

Research Article

Starch hydrolyzing enzyme production from *Aspergillus niger* EFRL-FC-024 using molasses as carbon source

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Abstract

Amylase is one of the important industrial enzyme due to its application in detergents, food and textile industries. In present study, the production of amylase, a starch hydrolyzing enzyme, was optimized using *Aspergillus niger* strain EFRL-FC-024 under various conditions i.e. incubation time, carbon source, pH and temperature. Amylase production was recorded as 2.26 U/mL after 96 h of incubation in mineral medium containing glucose as carbon source. In contrast its concentration increased to 2.55 U/mL when molasses was added to mineral medium. However, the highest enzymes titer of 2.97 U/mL was achieved in optimized conditions (1.0% w/v molasses, 96 h, pH 6.0 and temperature 40°C). Molasses are a cost-effective carbon source for amylase production using *Aspergillus niger* strain EFRL-FC-024. Thus this enzyme can be produced cost effectively using low cost agro-industrial waste natively like molasses and could be utilized in detergent or textile industry or even for the saccharification of starchy materials i.e. potato waste to produce ethanol and other commodity products.

Key words: Amylase; *Aspergillus niger* EFRL-FC-24; Molasses; Starch hydrolyzing

Introduction

Amylase (EC:3.2.1.1) hydrolyzes starch to release glucose, maltose and dextrose [1-4]. It is utilized in food, detergent, paper, textile and pharmaceutical industries. It also plays an important role in cereal processing, baking, sweetener, syrup and beverages production in the food industry [5-12]. The annual sales of amylase, in the global market, are estimated to be \$11 million [10].

In 2015 the enzyme's market was \$8.18 billion and it is estimated that this market will reach to \$17.50 billion in 2024, globally [11]. Increasing demands of amylase have influenced local researchers to optimize amylase production using local inexpensive materials and novel strains [12, 13]. Based on the cleavage sites in starch, amylases have been categorized into three groups. α -amylase hydrolyze α , 1-4 glucosidic linkage,

β -amylase cleave α 1-4 glucosidic linkage and glucoamylase cleaves α ,1- 4, and 1-6 linkage releasing single glucose units [14].

Amylase can be obtained from microbes, plants and animals. However fungal and bacterial amylase are more promising due to less time required for their production, huge diversity, and low production costs [15,16]. Amylase could be produced through solid-state and submerged fermentation conditions [17]. However, submerged fermentation is routinely used for amylase production [16, 18]. It is advantageous because it provides a better growth environment to microbes, less contamination chances, easy process control, high productive yield, is less labor-intensive, and cost-effective [15]. Keeping in view the increasing demands of amylase a local fungal strain was used for the production of starch hydrolyzing enzyme and process conditions were optimized. Enzyme produced from this study could be utilized for the saccharification of starchy materials like potato waste etc. to produce ethanol and other commodity products. Further applications include use in detergent and textile industry in Pakistan.

Materials and Methods

Inoculum preparation

Aspergillus niger strain EFRL-FC-024 (*A. niger*) was obtained from Enzyme and Fermentation Research Laboratory at the Institute of Biotechnology and Genetic Engineering (IBGE), University of Sindh, Jamshoro. The culture was activated on synthetic medium containing 20 g/L of dextrose, 20 g/L of agar, 10 g/L of peptone and incubated at 37 °C for 7 days then subcultured on fresh glucose agar medium. Culture was grown for 7 days to obtain luxurious growth that was used as inoculum in all fermentation experiments. For the preparation of spore suspension, the surface of culture stock of *A. niger* was slightly scrubbed with a wire loop and 10 mL of

sterilized water was added. 1.0% v/v culture was inoculated in fermentation media [19].

Culture conditions amylase production

The fermentation medium was composed of glucose 20 g/L, Peptone 10 g/L, CaCl₂. 2H₂O 1 g/L, KH₂PO₄ 1 g/L, MgSO₄.7H₂O 1.0 g/L, MnCl₂.2H₂O 0.5 g/L, NH₄NO₃ 1 g/L. Fermentation medium composition was slightly modified according to the earlier reports [19]. This medium was used for amylase production. All chemicals were purchased from Sigma and/ or E. Merck. Molasses was collected from “Mehran Sugar Mill, Tando Allahyar”. The pH of the medium was adjusted to 6.0 using 1N HCL or 1N NaOH. The medium was inoculated with fresh *A. niger*, fungal culture and incubated at 37°C for 144 h.

Fermentation process of amylase

50 mL of sterilized fermentation medium in a 250 mL conical flask was inoculated with 2.0% v/v of spore suspension. The flask was incubated at 37°C without shaking. After an interval of 24 h as incubation time, the culture was filtered through Whatman filter paper (No.1) to remove biomass. The harvested biomass was dried out at 80°C and weighed in g/ 50 mL.

Optimization of fermentation conditions

Different sugars (glucose, molasses, sucrose, fructose, maltose, galactose and mannitol) were used as carbon sources to evaluate their effect on amylase production. The fermentation medium was supplemented with different sugars accordingly and flasks were incubated at 37°C for 144 h. Different parameters like effects of incubation time (24-144 h), pH (4-9) and temperature (20 to 60°C) were optimized for amylase production by *A. niger* under submerged condition. Further, final pH of the broth culture was determined by pH meter (Scientific Instrument). The culture broth was used for the determination of total protein, total sugar, reducing sugar and amylase activity.

Determination of amylase activity, total protein, reducing sugar and total sugar

Amylase activity was tested either by Shafique or Fischer and Stein method [20]. 1mL of culture broth, 1mL of substrate (1% w/v soluble starch) was added and kept in water bath for 15 min at 37°C. Then 2 mL of 3, 5 dinitro- salicylic acid (DNS) reagent was added and the reaction was stopped by putting tubes in boiling water bath at 100°C for 5 min. Then the reaction was cooled to room temperature, the absorbance was recorded by Spectrophotometer at 540 nm against the blank. Blanks were prepared by replacing culture broth and substrate with water. One unit of amylase activity is defined as the amount of enzyme required to release 1 micromole of reducing sugar under assay conditions.

Total protein was analyzed according to the Lowery *et al.* (1951) method using bovine serum albumin as standard [21]. Total sugar was determined according to Montgomery (1961) method using glucose as a standard [22]. Reducing sugar was measured according to Miller *et al.* (1959) method using glucose as standard [23].

Results

Effect of time of incubation on production of amylase

In present study, amylase production was recorded from 24 to 144 h using *A. niger* strain EFRL-FC-024. Amylase concentration increased with increase in fermentation time and maximum amylase activity of 2.26 U/mL was observed at 96 h when the culture was grown on glucose mineral medium, pH 6.0 and incubated at 37 °C. Results are depicted in (Fig. 1). Results suggest that the enzyme was produced in log phase to early stationary phase. More than 50% of enzyme activity decreased after 144 h of incubation. Highest total protein content was reached to 0.50 mg/mL at 48 h of incubation period. Microbial growth was also monitored from 24 to 144 h. Highest biomass was noted at the 4th day of incubation however, and then declined with increasing incubation period.

Residual total and reducing sugar during fermentation was observed from 24 to 144 h. The maximum total sugar was noted 1.92 mg/mL at 24 h and the lowest amount of total sugar was recorded after 144 h. The highest reducing sugar was also detected at 24 h of fermentation time 0.88 mg/ mL then declined with incubation time. These results suggest that sugar was consumed by microorganisms for its growth and enzyme production. Results are shown in (Fig. 2).

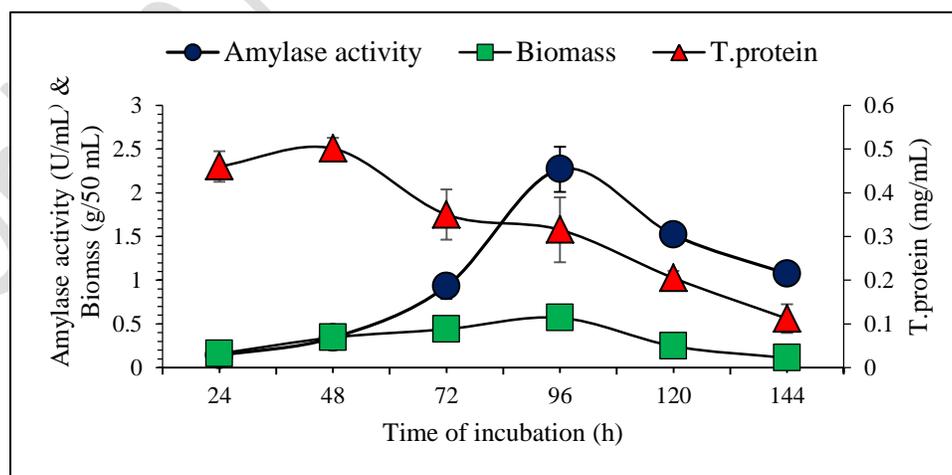


Figure 1. Effect of time of incubation on microbial growth and α -amylase production

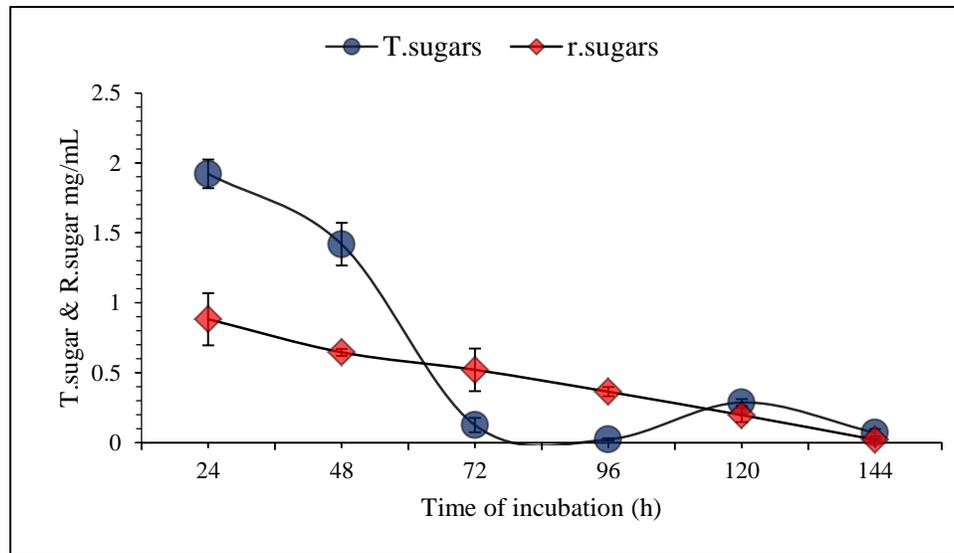


Figure 2. Residual total and reducing sugars at different fermentation time

Effect of carbon source on amylase production

Effect of different carbon sources (glucose, molasses, sucrose, fructose, maltose, galactose and mannitol) was tested on amylase production in mineral medium. Glucose was replaced with molasses, fructose, sucrose, maltose, galactose and mannitol at 1% w/v in fermentation medium for amylase synthesis and cell growth. Maximum amylase production was achieved in molasses mineral medium (2.55 U/mL) as compared to other sugars. Molasses is an industrial waste that contains sugars,

nitrogen compounds, vitamins and minerals that support microbial growth and enzyme production. The highest amount of total protein was recorded in molasses fermented medium (0.93 mg/mL) at 96 h. Results are depicted in (Fig. 3). Different carbohydrates (1% w/v) were incorporated in the fermentation medium. Residual total and reducing sugars were measured after 96 h. Maximum amount of total and reducing sugars were (4.11 mg/mL) and (2.25 mg/mL), respectively, in molasses fermented medium as compared to all other tested sugars. Results are displayed in (Fig. 4).

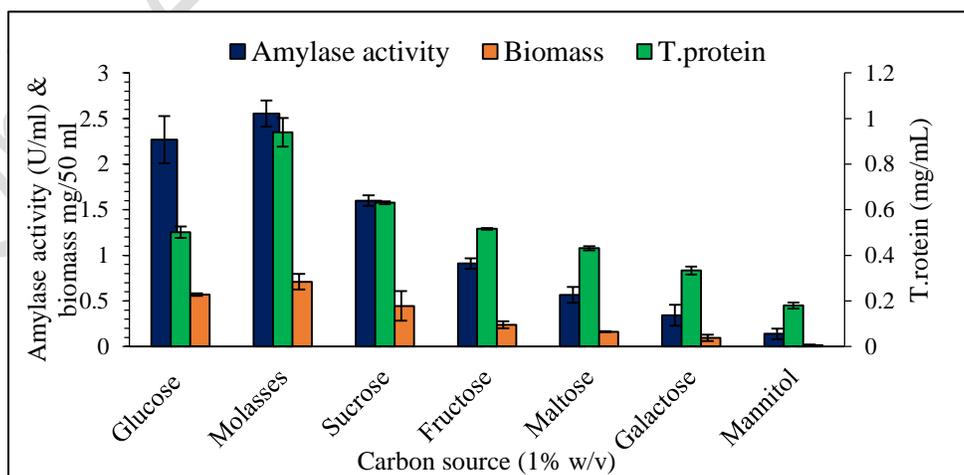


Figure 3. Effect of 1% carbon sources on production of amylase by *Aspergillus niger*

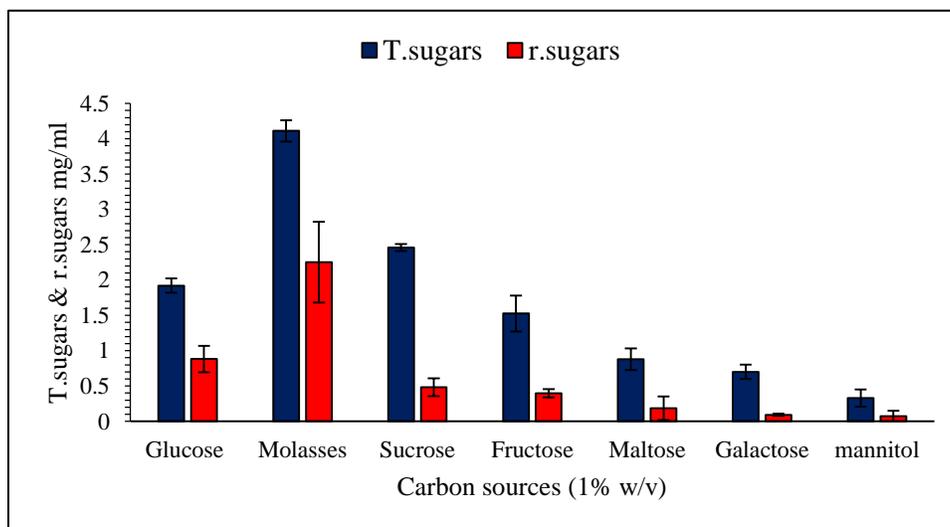


Figure 4. Residual total and reducing sugars from culture broth by *Aspergillus niger*

Effect of pH on the production of amylase

Initial pH is one of the important parameter responsible for certain changes in the enzyme activity and growth of organism. In current study, a range of pH (4-9) was analyzed to record its influence on amylase production. Results showed that the highest enzyme activity of 2.63 U/mL was achieved at pH 6.0. Further increase in the pH declined the enzyme titers. The minimum activity of amylase was noted at pH 9.0. Results are indicating that the amylase

production was highest when the strain was grown in the acidic pH range. During fermentation, culture medium was tested for the total protein and biomass and both were found highest at pH 6.0. Results suggest that there is a correlation between enzymatic activity, total protein and mycelium mass. (Fig.5). The highest amount of total sugar (2.45 mg/mL) and reducing sugar (0.88 mg/mL) were observed at pH 6.5. Results are presented in (Fig. 6).

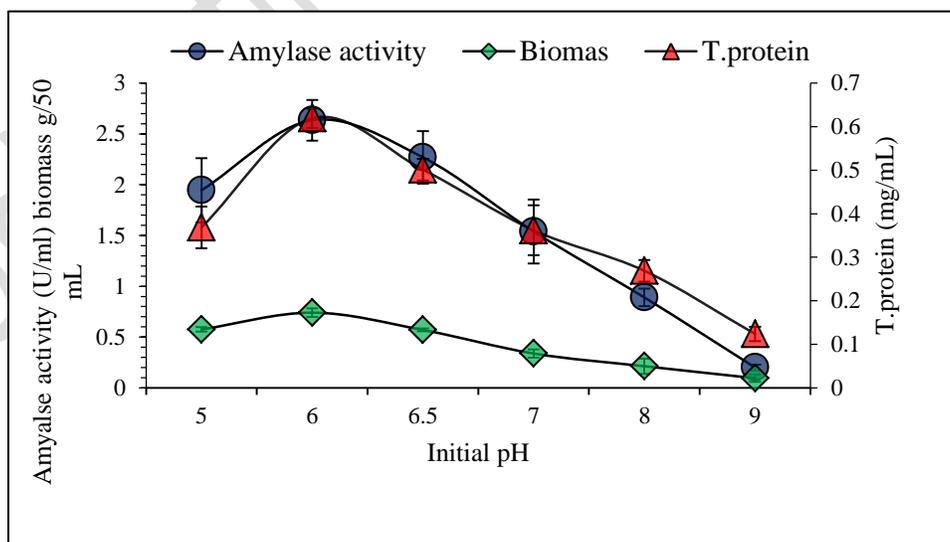


Figure 5. Effect of pH on carbon sources for production of amylase by *Aspergillus niger*

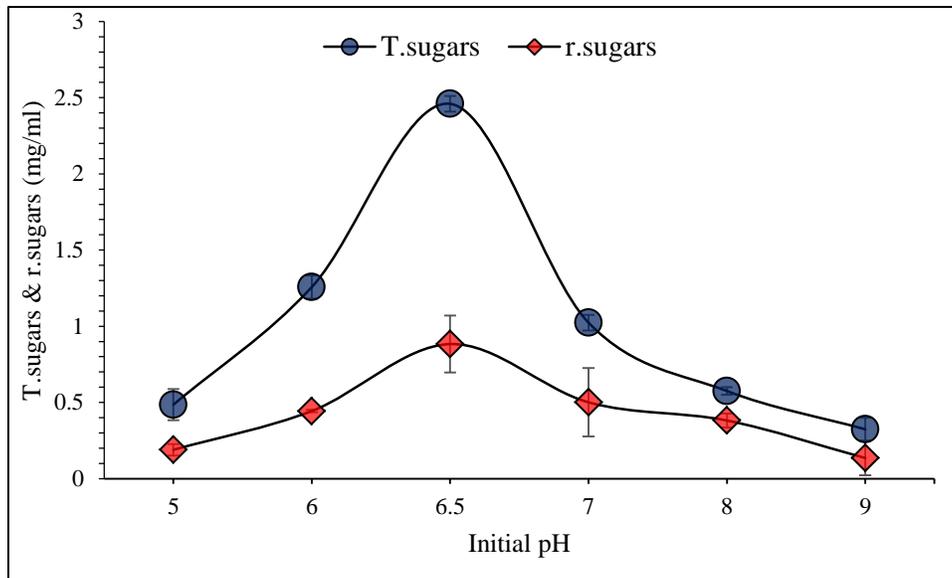


Figure 6. Residual total and reducing sugars from culture broth by *Aspergillus niger*

Effect of temperature on the production of amylase

Effect of incubation temperature (20 to 60 °C) on amylase production and microbial growth were investigated in molasses mineral medium. Amylase concentration increased with the temperature up to 40 °C. Highest enzymatic activity was recorded at

40 °C (2.97 U/mL). On further increase in the incubation temperature decreased the amylase production. Highest total protein content was recorded at 40 °C. Maximal total and reducing sugars were 2.56 mg/mL and 1.09 mg/mL, respectively at 30 °C. Results are shown in (Fig. 7 & 8).

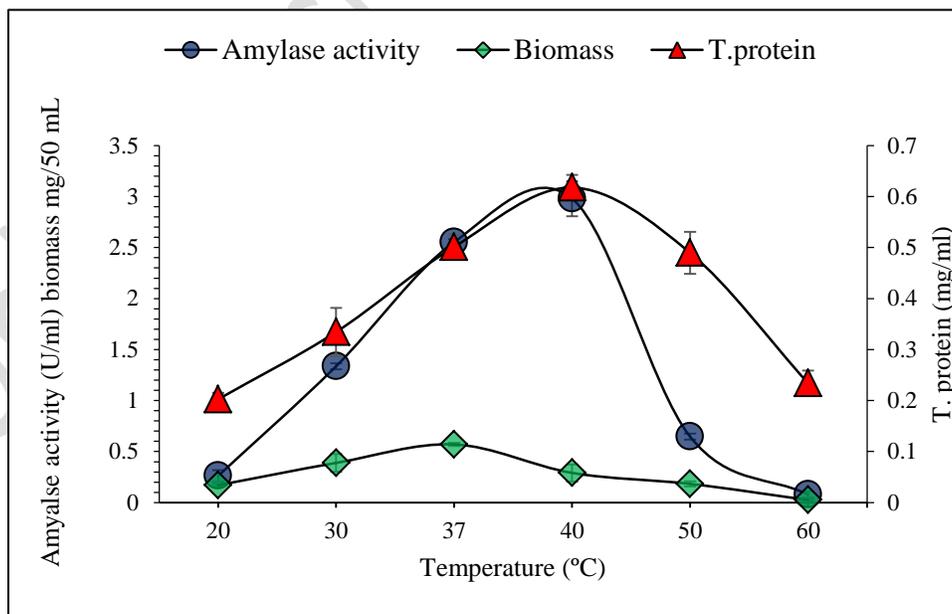


Figure 7. Effect of incubation temperature on the production of amylase by *Aspergillus niger*

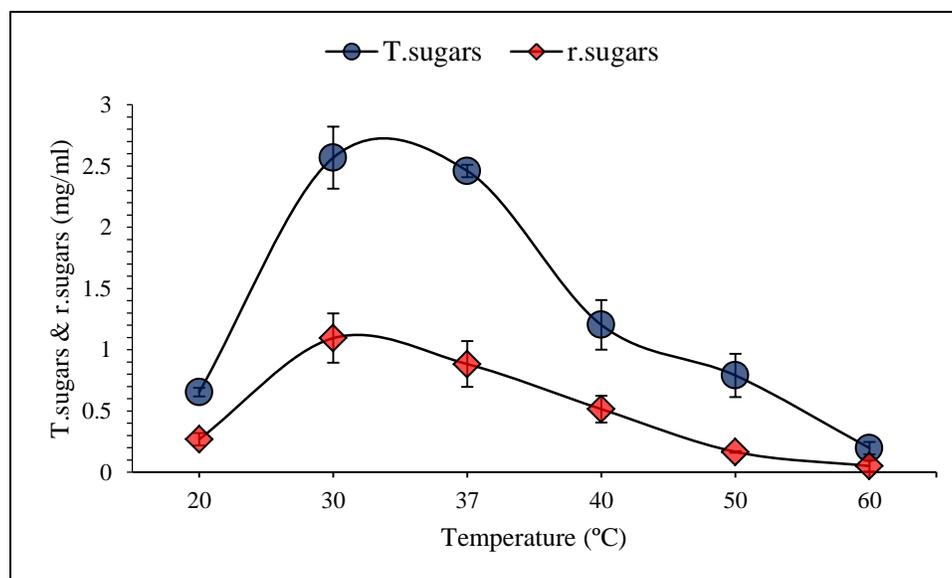


Figure 8. Residual total and reducing sugars from culture broth by *Aspergillus niger*

Discussion

Amylase is the 2nd most important hydrolytic enzyme which is used in dishwashing, laundry purpose, paper industry, brewing industry, removing wallpapers and pharmaceuticals [24]. This is why, in present work, amylase production from *A. niger* strain EFRL-FC-024 was optimized. Agro-industrial waste materials are an excellent source of energy for the production of biofuel, enzymes and other commodity products. Among different low cost agro-industrial waste materials, molasses is a by-product of sugar industry. Molasses enhances microbial growth and enzyme production largely due to its composition. Molasses contains sugars, nitrogen compounds, vitamins and minerals. The optimization of incubation time plays an important role for maximizing enzyme production and microbial growth. In present study, the maximum amount of α -amylase was noted at 96 h of incubation. Esam Ali reported maximum amylase concentration from *Aspergillus flavus* after 4-5 days of incubation [25]. Highest amylase production from *Penicillium fellutanum* was noted at 4th day of incubation [26]. Decline in enzyme

activity with an increase in fermentation time might be due to denaturation of enzyme or production of other inhibiting secondary metabolites.

Effect of different sugars (molasses, glucose, sucrose, fructose, maltose, galactose and mannitol) was tested on amylase production and microbial growth. Maximum enzyme production was noted when molasses was supplemented in the fermentation medium. This happened probably due to the presence of growth promoters in nutrients rich molasses (nitrogen compounds, vitamins and minerals). Fructose, lactose, sucrose, xylose and glucose additive results in catabolic repression of synthesis of enzymes [27]. Agriculture residues including orange peel, potato peel, corn, wheat and rice as flours have been evaluated as cost-effective carbon sources for amylase production that reduces the fermentation medium cost [28]. Pomegranate peel medium produced maximum amylase concentration [29]. The highest amylase production was obtained from *Penicillium rugulosum* when grown on starch mineral medium. However, maltose was found the best carbon source for

amylase production when *Bacillus licheniformis* was used as amylase producing strain [30].

In enzyme production, pH plays an important role. In order to determine the effect of initial pH on microbial growth and amylase production, different initial pH range (4-9) was used. In our study, maximum amylase production was noted at pH 6. Similar results were reported for maximum enzyme activity using *A. niger* at pH 6.0 [31]. The pH 6.0 is optimum for amylase production using *Aspergillus niger* [32]. Sivaramakrishnan *et al* [33] reported amylase production from *Aspergillus oryzae* when grown on wheat bran medium and maximal amylase concentration was noted at pH 5.0. *T. maculatum* produced highest amylase when grown from pH 6.0 to 7.0. Further increase in the pH declined amylase production that shows neutrophilic nature of the strain [34].

One of the most important factors for amylase production and microbial growth is temperature. Optimum temperature usually depends on the nature of microorganisms whether strain is thermophilic or mesophilic [35]. In our study, the optimum temperature for amylase activity was 40 °C and on further increase in temperature declined the amylase activity due to the degradation of enzyme. According to other researchers, maximum amylase production using *Aspergillus niger* was found at room temperature through submerged and solid state fermentation [36]. Optimum amylase titer was shown from 40 to 50 °C then decreased [34]. The highest amylase concentration was noted at 30 °C from *Penicillium fellutanum* [26]. The optimum temperature for amylase activity at 30, 37 and 40°C using *Aspergillus* spp was reported by Suganthi [37] Ugoh and Ijigbade, respectively [38]. Enzymatic activity declines due to protein degradation and

change in membrane composition related to high incubation temperature [39].

Conclusion

In current study, amylase production was optimized using *Aspergillus niger* strain EFRL-FC-024. Amylase production reached to 2.26 U/mL after 96 h of incubation in mineral medium containing glucose as a carbon source. Amylase concentration increased to 2.55 U/mL when molasses was used in mineral medium. The highest enzyme titer of 2.97 U/mL was achieved in optimized conditions (1.0% w/v molasses, 96 h, pH 6.0 and temperature 40°C). Molasses found a cost-effective industrial waste for amylase production. Amylase produced from this study could be applied in saccharification of starchy material (potato waste) to produce ethanol and other commodity products and can be used in detergent and/ or textile industries.

Authors' contributions

Performed the experiments, data analysis, results interpretations and manuscript write up: MA Jokhio, Supervised the work and designed the experiments: HA Naqvi, Helped in manuscript write up, editing and review: A Yasmin, Reviewed the manuscript: N Channa, Edited and reviewed manuscript: AS Qureshi, Given technical suggestions: I Khushk.

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