleted and other secondary metabolites are produced altering the pH of the fermentation medium, and eventually inhibiting both the growth of the fungi as well as enzyme secretion. It was observed that pH 5.0 is the optimum pH for the growth of the Aspergillus species, as well as the optimum pH for the production of glucoamylase. This is in agreement with earlier studies that fungi require slightly acidic pH (4.0 – 6.5) for optimum growth (Sun et al., 2009). The medium pH is an important factor that determines growth and morphology of microorganisms since they are sensitive to the concentration of hydrogen ion present in their medium of growth (Sun et al., 2009).

The adaptation of microorganisms to the thermal environment is also very important during fermentation because microorganisms grow optimally at certain degrees of temperature above or below which they do not thrive well. Therefore, the temperature and other cultural parameters of the fermenting medium must be carefully monitored and controlled for the optimum production of enzymes by microorganisms.

**Conclusion**

The environment in which humans live to carry out their day-to-day activity is endowed with so much naturally occurring compounds and organisms that can be harnessed and channeled properly to the development of technologically important products such as enzymes employed to drive biotechnological processes. The local sourcing and
production of industrially viable enzymes can help to conserve foreign exchange and also serve to provide platform for indigenous industrialization in Nigeria.

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