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Jacobs Journal of Enzymology and Enzyme Engineering

Research Article

Enzyme Production by *Rhizophus Stolonifer* Isolated from Bread and Kinetic Properties of the Extracellular Amylase

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Received: 06-21-2015

Accepted: 07-24-2015

Published: 07-29-2015

Abstract

Rhizophus species are widely distributed thread like mold, categorized under Kingdom Fungi. It is capable of producing diverse group of enzymes including amylases, proteases and xylanases. The study was aimed to determine the kinetic properties and stability of amylase enzyme produced by *Rhizophus stolonifer* strain purified from bread. A *Rhizophus stolonifer* strain than can grow and produce amylase enzyme on moist bread at room temperature at neutral pH, was selected for this study and screened for the enzyme production. Selected *Rhizophus stolonifer* strain grew well and produced amylases, proteases and xylanases at room temperature and pH 7.0 and a pure form of this strain was used to study the kinetic properties of the crude amylase. The amylase was extracted with 0.1M phosphate buffer of pH 7.0 and at 30°C and it showed zero order kinetics for 10 minutes. The crude amylase activity was higher at pH 7.0 and 40°C. Vmax for the crude enzyme to soluble substrate was 2.81 UmL^{-1} at pH 7.0 and 40°C.

Key words: Rhizophus Stolonifer; Amylase; Kinetic Property; Vmax

Introduction

Rhizophus stolonifer is a widely distributed thread-like mucoralean mold. It is commonly found on bread surfaces, thus takes food and nutrients from the bread and causes damage to the surface where it lives. The Black Bread Mold causes rotting of fruits and infects humans. *Rhizopus stolonifer* acts as decomposers in soil, dung, and in many foods [1]. They grow inside food and absorb nutrients and dissolve the substrate with diverse extracellular enzymes including amylases, xylanases and proteases.

On the basis of various economical, technological and ethical issues, microorganisms are the best sources of enzymes [2]. Microbes serve as the ideal source of enzymes because of their rapid growth, limited space required for their growth and cultivation and the ease of producing enzymes with altered culture conditions [3]. Amylases are starch degrading enzymes, produced by wide range of animals, plants and microorganism. Amylase enzyme is one among the valuable commercial enzymes, is the single largest class of enzymes occupying a pivotal position due to their wide range of application in the industrial processes. Amylases hydrolyze starch, glycogen and related polysaccharides by randomly breaking the internal α-1,4-glucosidic bonds to produce different sizes of oligosaccharides. Amylases are of different categories and are employed mainly in starch processing, brewing, baking and textile industries [4]. There are a lot of factors that

influence the metabolic processes and amylase synthesis and function. Amylases contribute approximately one fourth of the world enzyme market.

Rhizophus species produce a huge variety of extracellular enzymes such as protease, amylase and xylanase which are of industrial importance. It is important to have the kinetic information for any enzyme used in the industry. Poor enzyme stability and quality under standard conditions, will affect the end product and the yield [5,6]. The Objective of this study was to determine the kinetic properties and stability of amylase enzyme produced by *Rhizophus stolonifer* strain purified from bread.

Materials and Methods

Isolation and Purification of *Rhizophus*

The 24 hour old moist bread was kept in the laboratory for one day and the colonies were streaked on a Potato Dextrose Agar (PDA) plate and allowed to grow for 48 hours at room temperature. Colonies grown were transferred on to new sterile PDA plates and incubated. Selection of *Rhizophus stolonifer* was done by streak plate technique, on PDA plates and the plate was incubated for 48 hours at room temperature. Eight pure colonies of *Rhizophus* were selected based on the colour and shape of the observed fungal structures such as sporangium, stolen and rhizoids seen through light microscope.

Screening of *Rhizophus Stolonifer* for the Production of Amylase, Protease and xylanase

The selected pure strains were screened for the production of extracellular protease, amylase and xylanase enzymes. The pure cultures were streaked as a line on the skim milk agar plates and plates were incubated at 25°C for 48 hours. The isolates producing clear zones of hydrolysis were considered as protease producers. Starch agar plates were prepared and inoculated with 5mm diameter cork bore inoculum of the selected isolate. Amylase production was detected by the development of clear zone with iodine after 2 days of incubation at room temperature [7]. Liquid xylan broth (490 mL) was prepared and 10 mL of overnight liquid culture of *Rhizophus stolonifer* was inoculated and maintained at 25°C for 48 hours in a shaker incubator [8]. Xylanase activity was determined by measuring the amount of xylose produced. Here the xylose produced was measured by Dinitrosalicylic acid (DNSA) method [9]. One unit of xylanase activity is defined as the amount of enzyme that produces one µmol of reducing sugar in one minute at pH 7.0 and 30°C with 20 gL^{-1} xylan.

Small Scale Crude Amylase Production by *Rhizophus Stolonifer* and Enzyme Assay

The nutrient agar medium used in the study contained (L^{-1}) 25.0 g nutrient agar, 3.0 g soluble starch. The activation medium used in the study contained (L^{-1}) 25.0 g nutrient broth, and 3.0 g soluble starch at pH 7.0. The fermentation medium contained (L^{-1}) 4.0 g soluble starch, 5.0 g $(NH_4)_2SO_4$, 6 g peptone; 0.01g $FeCl_3$; 0.01 g MgCl2.6H_2O; 0.01g CaCl2.2H2O; 4.0 g of KH_2PO_4, and 7.5 g of K_2HPO_4 at pH 7.0. A loopful of *Rhizophus stolonifer* spore grown in PDA plate with 0.3 % soluble starch at room temperature for 24 h was transferred to 10 ml activation medium and incubated at 40°C in a rotary shaker (100 rpm) for 12 h and used as the inoculum. The fermentation medium was inoculated with 20% (v/v) inoculum and the inoculated flasks were incubated for 48h at 40°C and spun at 100 rpm. The culture filtrate was used as the source of amylase.

Kinetic Studies of the Crude Amylase from *Rhizophus Stolonifer*

Activity of Amylase with Time

Soluble starch (20gL^{-1}, pH 7.0, 0.5 mL) was mixed with amylase (0.5 mL) at 40°C and the amount of glucose produced was monitored.

Effect of Temperature on Amylase Activity

The effect of temperature on amylase activity was determined by incubating the appropriately diluted enzyme (20 mM phosphate buffer at pH 7.0) for optimized amount of time with 0.5mL of soluble xylan at pH 7.0 (20 gL^{-1}) and at different temperatures.

Effect of pH on Amylase Activity

The effect of pH on amylase activity was measured by preparing 2 gL^{-1} soluble starch in buffers at different pH values (pH 3-6 Citrate-phosphate buffer; pH 8.0 Tris- aminomethane buffer; pH 9.0 Glycine-NaOH buffer and pH 10 to 12 Phosphate buffer) and incubated at optimized conditions.

Effect of Substrate Concentration

Different concentrations of starch solutions were prepared (0.25 to 40 gL^{-1}) in 20 mM phosphate buffer at optimum pH and they were allowed to react with the diluted enzyme at optimum temperature for optimized time. The enzyme activity was measured.

Effect of Thermal Stability of the Enzyme

Crude, purified and commercial amylases were pre-incubated at 85°C and at pH 7.0 and the activities of the enzymes were monitored.

Results

Screening Based on Enzyme Production by *Rhizophus Sto-*

***lonifer* Strain**

The selected *Rhizophus stolonifer* strain was screened for the production of amylase, protease and xylanase by growing with the appropriate substrate. Among the eight purified colonies, colony numbers 1 and 2 were selected first because of the production of three types of enzymes and then it was decided to proceed with colony number 2 for its increased amount of enzymes based on the diameter/length of the clear zones on the skim milk agar and starch agar plates. The activity of crude protease, amylase and xylanase of the selected colony number 2 were 78.4, 55.2 and 31.5 pmole/min/m respectively. On the basis of these screening tests, the examined strain produced all the enzymes tested. As the amylase activity was decided to study further in this research, kinetic properties of the amylase were analyzed.

Effect of Time

Purified amylase showed zero order kinetics for 10 minutes (Figure 1).

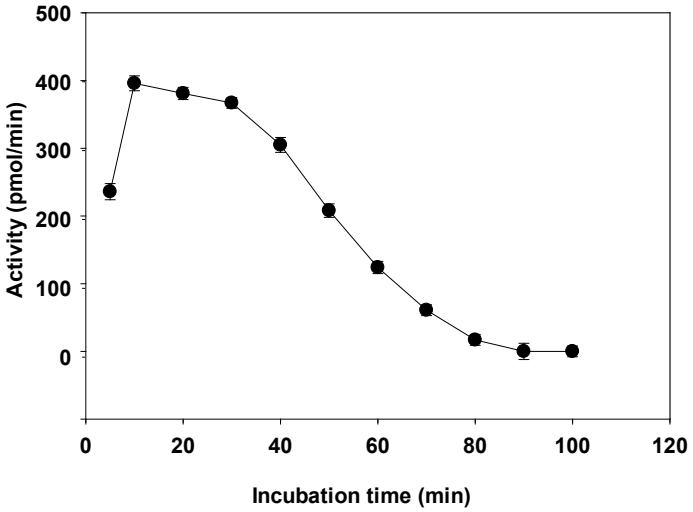

Figure 1. Effect of time of incubation on crude amylase activity.

Effect of Temperature

The amylase activity was assayed at different temperatures ranging from 40 - 100°C at pH 7.0. The activity of amylase increased up to 40°C and further increase in temperature decreased the enzyme activity. Amylase from the isolate exhibited a temperature profile with a sharp peak of maximal activity at 40°C and showed activity between 40 - 90°C (Figure 2). The optimum temperature for the activity depends on the type of organisms.

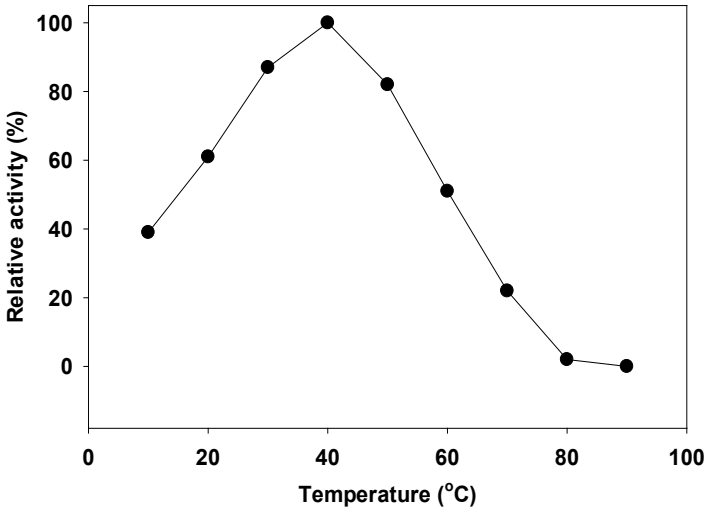

Figure 2. Effect of temperature on the crude amylase activity at pH 7.0 and 20 gL^{-1} soluble starch.

Effect of pH

When the pH was varied from 3.0 to 12.0 the activity of amylase was increased up to pH 7.0 and further increase of pH decreased the enzyme activity (Figure 3). Amylase showed activity between pH 3.0 to 12.0. However the enzyme showed 75% of its original activity at pH values between 5.0 and 9.0. Since the highest activity was obtained at pH 7.0, it was selected for further studies (Figure 3).

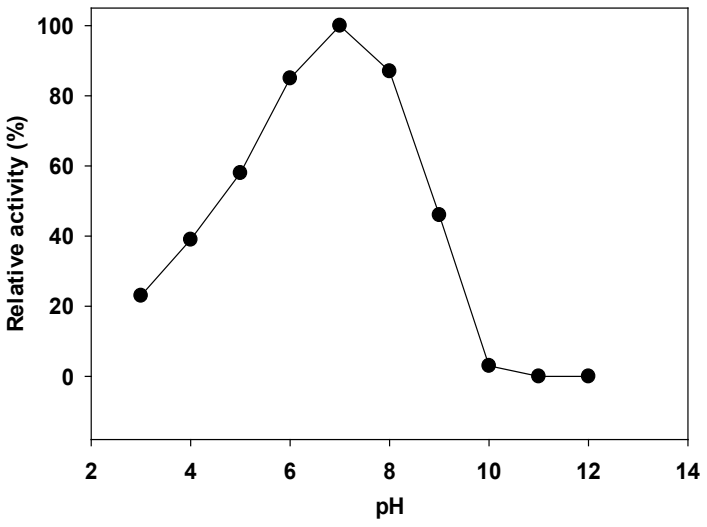

Figure 3. Effect of pH on the crude amylase activity at 40°C at 20 gL^{-1} soluble starch.

Effect of Substrate Concentration

Lineweaver-Burk plot (Figure 4) was used to determine the Km and Vmax of the crude extract of amylase enzyme and is shown in Figure 5. When the substrate concentration was increased from 0.25 to 40 gL^{-1} at pH 7.0 and the activity of amylase increased up to 10 gL^{-1} and reached the maximum velocity at 40 gL^{-1}. Michaelis constant for the crude enzyme to soluble starch was 6.95 $g\cdot L^{-1}$ and Vmax was 2.81 UmL^{-1} at pH 7.0 and at 40°C (Figure 4). Therefore 20 gL^{-1} substrate was used for the assay for further studies of enzyme activity.

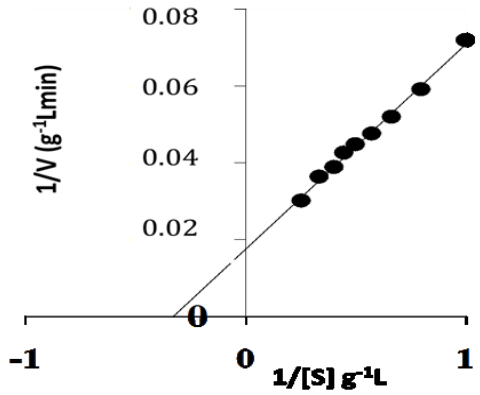

Figure 4. Lineweaver-Burk plot of the crude amylase activity at pH 7.0 and 40°C using different concentrations of substrate.

Stability of Enzymes with Temperature

The residual activities of crude amylase incubated at different temperatures for a period of 1 hour were estimated. When the crude enzyme was preincubated at different temperatures (30, 40 and 50°C), the activity decreased with time. The enzyme was stable for at least 40 minutes at all the temperatures tested. When the crude enzyme was preincubated at 30, 40 and 50°C for 40 min it retained 21.7, 1.9 and 0% of the original activity respectively. The enzyme showed optimum activity at 40°C. Half-life of amylase indicates that amylase is more stable at 30°C.

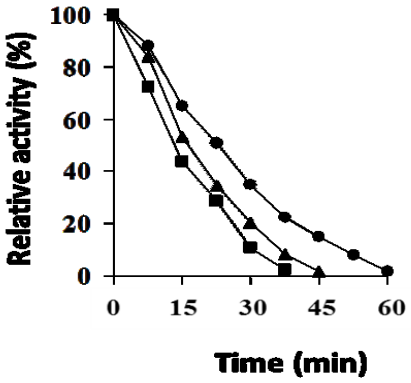

Figure 5. Stability of amylase at different temperature of (●), 30; (▲), 40 and (□), 50°C. Amylase activity was measured at pH 7.0, using 20 gL^{-1} starch as substrate by incubating for 5minutes.

Stability of Enzymes with pH

When the crude enzyme solution, at pH 6.0 –8.0 was preincubated at 40°C, the enzyme activity decreased with time. The enzyme was stable for at least 48 minutes at all the pH values tested. The crude enzyme retained 9.2 (pH 6.0), 7.2 (pH 7.0), and 0.2 (pH 8.0)% of the activity at 48 minutes at 40°C (Figure 6). The half-life of amylase indicates that amylase is more stable in pH 7.0 than the other pH values. It is also important to note that the enzyme showed optimum activity at pH 9.0.

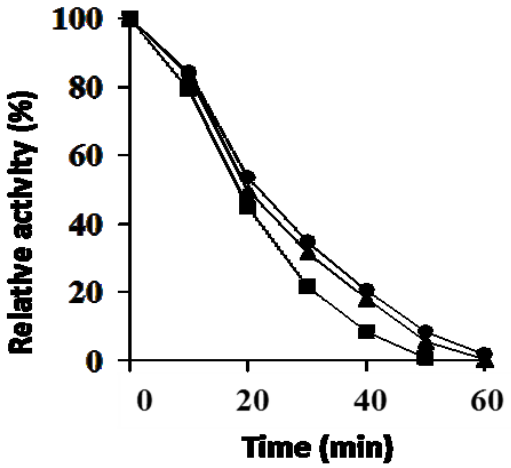

Figure 6. Stability of amylase at different pH values of (▲), 6; (●), 7 and (□), 8. Amylase activity was measured at 40°C, using 20 gL^{-1} starch as substrate by incubating for 5minutes.

Discussion

The optimal pH of amylase was 7.0 that suggest it can be classified as neutral amylase. The optimal temperature for the protease activity was 40°C. The activity remained at 90°C, so amylase is thermostable, this may be due to the presence of higher amount of cysteine residues [10]. The increase in temperature causes increase in collision between enzymes and substrates [11]. Beyond these optimal temperatures the activities were decreased due to denaturation of amylases due to heat [12]. The incubation time was found to be most effective only for 90 minutes. The enzyme activity gradually fell down indicating inactivation of enzyme with time. Slightly linear line was obtained for the effect of enzyme concentration on amylase activity indicated that the enzyme concentration enhances the rate of reaction. The linear line obtained for the effect of substrate concentration on amylase activity indicated that the rate of reaction increases with the increase in substrate concentration. The low Km value of amylases indicated that the substrate is tightly bound to the enzyme. These stabilizing electrostatic in-

teractions reduce the extent of unfolding of the enzyme molecule at high temperatures and it will make the enzyme less prone to forming incorrect structures and thus the chances for the thermal inactivation will be low [13] .

Conclusion

Rhizophus stolonifer strain isolated from moist bread grew well in submerged fermentation system and produced amylases, proteases and xylanases at room temperature and pH 7.0. The activity of crude amylase produced by *Rhizophus stolonifer* was higher at pH 7.0 and temperature of 40°C. Amylase showed zero order kinetics for 10 minutes and it was active even at 90°C. The Michaelis constant for the crude enzyme to soluble starch was 6.95 gL^{-1} and Vmax was 2.81 UmL^{-1} at pH 7.0 and at 40°C.

Acknowledgements

The author is sincerely grateful to the MRC, Chiang Mai, Thailand for the financial support and laboratory facilities.

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