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Effect of carbohydrates and amino acids on the production of amylase enzyme by *Rhizopus oryzae* (Went & Prins Geerl.)

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Abstract
Amylases are among the most important industrial enzymes and also have great significance in biotechnological studies. In this study effect of different concentrations of various carbohydrates and amino acids on the production of amylase enzyme by *Rhizopus oryzae* were studied. The activity of amylase enzyme was determined by cup plate assay method as described by Chaurasia *et al*. (2015, 2017b). [9, 11]

Five carbohydrates, viz., Glucose, Fructose, Galactose, Sucrose and Starch were used in different concentrations to study their effect on the production of amylase enzyme. The amylase enzyme production was increased with the increase in the concentrations of carbohydrate. The higher concentration (i.e, 1.5%) of carbohydrates proved to be the most effective for the maximum production of amylase enzyme. Among the carbohydrates, Starch and Glucose were found to be most favourable for maximum production of amylase enzyme while Galactose proved to be poor in this respect. In control (without carbohydrate) no trace of amylase enzyme activity was detected.

Six amino acids, viz., DL- Iso-Leucine, DL-Methionine, DL-β–Phenylalanine, L-Asparagine, L-Glutamic acid and L-Lysine were also used in different concentrations to study their effect on the production of amylase enzyme. Among the amino acids, L-Asparagine (at 5000 ppm concentration) was found to be the most effective for the maximum production of amylase enzyme. DL-Iso-Leucine, DL- β-Phenylalanine and L-Lysine proved to be unfavourable for amylase production in which no trace of amylase enzyme was detected. Therefore, these amino acids have not influenced the production of amylase enzyme.

Keywords: *Rhizopus oryzae*, amylase enzyme, carbohydrates, amino acids

Introduction

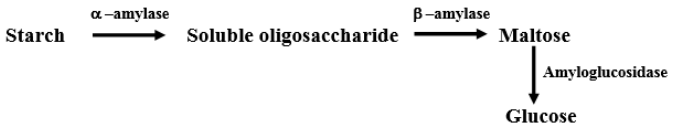
Starch is the major polysaccharide produced by plants as an energy store and is considered as the third biomass source on earth after lignocelluloses and chitins (Choubane *et al*., 2015) [13]. Starch occurs mainly in the seeds, roots and tubers of higher plants. Plants synthesize starch as a result of photosynthesis. It is synthesized in plastids as a storage compound for respiration during dark periods. It is also synthesized in amyloplasts found in tubers, seeds and roots as a long-term storage compound.

Starch is a polymer of glucose linked to one another through the C_1 oxygen by a glycosidic bond. Two types of glucose polymers are present in starch (i) amylose and (ii) amylopectin. Amylose is a linear polymer consisting of upto 6000 glucose units with α, 1-4 linked glucosidic bonds. The amylopction consists of short α,1-4 linked linear chains of 10-60 glucose units and α, 1-6 linked side chains with 15-45 glucose units. These glucose polymer degraded predominantly by hydrolytic enzymes called amylase enzymes.

Amylase enzymes can be divided into following three enzymes:

1. **α –amylase (Alpha-amylase):** α–amylase reduces the viscosity of starch by breaking down the bonds at random, therefore producing varied sized chains of glucose.
2. **β –amylase (Beta-amylase):** β–amylase breaks the glucose- glucose bonds down by removing two glucose units at a time, thereby producing maltose.
3. **Amyloglucosidase (AMG):** Amyloglucosidase breaks successive bonds from the non-reducing end of the straight chain, producing glucose.

Degradation of starch into useful byproducts is a complex process which takes place as under:



In this way, the enzyme amylase helps in the degradation of starch and after enzymatic action, the glucose is a final product (Chaurasia, *et al*., 2015) [9].

Amylases are among the most important enzymes and are of great significance for biotechnology, constituting a class of industrial enzymes having approximately 25-30% of the world enzyme market (Azad *et al*., 2009; Rajagopalan and Krishnan, 2008) [6, 34]. Amylase enzymes are employed in starch processing industries for hydrolysis of polysaccharides such as starch into simple sugar constituents (Fogarty and Kelly, 1980; Nigam and Singh, 1995; Akpan, *et al*., 1999.) [15, 31, 2]

They have diverse application in wide variety of industries such as food, fermentation, textile, paper, detergent, brewing, baking, drink, animal feed, distilling, pharmaceutical and sugar industries (Gupta *et al*.,2003; Kammoun *et al*., 2008; Sharma and Satyanarayana, 2012) [18, 22, 41]. They are principally used for starch liquefaction to minimize viscosity, production of maltose, oligosaccharide mixture, high fructose syrup and maltotetrose syrup. They are used in improving cleaning effect in detergents and starch de-sizing in textile industry (Haq *et al*., 1997) [19]. They are also used in saccharification of starch for fermentation process (Sivaramakrishnan, *et al*., 2006) [44]. Amylase enzymes are also used in the baking industry to give products a larger volume, better colour and smooth texture. Nowadays, spectrum of applications of amylase enzymes are also extending in many other areas such as analytical chemistry, clinical and medicinal diagnosis eg. diagnosis of acute inflammation of pancreas, macroamylasemia, perforated pelvic ulcer and mumps (Muralikrishna and Nirmala, 2005; Anto *et al*., 2006; Nimkar *et al*., 2010; Chimata *et al*., 2010) [29, 5,32, 12].

Amylase enzymes can be procured from various natural resources such as plants, animals and microorganisms (Pandey *et al*., 2005; Muralikrishna and Nirmala, 2005; Mageswari *et al*., 2012) [33, 29, 25]. The enzymes from microbial sources are generally used for industrial applications because they are cheaper to produce and their contents are more predictable, controllable and reliable (Burhan *et al*., 2003) [7]. Amongst microbial sources, fungal amylases are very important because of their more acceptable GRAS (generally regarded as safe) status, the hyphal mode of growth and good tolerance to low water activity and high osmotic pressure conditions which make fungi most efficient for bioconversion of solid substrates (Raimbault, 1998) [36] and thus attracting increasing attention as source of amylolytic enzymes suitable for industrial applications (Mishra and Maheshwari, 1996; Kathiresan and Manivannan, 2006; Hernandez *et al*., 2006) [26, 23, 20]. Amylase of fungal origin are more stable than the bacterial enzymes on a commercial scale (Abu *et al*., 2005; Sanghvi *et al*., 2011) [1, 38].
Major advantage of using fungi for the production of amylase enzymes is the economical bulk production capacity and ease of manipulation. Several fungal species produce amylase enzymes including *Acremonium, Aspergillus, Penicillium, Mucor, Rhizopus, Neurospora* and *Thermomyces. Aspergillus niger, Aspergillus flavus, Aspergillus oryzae* and *Aspergillus awamori* are important sources used among the fungal sources (Ramachandran *et al*., 2004; Sivaramakrishnan *et al*., 2006; Gupta *et al*., 2008) [17, 44, 35]. Other fungal species producing amylase enzyme include *Sclerotium rolfsii* (Chaurasia *et al*., 2015) [9], and *Rhizopus oryzae* (Chaurasia *et al*., (2017b) [11].
Low yield of enzyme has always been a problem in the commercial production of amylases. It is well known that extracellular enzyme production in microorganisms is greatly influenced by nutritional factors like carbohydrates and amino acids (Sema *et al*., 2000; Monga *et. al*., 2011; Vengadaramana *et al*., 2012; Saleem and Ebrahim, 2014) [40, 27, 48, 37]. Carbohydrates and amino acids are known to stimulate the production of amylase enzymes (Zhang *et al*., 1983; Kilingar *et al*., 2012; Vengadaramana *et al*., 2011; 2012) [49, 24, 47, 48].
Considering the above factors, studies were carried out to evaluate the effect of different concentrations of various carbohydrates and amino acids on the production of amylase enzyme. The aim of this work was to study the effect of carbohydrates and amino acids on amylase production by *Rhizopus oryzae*, in order to increase the level of this enzyme in production medium by suitable concentration of carbohydrate and amino acid.

Materials and Methods

Microorganism and Culture Maintenance

Rhizopus oryzae (IMI No. 223116) was used in this study for amylase production, which was isolated from diseased brinjal (*Solanum melongena*) fruit (Chaurasia *et al*., 2017a; 2017b) [10, 11].

The culture was maintained on potato dextrose agar (PDA) slants. The slants were grown at 30^0C for five days and stored at 5^0C. The slants were sub-cultured routinely at an intervals of four-five week.

Production Medium

The production of amylase enzyme was carried out in broth Fernando's medium. The pH of the broth medium was adjusted to pH 5.0 with the help of Backman's pH meter by using 1N HCl or 1N NaOH. This broth fernando's medium was found to be most suitable for the maximum production of amylase enzyme (Chaurasia *et al*., 2017b) [11].
Fernando's medium was of the following composition:

$MgSO_4$. $7H_2O$	…	…	5.00 g
KH_2PO_4	…	…	6.80 g
Asparagine	…	…	5.00 g
Glucose	…	…	15.00 g
Distilled water	…	…	1000 ml

(i) Effect of carbohydrates on amylase production

The various carbohydrates such as Glucose, Fructose, Galactose, Sucrose and Starch in different concentrations were evaluated for their effect on the production of amylase enzyme. Each carbohydrate was used individually in three different concentrations i.e., 0.5, 1.0 and 1.5% by replacing Glucose in the production medium. The production medium without the addition of carbohydrate was used as control.
Twenty five ml of carbohydrate treated production medium was transferred to each 150 ml cotton plugged Erlenmayer flask. The flasks were sterilized in the autoclave at 121^0C for 20 minutes and cooled at room temperature. After cooling each flask was inoculated by a 7.0 mm diameter of mycelial disc taken from the periphery of two days old colony of *Rhizopus oryzae* growing on potato dextrose agar (PDA) medium. The flasks were then incubated at 30^0C for 9 days. After incubation, the culture filtrate of each set was used for enzyme extraction. Each set was run in triplicates.

(ii) Effect of amino acids on amylase production

The various amino acids viz., DL-Iso-Leucine, DL-Methionine, DL-β Phenylalanine, L-Asparagine, L-Glutamic acid and L-Lysine in different concentrations were evaluated for their effect on the production of amylase enzyme. Each amino acid was used individually in four different concentrations i.e., 625, 1250, 2500 and 5000 ppm by replacing Asparagine in the production medium. The

production medium without the addition of amino acid was used as control.

Twenty five ml of amino acid treated production medium was taken in a 150 ml cotton pluged Erlenmayer flask. The flasks were sterilized in autoclave at 121^0C for 20 minutes and after cooling the flask was inoculated by a 7.0 mm diameter of mycelial disc taken from the periphery of two days old colony of *Rhizopus oryze* growing on potato dextrose agar (PDA) medium. The inoculated medium was incubated at 30^0C for 9 days. At the end of the incubation period, the culture filtrate of each set was used for enzyme extraction. Each set was run in triplicates.

Extraction of Amylase

The method of enzyme extraction described by Chaurasia, *et al*., (2015; 2017b) [9, 11] was used. After desired incubation, fungal mat was removed from the medium and the culture filtrates of *Rhizopus oryzae* were collected in separate flasks by filtration under suction. The culture filtrates thus obtained were centrifuged at 10,000 rpm at 4^0C for 20 minutes. After centrifugation, the clear supernatant liquids obtained decanted and used as the crude enzyme preparations.

Assay of Amylase Activity

Enzyme preparations thus obtained were assayed for the activity of amylase. The activity of amylase was determined by cup plate assay method as described by Chaurasia *et al*., (2015; 2017b) [9, 11].

Starch agar medium of the following composition was used for cup plate assay method:

Soluble starch	….	….	10.00 g.
Na_2HPO_4	….	….	2.84 g
NaCl	….	….	0.35 g
Agar agar	….	….	20.00 g
Distilled water	….	….	1000 ml

Twenty five ml of melted starch agar medium was poured into 90 mm diameter sterilized Petridishes and allowed to solidify at room temperature. Then a cavity (10 mm diameter) was made in the center of each Petridish with the help of cork borer. After this, the bottom of the cavity of each Petridish was sealed by adding two drops of melted agar. 0.2 ml of anzyme extract (culture filtrate) of *Rhizopus oryzae* was added to the cavity with the help of micropipette and incubated at 30^0C temperature. After 24 hours, the Petridishes were treated with Logol's iodine solution (Iodine, 5.0g; Potassium iodide, 10.0g; Distilled water 1000 ml). After iodine treatment a clear non-blue zone was measured in mm and the activity of amylase expressed after subtracting the diameter of cavity from the diameter of non-blue zone. Each set was run in triplicates. The amylase activity was calculated by the following formula:

$$\mathbf{AA = D\text{-}d}$$

Where,

AA = Amylase activity.

D = Diameter of cavity in mm plus diameter of non-blue zone in mm

d = Diameter of cavity in mm.

Results and Discussion

1. Effect of Carbohydrates on Amylase Production

Five carbohydrates viz., Glucose, Fructose, Galactose, Sucrose and Starch in different concentrations ware used to study their effect on the production of amylase enzyme. Each carbohydrate was used in three different concentrations i.e., 0.5, 1.0 and 1.5% (W/V) in production medium. The results thus obtained during the present investigation, are presented in Table 1 and Fig. 1.

Table 1: Effect of different concentrations of various carbohydrates on the production of amylase enzyme

Carbohydrates	Amylase activity (width of non-blue Zone in mm)*		
	Concentration (%)		
	0.5	1.0	1.5
Glucose	11.0	13.5	15.0
Fructose	7.6	8.5	11.0
Galactose	5.0	6.0	8.3
Sucrose	10.0	12.0	13.8
Starch	13.0	14.0	15.0
Control (No Carbohydrate) : 0.0			

*After deducting the cavity of 10.0 mm diameter.

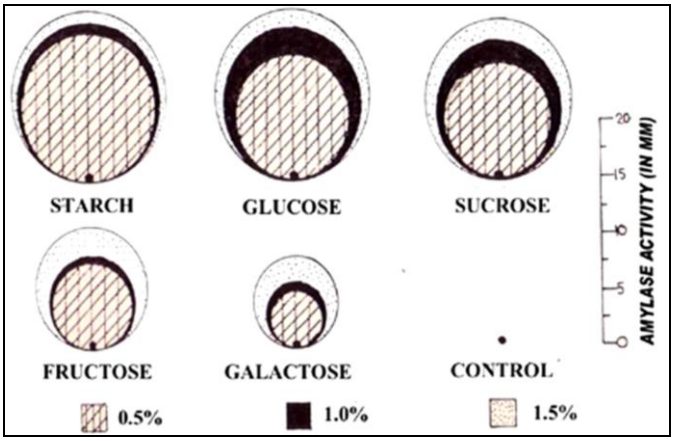


Fig 1: Effect of Different Concentrations of Various Carbohydrates on the Production of Amylase Enzyme

It is evident from the results that all the tested carbohydrates showed stimulatory effect on the production of amylase enzyme in comparison to control. The stimulatory effect of carbohydrates may be due to the way it acts as inducer to stimulate *Rhizopus oryzae* for the production of amylase enzyme. In control (without carbohydrate), no trace of amylase enzyme activity was detected. Lack of amylase production in control medium could be due to carbohydrate deficiencies. In the present study, it was also observed that the amylase enzyme activity increased with the increase in the concentration of carbohydrate from 0.5% to 1.5% (W/V). The results indicate that higher concentration (i.e., 1.5%) of carbohydrate produced more amylase than the lower concentration of carbohydrate. Among the carbohydrates, the Starch and Glucose were found to be the best carbohydrate for the maximum production of amylase enzyme in which 15.0 mm non-blue zone was recorded at 1.5% concentration.

Charya *et al*., (1983) [8] have also reported the maximum production of amylase enzyme when Starch or Glucose was

added to the production medium. Amadioha (1998) [4] has also reported that Starch and Glucose are the best carbohydrates for amylase production. The addition of carbohydrates in the form of either Starch or Glucose had earlier been reported to induce the production of amylase enzyme in *Aspergillus sp.* JGI 12 (Alva *et al.*, 2007) [3], in *Aspergillus sp*. (Sasi *et al*., 2010) [39], in *Aspergillus awamori* (Negi and Banerjee, 2010) [30], in *Aspergillus* niger ML-17 and in *Rhizopus aligosporus* ML-10 (Irfan *et al*., 2012) [21]. Many other workers also reported that Starch is the best carbohydrate for the production of amylase enzyme (Gigras *et al*., 2002; Dharani, 2004) [16, 14]. It is because of the fact that amylase is an extracellular enzyme and its production is increased by its substrate (Varalakshmi *et al*., 2009; Chiamata *et al*., 2010) [46, 12]. In the present study, Sucrose and Fructose (at 1.5% concentration) have been found to be appreciable for amylase production where 13.8 mm and 11.0 mm non blue zone were recorded respectively. Comparatively, Galactose (at 1.5% concentration) was found to be poor for the production of amylase enzyme (8.3 mm non-blue zone). These results indicate that Galactose was poor inducer for amylase production in comparison to other carbohydrates. This may be due to the catabolic repression of amylase production in presence of this Galactose.

From the above results it can be concluded that Starch and Glucose were found to be most favourable for maximum production of amylase enzyme while Galactose proved to be poor in this respect.

2. Effect of Amino acids on Amylase Production

Six amino acids viz., DL-Iso-Leucine, DL-Methionine, DL-β-Phenylalanine, L-Asparagine, L-Glutamic acid and L-Lysine were tried in different concentrations to study their effect on the production of amylase by *Rhizopus oryzae*. Each amino acid was used in four different concentrations i.e., 625, 1250, 2500 and 5000 ppm in production medium. The results obtained during present investigation are presented in Table 2 and Fig. 2.

Table 2: Effect of different concentrations of various amino acids on the production of amylase enzyme

Amino Acids	Amylase activity (width of non-blue Zone in mm)*			
	Concentration (ppm)			
	625	1250	2500	5000
DL-Iso-Leucine	0.0	0.0	0.0	0.0
DL-Methionine	0.0	2.0	5.5	9.0
DL-β-Phenylalanine	0.0	0.0	0.0	0.0
L-Asparagine	3.0	7.0	14.0	15.0
L-Glutamic acid	0.0	0.0	3.0	5.5
L-Lysine	0.0	0.0	0.0	0.0
Control (No amino acid) :	0.0			

*After deducting the cavity of 10.0 mm diameter.

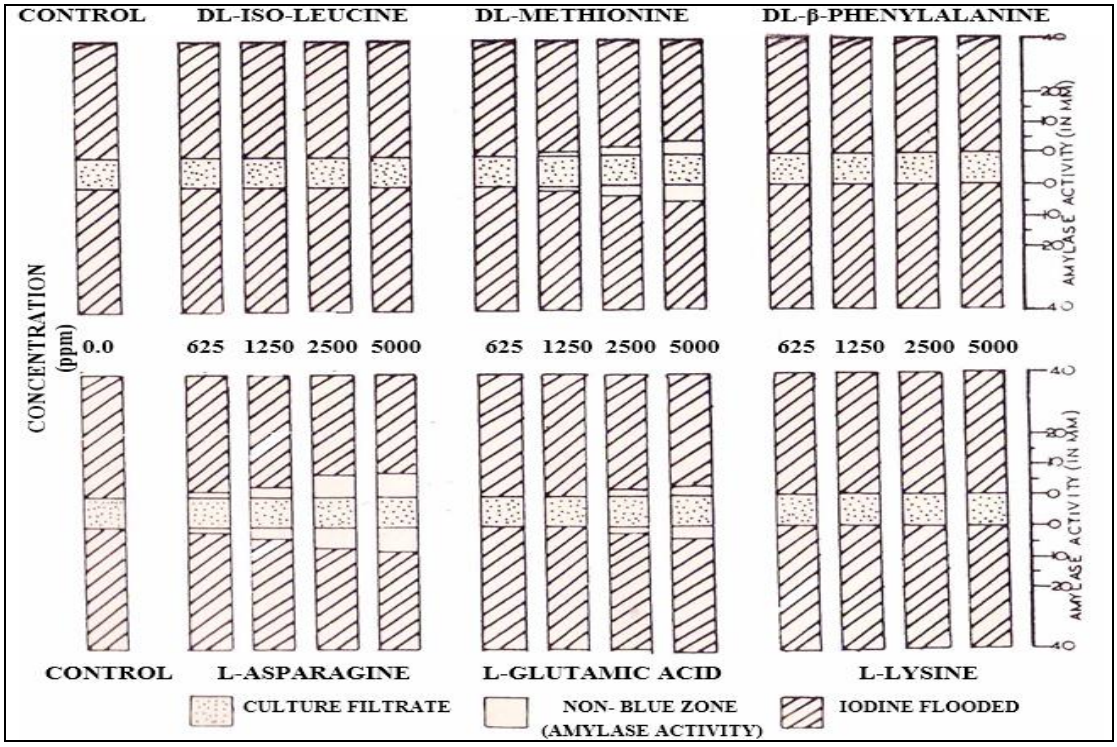


Fig 2: Effect of Different Concentrations of Various Amino Acids on the Production of Amylase Enzyme

It is clear from the results that the production of amylase by *Rhizopus oryzae* was found to be either stimulated and or repressived in presence of amino acid. Out of six amino acids, L-Asparagine was found to be the best for the production of amylase enzyme. The amylase activity was gradually increased with the increase in the concentration of L-Asparagine i.e., from 625 to 5000 ppm. In 5000 ppm concentration of L-Asparagine, maximum amylase activity (15.0 mm non-blue zone) was recorded. The increased production of amylase enzyme might be due to the enhanced

synthesis of amylase enzyme in presence of L-Asparagine. Irfan *et al*., (2012) [21] have also reported the maximum production of amylase by *Rhizopus aligosporus* when L-Asparagine was added to the culture medium. Next to L-Asparagine, DL-Methionine have been found to be satisfactory in which appreciable amylase activity (9.0 mm non-blue zone) was recorded at 5000 ppm concentration. The lower concentration (i.e, 625 ppm) of DL-Methionine proved to be unfavourable for amylase production. Sidkey *et al*., (2010) [42] reported that supplementation of Methionine as an amino acid content to the medium effectively increased the amylase production by *Aspergillus flavus*. In case of L-Glutamic acid, the poor amylase activity i.e., 3.0 and 5.5 mm non-blue zone was detected at 2500 and 5000 ppm concentrations respectively. Below 2500 ppm concentrations of L-Glutamic acid showed no effect on amylase production. Poor amylase production may be due to the poor metabolism of *Rhizopus oryzae*. Some workers (Sidkey *et al*., 1996; Moustafa, 2002) [43, 28] reported that acidic amino acids like Glutamic acid and Aspartic acid are the best inducers for amylase production.

Remaining other three amino acids namely, DL-Iso-Leucine, DL-β-Phenylalanine and L-Lysine have been found to be unfavourable for amylase production in which no trace of amylase enzyme was detected like control (without amino acid). Therefore, these three amino acids did not influence the production of amylase. These results are more or less coincide with the findings of Vengadaramana *et al*., (2012) [48], who have reported that the production of amylase enzyme in amino acids containing medium were almost same as that in the control medium.

From the above results, it can be concluded that L-Asparagine was most favourable for the maximum production of amylase anzyme while DL-Iso-Leucine, DL-β-Phenylalanine and L-Lysine proved to be unfavourable for amylase production.

Conclusion

Fungal amylases are the most important enzymes and have great significance in present-day biotechnology. The production of an amylase enzyme is very sensitive to carbohydrate and amino acid concentrations. Therefore, the selection of suitable concentration of various carbohydrates and amino acids are essential for the production of amylase enzyme. In this study the effect of different concentrations of various carbohydrates and amino acids on the production of amylase enzyme by *Rhizopus oryzae* were studied.

The results obtained in this study show that all the carbohydrates have stimulated the amylase production. The 1.5% concentration of Starch and Glucose were found to be the most effective for the maximum production of amylase enzyme. Out of six amino acids only three amino acids i.e., DL- Methionine, L-Asparagine and L-Glutamic acid were found to be the stimulated for the production of amylase enzyme. The 5000 ppm concentration of L-Aspargine was found to be the most effective for the maximum production of amylase enzyme. This suggests that Starch, Glucose and L-Asparagine can be a potential inducer to stimulate *Rhizopus oryzae* for the production of amylase enzyme which could find applications in industry and biotechnology.

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