Indian Journal of Traditional Knowledge
Vol 19(1), January 2020, pp 158-163

Production of extracellular amylase by *Aspergillus niger* under submerged fermentation using jack fruit rag as the carbon source

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Received 15 December 2017; revised 23 September 2019

Production of extracellular amylase by *Aspergillus niger* was studied under submerged fermentation using jackfruit rag as the carbon source. Different parameters, such as incubation period, pH of the culture broth and level of substrate were changed to optimise the conditions for amylase production. Maximum enzyme production ~ 8400 units/g was obtained in 5 days old cultures, grown at pH, 6.5 and 30°C with substrate level 20 gL^{-1}. As nitrogen sources NH_4Cl, KNO_3 casein, peptone and beef extract were tested. Except NH_4Cl all other sources enhanced the amylase production. Study on the kinetics of extracellular and intracellular amylase production revealed that extracellular amylase production was always higher than that in intracellular. Crude amylase obtained from culture broth was partially purified by ammonium sulphate fractionation followed by DEAE Cellulose chromatography. Partially purified enzyme exhibited optimum pH and incubation temperature at pH 6 and 60°C respectively and higher thermal and pH stability at 50-60°C and pH 5-7 respectively and enhanced activity with Ca^{2+}. These unique features of the enzyme indicates its suitability for various industrial applications. Shorter incubation period and lower substrate cost offer the potential for inexpensive production of amylase, making the process industrially and economically feasible.

Keywords: Amylase, *Aspergillus niger,* Jackfruit rag, Submerged fermentation
IPC Code: Int. Cl.[20]: C12Q 1/40, C12R 1/685, A61K 36/00, A23F 3/10

Amylase in one of the important and well known industrial enzyme that can use to breakdown starch and glycogen. Though it can be derived from several sources such as plants, animals, and microorganisms, the microbial sources generally meet the industrial demand owing to their rapid growth rates that lead to short fermentation cycles and bulk production capacity. Apart from that, microbes are easy to manipulate to obtain enzymes of desired characteristics[1].

To date a large number of microbial amylases are available commercially and most commonly used organisms are *Bacillus licheniformis, Bacillus amyloliquifaciens* and *Aspergillus niger*. Fungal amylases particularly from *Aspergillus species* find various application in food industry as an anti-staling agent in baking industry, for haze clarification in fruit juices, alcoholic beverages, glucose and maltose syrup production and other food products. Several methods such as submerged fermentation and solid-state fermentation have been successfully used for amylase production from various microorganism. Since, the contents of synthetic medium used for amylase production are very expensive and uneconomical, they need to be replaced with more economically available agricultural and industrial by products. Agro industrial residues such as wheat bran, spent brewing grain, maize bran, rice bran, rice husk coconut oil cake, mustard oil cake, corn bran *etc*. have been used as substrates for amylase production[2,3,4]. Present study deals with use of inexpensive highly abundant jackfruit rag powder as the substrate for amylase production by *A. niger* under submerged conditions. In this work, growth conditions will be optimised to achieve maximum amylase production. Amylase, thus produced will be partially purified and characterised.

Materials and methods

All the chemicals used were obtained from Fluka, Chemi new Ulm, Switzerland. *Aspergillus niger* strain was obtained from culture collection of Department of Microbiology, University of Kelaniya, Sri Lanka. The culture was maintained by sub-culturing fortnightly on Potato Dextrose Agar (PDA) slants and stored at 4°C in a refrigerator.

Culture conditions and growth

The growth medium for the fungal strain consisted of KH_2PO_4 (1.40 g), $(NH_4)_2SO_4$ (1.00 g),

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KCl (0.50 g), $MgSO_4.7H_2O$ (0.10 g) and $FeSO_4.7H_2O$ (0.01 g) dried, powdered jack fruit rag powder (instead of starch) 15.00 g in 1liter[5], pH of the culture media was adjusted to 6.0 and sterilized at 121°C, 12 psi for 15 min. Sub inoculum was prepared inoculating 50 mL of above culture medium with *Aspergillus niger* and incubated shaken at 120 rpm on an orbital shaker at room temperature for 24 h. Sub inoculums were used to inoculate the culture media.

Optimization of the condition for extracellular amylase production

Al the experiments were done in triplicate.

Kinetics of amylase production at different pH level

A. niger cultures were grown at 30°C varying pH of the culture media from pH 4.0 to pH 7.5. Culture filtrates of 4^{th}, 5^{th}, 6^{th}, 7^{th} and 8^{th} days old cultures were obtained and assayed for amylase activity.

Determination of optimum concentration of carbon source.

Cultures were grown varying the levels of carbon source, from 5 gL^{-1} to 30 gL^{-1} level. Amylase activity of culture filtrate was assayed at 4^{th}, 5^{th}, 6^{th}, 7^{th} and 8^{th} days of incubation.

Effect of nitrogen source on amylase production.

Cultures were grown (pH 6.5, culture media supplemented with jackfruit rag powder, 20 gL^{-1}) changing the nitrogen source. NH_4Cl, KNO_3, peptone casein and beef extract were selected. (Each nitrogen source, 20 gL^{-1}). Amylase activity of 4^{th} to 8th days old, cultures were assayed.

Comparison on the extracellular and intracellular amylase activity of the fungus

Cultures were grown under optimised conditions (pH 6.5, 30°C, jack fruit rag powder 20 $gL^{-1,}$ KNO_3 20 $gL^{-1,}$). Extracellular and intracellular amylase activity were monitored in 4 to 8 day old cultures. Culture filtrate was used for extracellular enzyme. Fungi mycelium was used to obtain Intracellular enzyme

Protein estimation

Protein assays were done by Biuret method[6] using Bovine serum albumin as the standard.

Amylase assay

Estimation of amylase activity was carried by DNS (3,5-dinitro salicylic acid) method using 1% starch as the substrate. One unit of enzyme activity is defined as the amount of enzyme, which releases 1 mole of reducing sugar as glucose per minute, under the assay conditions. The experiments were carried out in triplicates, mean and standard error was calculated.

Enzyme purification

Cultures were grown in bulk under the optimized conditions. At day 5, culture filtrate was fractionated with $(NH_4)_2SO_4$, 50-75% ammonium sulphate pellet which had highest amylase activity was desalted and purified by DEAE cellulose chromatography eluting with phosphate buffer (0.01 M, pH 6.5) containing 1 M NaCl concentration.

Protein assays and enzyme assays were carried out at each purification step.

Characterization of the enzyme

Following experiments were carried out using the enzyme fraction obtained from above DEAE step. All the assays were done in triplicate.

Optimum incubation temperature for amylase

Amylase enzyme assay was carried out at different incubation temperatures ranging from 25°C to, 90°C.

Optimum pH for amylase

Amylase enzyme assay was carried out using different buffers ranging from pH 3-9.

Thermo-stability of Amylase

The thermal stability of the enzyme was assessed by pre-incubating the enzyme without the substrate at various temperatures between 50 to 100°C for 3 h. Enzyme fraction was taken out at every 30 min intervals, cooled to room temperature and assayed to determine residual amylase activity.

pH stability

The stability of the enzyme at different pH level was assessed by incubating the enzyme for 3 h in buffers of different pH ranging from 5 to 9. (Citrate phosphate buffer for pH 5-8 and Tris-HCl buffer for pH 9). Residual enzyme activity at each pH level was assayed at every 30 min.

Effect of Metal Ions

Enzyme activity was assayed in the presence of 5 mM and 10 mM concentrations of various metal ions (Na^+, Mg^{2+}, $Ca^{2+}$and Co^{2+}) in chloride salts. The relative activity of the enzyme was compared with the activity obtained in the absence of metal ions.

Results and discussion

In recent past, great interest has been shown for value addition to agro- industrial bio wastes in order to develop industrial products such as enzymes, organic acid, mushrooms, flavour and aroma compounds, pigments, polysaccharides, hormones and animal wastes. The selection of an ideal agro biotech waste for enzyme production in submerged

fermentation process depends upon several factors related to availability and cost of the substrate material and, thus involves screening of several agro-industrial residues. In the present study, potential of using jackfruit rag powder as a substrate for the production of amylase by *Aspergillus niger* under submerged fermentation was investigated. Jackfruit rags contain high concentration of carbohydrates (18%), protein (3%) and crude fibre (6%), which makes it a good source of carbohydrates. Most of the carbohydrates are in the form of starch. Apart from that, rags contain minerals like calcium (3.4%) and potassium (2.5%). Since calcium is required for α amylase activity, jackfruit rags makes a good substrate for α amylase production[8].

Kinetics of amylase production at different pH level

As the data given in (Fig. 1.a) enzyme activity was increased in a time dependent manner and the maximum amylase activity was observed in 5 days old cultures. Same behaviour was reported for *A. niger* grown on wheat bran, black gram bran and rice bran[3,9]. In another work maximum amylase production has been seen on 6th day for mycelia grown on cassava peel and groundnut oil cake and on 4th day for gingelly oil cake[3,10]. All these evidence, suggest findings of the present study is well compatible with previous reports. However, somewhat different results had been observed with banana peel and with rice bran which had shown highest activity after 3 and 7 days respectively[2,3].

Shorter incubation period offers potential for inexpensive production of enzyme[3] (suganthi *et al*., 2011). Hence, five-day incubation period in the present study gives promising results for the industrial production of amylase by *Aspergillus niger***.** Results given in (Fig. 1.b). Indicate amylase production was progressively increased reached to maximum level at pH 6.5. Above this level, (pH 6.5) amylase production slowly decline and reach to a lower value at pH 7.5.

This characteristic is most important to fermentation process because under these conditions most of the bacteria responsible for contamination of the fermentation processes are inhibited[3]. As the data indicated (Fig. 1.b) amylase production is active in the pH range 6.0-7.0, suggesting that the enzyme would be useful in processes that required range of pH which span through slightly acidic to slightly alkaline. Maximum amylase production observed at pH 6.5 in the current study is well consistent with the optimum pH reported for *A. niger* grown on rice bran, sweet potato[11] wheat bran[9] and cassava peel[10]. Accordingly, maximum amylase production observed at pH 6.5 in the present study is also well compatible with the previous findings. However *A. niger* grown in Czapeckdox medium reported lower pH value at pH 4.5 as optimum pH for amylase production[12].

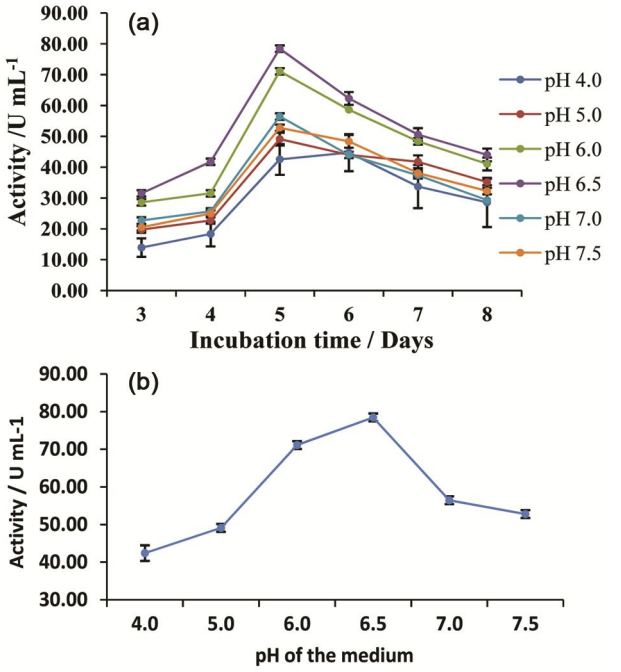

Fig. 1. — (a) Kinetics of amylase production at different pH level; (b) Amylase production on 5th day at different pH level

Kinetics of amylase production with different Substrate levels

Maximum amylase production (Fig. 2) was observed at 20 gL^{-1} concentration. At this 20 gL^{-1} concentration level, amylase production was slowly increased with increasing incubation period and reached to maximum at day 5. Above this time period amylase production was slowly declined. Similar results has been observed with *Aspergillus niger* grown on cassava peel as well as that on soluble starch[10].

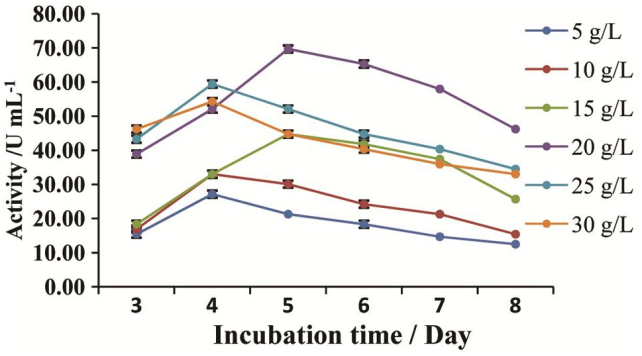

Fig. 2 — Kinetics of amylase production at different substrate levels

However, somewhat higher value (30 gL^{-1}) than this has been observed for *Aspergillus niger* grown on raw tube starch[3]. All these findings implies that jack fruit rag may serve as ideal fermentation bases for obtaining high yields of amylase from *Aspergillus sp.*

Effect of nitrogen source on amylase production

KNO_3 showed the maximum amylase (Fig. 3) production with respect to control. Beef extract showed similar production to the control. Casein, peptone showed higher production than control, but lower than that with KNO_3. Previous findings have shown that peptone, sodium nitrate and casein hydrolysate are good nitrogen supplements for amylase production[3]. Amylase production with NH_4Cl was very much less than the control**.** Contrary to this observation, Suganthi *et al*[3]. (2011) reported enhanced amylase production for the coconut oil cake substrate supplemented with ammonium chloride. However, in agreement with the above reports present study also demonstrated remarkable increase in the amylase production upon supplementing the medium with KNO_3. Previous work also reported that supplementing the fermentation media with ammonium nitrate as well as with $NaNO_3$ enhanced the amylase production[3].

Production of extracellular and intracellular amylase

Extracellular amylase production (Fig. 4) found to be higher than intracellular amylase production. Intracellular amylase production reached to maximum at 4th day after incubation where as extracellular production reached to maximum at 5th day. Most probably, this is due to the extra cellular digestion of fungi. They digest the food first and then ingest the food, to accomplish this by producing exo-enzymes.

Characterization of partially purified amylase

Amylases are known to be active in a wide range of temperature (40-90ºC) and pH (4–11). As the data shown in (Fig. 5) current enzyme exhibits optimum activity in the rage of 50-60ºC. This value is well compatible with the values that reported for amylases described in Omemu *et al.*[13] and Tiwari *et al.*[14]. Same behaviour has been reported for amylases described in Saxeena *et al.*[15] and Suagnthi *et al*[3]. However, amylase produced by *Aspergillus niger* grown on wheat bran (40ºC) has been demonstrated somewhat lower value than the amylase in the current study[16]. Amylases are known to be active and stable over a wide range of pH (3.5-12) though some are only stable within a narrow pH range[1,3,12].

As the data represent in (Fig. 6), amylase in the present study exhibits higher activity in the range of pH 5-7 showing optima at pH 6. Similar trend has been observed for amylases produced by *Aspergillus niger* grown on mustard oil cake as well as on wheat bran[13,16]. This evidence suggest higher activity of the current enzyme in the range of pH 5-7 is quite comparable with previous findings. However, amylase produced by *Aspergillus niger* grown on raw tuber starch has been shown[3] optimum pH at 4.

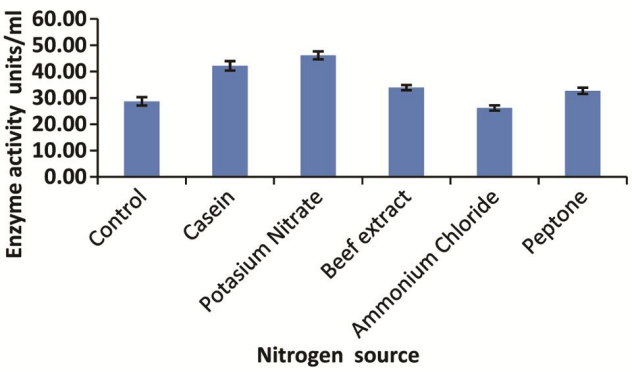

Fig 3 — Amylase production with different nitrogen sources

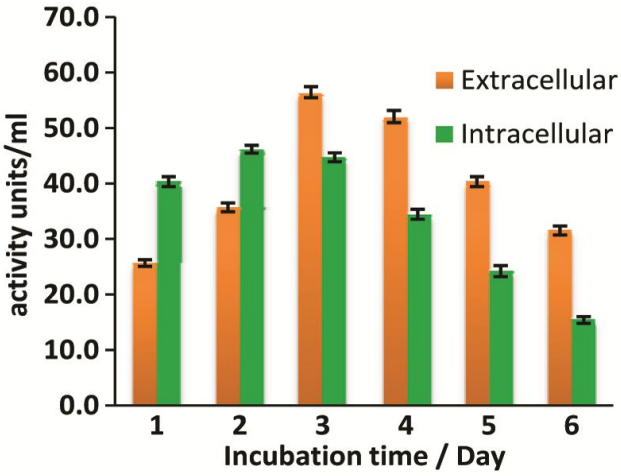

Fig. 4 — Comparison on extracellular and intracellular amylase production with the incubation time

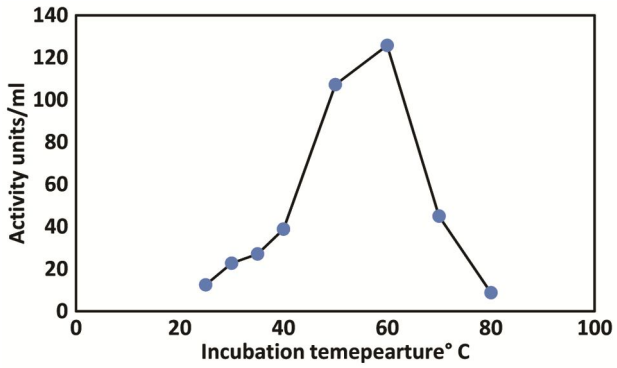

Fig. 5 — Effect of incubation temperature on amylase activity

Thermal stability

Studies on thermal stability revealed, enzyme remains very stable at 50–60°C nearly for 2 h. Thereafter enzyme retained ~70% of its activity even up to 3 h (Fig. 7). At 70°C also enzyme remained very stable for 1 h and retained ~ 85% and 65% of residual activity even up to 2 and 3 h respectively. At 80 ºC and 90 ºC thermal stability of the enzyme found to be very poor. Similar trend has been reported for amylase described in Suganthie *et al*.[3]

pH stability

As shown in (Fig. 8) enzyme displayed remarkable stability at pH 6 retaining nearly 92% residual activity even up to 3 h (Fig. 8). At pH 5 and 7 enzyme maintained ~100% residual activity up to 1 h. Above that level residual activity slowly decline retaining nearly 75% residual activity even up to 3 h. At pH values above 7, enzyme shows lower activity retaining very poor stability. Similar findings have been reported in Saxeena *et al.* [16] and Suganthi *et al*. [3]

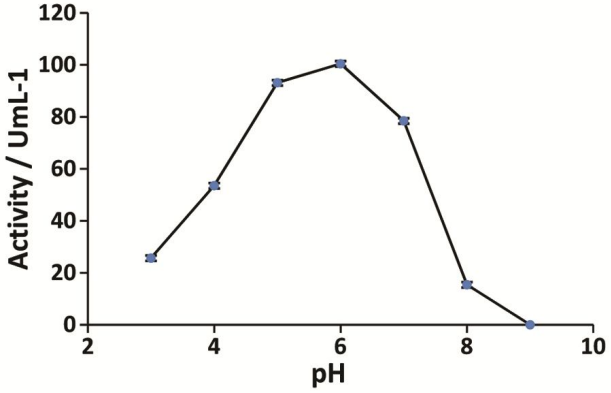

Fig. 6 — Effect of pH on amylase activity

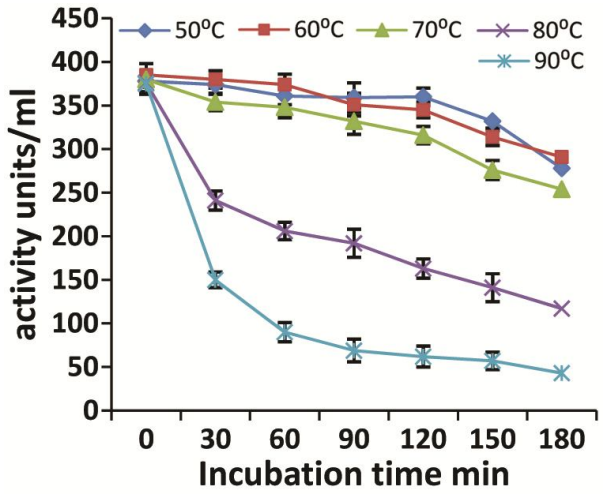

Fig. 7 — Thermal stability of partially purified amylase

As data present in Table 1, enzyme activity was found to be enhanced by Ca^{2+}, Cobalt and sodium ions display no significant effect on enzyme activity[3]. Suganthi *et al*. also reported that sodium ions has no significant effect on enzyme activity. Mg^{2+}shows slight enhancement activity at 10 mM concentration. However, at 5 mM and 10 mM concentration of Ca^{2+}, enzyme activity has been increased by 30% and by two fold respectively. Similar observation has been observed by Saxeena *et al*.[15] for amylase produced using agricultural residues as the carbon source and in Suganthi *et al*. [3]

In conclusion, jackfruit rag seems to be a better carbon source for amylase production. Maximum enzyme production ~ 8400 units/g can be obtained in day 5 old cultures grown at pH of 6.5, 30°C with the substrate level 20 g/L and the nitrogen sources KNO_3 Casein, peptone and beef extract enhance the amylase production. Of them KNO_3 found to be the better nitrogen source. Extracellular amylase production was always found to be higher than that of intracellular. Partially purified enzyme exhibited optimum pH and incubation temperature at pH 6 and 60°C respectively. The unique capability of present amylase to retain about 80% enzyme activity at 70°C compared to the optimum at 60°C makes it useful for various industrial applications like starch liquefaction which carried out at higher temperatures of 70–90°C.

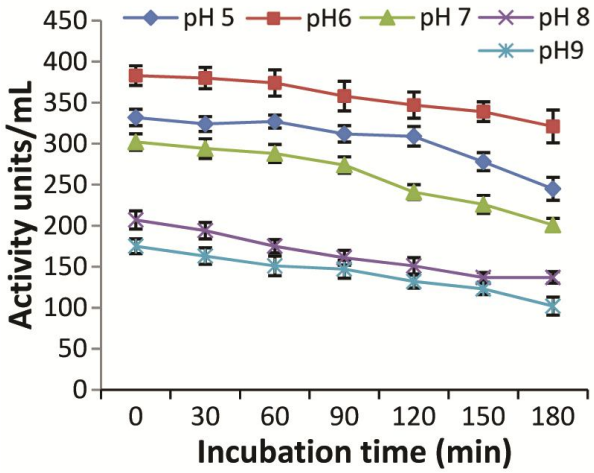

Fig. 8 — pH stability of partially purified amylase

Table 1 — Effect of metal ions on amylase activity

		Amylase activity units/mL			
		Ca^{2+}	Mg^{2+}	Co^{2+}	Na^{+}
Without metal ions		410±10	379±12	389±7	424±11
With metal ions	5 mM	410±10	379±12	389±7	424±11
t	10 mM	1060±9	410±8	407±10	415±8

Furthermore, the enzyme was active under acidic to neutral condition (pH 6-7), which facilitates its use in dough preparation in the food industry, juice and fruit processing, baking and brewing industry. Further, shorter incubation period and lower substrate cost offer the potential for inexpensive production of amylase, making the process industrially and economically feasible.

Acknowledgement

Research grant scheme, University of Kelaniya, Sri Lanka is highly acknowledged for the financial support.

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