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RESEARCH ARTICLE

FUNGAL AMYLASE

Starch Hydrolysis and α-amylase Activity of *Trichoderma viride* (AUFS1)

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Abstract

Quantification of starch hydrolysis and alpha-amylase activity of *Trichoderma viride* under laboratory assay conditions was studied by culturing the AUFS1 strain of *T. viridi* in fungal growth medium. Two percent starch was used as the substrate for amylase activity. The pH and salt concentration were maintained at 6.5 and 1.2%, respectively, and the incubation temperature was maintained at 30°C. Starch hydrolysis activity was measured based on the decrease in iodine staining of starch, and α-amylase activity was measured using dinitro salicylic acid. Color intensity was measured at 660 nm for iodine and 540 nm for α-amylase activity, respectively. Starch hydrolysis activity was found to be maximum after the 9^{th} day of incubation, but the crude enzyme extract of the 10^{th} day-old culture showed maximum activity. In the case of α-amylase, maximum activity was recorded from the 9^{th} to the 12^{th} days of incubation, while the crude enzyme extract of the 6-8 days old culture showed maximum activity. Protease activity was found to be positively correlated with starch hydrolysis and α-amylase activity. Amylase activity of the *T. viride* strain (AUFS1) was enhanced with an increase in the incubation period. Higher amylase activity was recorded between the 8^{th} and 11^{th} days of incubation, and the crude enzyme extract of the 6-8 days old culture had maximum amylase activity. Starch hydrolysis activity was positively correlated with α-amylase activity. This initial study shows that the above strain of *T. viridi* has the potential to be used for industrial applications.

Keywords: *Garden soil, α-amylase, Starch hydrolysis, Trichoderma viridi*

1 Introduction

Starch-degradation enzymes are gaining more importance among the industrial enzymes because of the importance of starch, sugar and other products in modern biotechnology era(1).Microbiol amylase has completely replaced chemical hydrolysis in starch processing industry(2). They are also potential candidates in the medicinal, clinical and fine chemical industries(2; 3). Industrial demand for these enzymes is limited with specific applications as in the food industry, where fungal amylases are preferred over other microbial sources mainly because of their more acceptable GRAS status(1). Hence, fungi are being considered as important microbes for production of amylolytic enzymes suitable for the industrial conversion of starch into maltose or glucose(4). *Trichoderma viridi* Pers ex Grey (1821) can be ranked as one of the most widely distributed of all soil fungi. It is found in the entire habitat from tropical to alpine regions. They are also found in extreme habitats such as salt-marshes, saline soil, mangrove swamps, dunes and desert, soils-under burnt chaparral(5). In the present work, starch hydrolysis and α- amylase activity of *Trichoderma viridi* strain (AUFS1) was measured from 5^{th} day to 11^{th} day of incubation period at 30°C temperature levels keeping pH and salt concentration constant at 6.5 and 1.2% respectively.

2 Materials and Methods

2.1 Sample Collection

To isolate and screened out amylase producing fungi, soil samples were collected from Botanical Garden of Rajiv Gandhi University, Arunachal Pradesh, India. Isolation was carried out by serial dilution plate method using Rose Bengal agar media containing 0.01% glucose, 0.5% peptone,

0.1% K_2HPO_4, 0.05% $MgSO_4 \cdot 7\,H_2O$, 1.5% agar, 0.003% rose Bengal and 1% soluble starch. Selections of amylase positive fungi were done on the basis of transparent zone created due to the hydrolysis of starch by staining the culture plate with iodine solution (containing 0.3% iodine and 0.6% KI). Subsequently, the amylase positive fungal colonies were pure cultured in potato dextrose agar media and identified. *Trichoderma viridi* was found to have higher starch hydrolyzing zone and was finally selected for further analysis.

3 Identification of Fungal Strains

Species level identification of *Trichoderma viridi* as done with the help of manuals of fungi available(5).

3.1 Preservation of Cultures

One copy of both the strains is preserved by dry preservation at -80°C in PDA media containing 20% glycerol and fresh cultures are maintained for different biochemical and molecular evaluations.

3.2 Minimal Medium for Amylase Production

The minimal medium for amylase production contained: 0.14% KH_2PO_4, 1% NH_4NO_3, 0.05% KCL, 0.01% $MgSO_4 \cdot 7\,H_2O$, 0.001% $FeSO_4 \cdot 7\,H_2O$ and 2% Starch (pH 6.5).

3.3 Amylase Production

Erlenmeyer flask (100 ml) containing 50 ml of medium was inoculated with approximately one loopfull of inoculum. After 5 days of growth, the culture was examined and diluted with the required quantity of autoclaved distilled water so as to get approximately $3x10^6$ numbers of spores per ml of the culture. 1ml of the culture was added per 50ml of medium and incubated at 30°C temperature for 11 days with continuous shaking to avoid clumping of mycelium.

4 Extraction of Enzymes

Cultured fungal medium was filtered using Whatman filter paper no. 42. The supernatant containing crude enzyme extract was considered for further analysis.

5 Amylase Assay-Starch Hydrolysis

Amount of starch hydrolyzed to simple sugar by AUFS1 strain was assayed based on the decrease in iodine staining of starch. One ml each of crude and heat killed enzyme extract was taken for each sample. To it one ml of 1% soluble starch in citrate-phosphate buffer (pH 6.5) was added. Tubes were incubated in water bath at 30°C for 30 minutes. The reaction was stopped by adding 1ml of 0.5N HCl. One ml of the above acidified solution was added to 1ml of 0.5N HCl. From this solution, 1ml was be added to 1ml iodine solution (containing 0.05% iodine in 0.5% KI). To it 20ml of distilled water was added and colour intensity was measured at 660 nm using spectrophotometer (Systronic, 106). An enzyme unit is defined as the amount of enzyme reducing 1µg of starch under assay condition.

6 α-Amylase Activity

α-amylase activity was measured by using dinitro-salicylic acid as describe by(6). One ml each of crude and heat killed enzyme extract was taken for each sample. To it one ml of 1% soluble starch in citrate-phosphate buffer (pH 6.5) was added. Tubes were incubated in water bath at 30°C for 30 minutes. The reaction ceased after 30 minutes of incubation by adding 2 ml of DNS reagent (containing 1% 3,5, dinitro salicylic acid; 20% potassium-sodium tertrate; 0.2% phenol; 0.05% Sodium carbonate and 1% NaOH). For colour development, the tubes were finally placed in boiling water bath for 5 minutes. After cooling, 20ml of distilled water was added to each tubes and colour intensity was determined at 540nm using spectrophotometer (Systronic, 106). The optical density value of killed samples gives an idea of reducing sugar on diurnal basis, whereas the value recorded by incubation non-killed enzyme extract of different growth intervals in starch solution (as substrate) for 30 minutes minus the value of killed samples gives an idea of reducing sugar produced in 30 minutes of incubation period. Maltose calibration curve was used to convert colour to reducing sugar equivalent. An enzyme unit is defined as amount of α- amylase releasing 1 µg maltose or maltose equivalent from the substrate under assay condition.

6.1 Protease Activity

Protein in the culture filtrate was measured by the method as described(7) using bovine serum albumin as standard. A unit of protease activity is defined as amount of protein releasing 1µg tyrosine from BSA under assay conditions.

6.2 Measurement of Biomass

Cells were harvested by filtration through pre weight Whatman no. 42 filter paper and dried to a constant weight at 110°C for 24 hours. The biomass was then determined as mg/10ml culture.

7 Results and Discussion

Diurnal activity on starch hydrolysis by AUFS1 strain was measured from 5^{th} day upto 11^{th} day of incubation at 30°C. Unit of starch reduced to simpler form after 5th day of incubation was found to be 423U/ml and that of 11^{th} day was 837U/ml. Highest activity was recorded after 9^{th} day of incubation (1159U/ml) followed by 11^{th} day (837U/ml) respectively. When the crude enzyme extracts of different growth interval were incubated with starch as substrate at 30°C for 30 min, maximum activity was recorded with 10^{th} day old enzyme extract (285U/ml) Figure 1

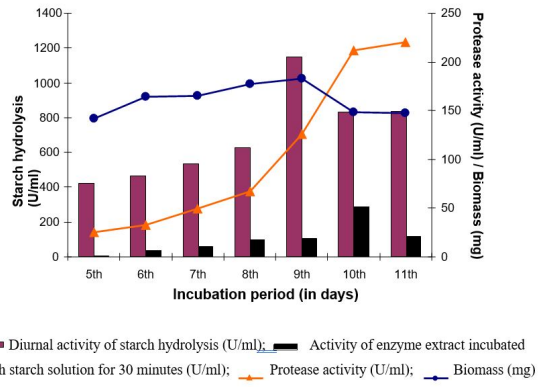

Figure 1: Growth and Starch Hydrolysis Activity of *T.viride* At 30°C Using Starch As Substrate

In case of alpha amylase, a sharp incline in activity was recorded from 5^{th} to 10^{th} day of incubation period after which the activity starts declining. Maximum activity was recorded after 10^{th} day of incubation (974U/ml) followed by 9^{th} day (952U/ml) and 11^{th} day (944U/ml) respectively. Similarly, when the alpha amylase activity measured with crude enzyme extracts, highest activity was recorded with 6^{th} to 8^{th} day old culture extract (305U/ml-369U/ml respectively) Figure 2.

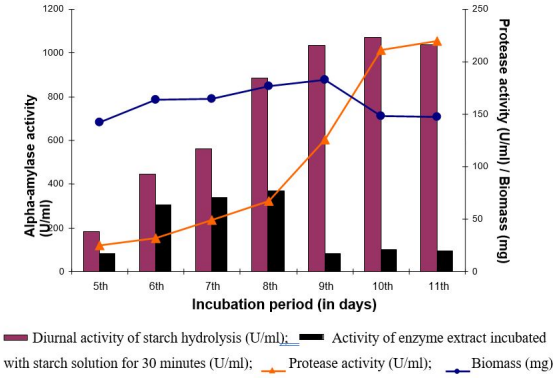

Figure 2: Growth and α-Amylase Production By *T.viride* At 30°C Using Starch As Substrate

The protease activity was found to be lower during the first two days of analysis (i.e, 5^{th} and 6^{th} day). After 7^{th} day of incubation, a sharp increase in protein concentration was recorded and it continues upto 11^{th} day. Maximum protease activity was recorded with sample extract of 10^{th} and 11^{th} day old culture (211.4 U/ml and 219 U/ml respectively). From Figure 1 it is also evident that there was increase in biomass content of *T.viridi* strain from 5^{th} day to 9^{th} day of incubation after which a decline in dry mass was recorded. Maximum dry weight was measured in 9^{th} day old culture (183 mg) followed by 8^{th} day (177mg), 7^{th} day (165 mg) etc, respectively. Statistical analysis showed significant positive correlation between starch hydrolysis activity and α-amylase activity (r=0.844). Protease activity was found to have significant correlation with the starch hydrolysis and α-amylase activity, but showed negative correlation with biomass and α-amylase activity of crude enzyme extract. Similarly, the diurnal changes in biomass were found to have insignificant correlation with starch hydrolysis and α-amylase activity Table 1.

Supporting the above findings, report(8) is available describing that the amylolytic enzyme system is completely extracellular, equally well induced by starch, amylose or amylopectin and that it consists mainly of enzymes of the glucoamylase type which yield glucose as the main product of starch hydrolysis. Supporting the present findings, there is a report(9) of maximum amylase and protease activity in Trichoderma species from 72-120 hrs of growth in the presence of specific substrate. Moreover, in accordance to the previous findings which stated that enzymatic activity in microbes can be correlated as a function of incubation temperature, incubation time, dilution factor and measurement method(10), the present study have shown maximum enzymatic activity of *T.viridi* strain (AUFS1) at optimum incubation temperature of 30°C, pH 6.5 and 1.2% salt concentration. It has also been reported(5) that the optimum temperature for maximum growth of most isolates of *T.viride* is 30°C and there is a report(11) indicating maximum alpha amylase production by *T.viridi* at 30°C. Higher amylolytic activity of AUFS1 strain of *T.viridi* indicates that this strain has wide scale application in starch industry and can also be utilized as a good source for decomposition of polysaccharides present in organic waste to simpler form. Role of *T.viride* in decomposition of starch and amylase production is well documented along with the report of wide scale occurrence of *T.viride* in sewage treatment plant, waste stabilization pond, composed house hold refuge etc. (5). Further studies on its fermentation kinetics and molecular characterization is essential to get better understanding on its industrial applicability.

8 Conclusion

Quantification of starch hydrolysis and alpha-amylase activity of Trichoderma viride under laboratory assay condition was studied culturing AUFS1 strain of *T.viridi* in fungal growth medium. Two percent starch was used as the substrate for amylase activity. Amylase activity of Trichoderma viride strain (AUFS1) got enhanced with increase in incubation period. Higher amylase activity was observed between 8-11 days of incubation while crude enzyme of 6-8 days old culture extract was found to have maximum amylase activity. Starch hydrolysis activity was found to be positively correlated with the α-amylase activity. The study showed that the AUFS1 strain of *T. viridi* has potential to be used for industrial applications.

Conflict of Interest

The authors declare no competing interest with any person or organization.

Table 1: Karl Pearson Correlation Coefficient, Showing Correlation of Starch Hydrolysis and α-Amylase Activity with Protease Activity and Biomass

Factors	Correlation with protease activity	Correlation with biomass
Diurnal starch hydrolysis activity of sample extract	0.7(p= 0.05)	0.365(p= NS*)
Starch hydrolysis activity of crude enzyme extract in 30 minutes of incubation period	0.798(p= 0.02)	-0.119(p= NS)
Diurnal α-amylase activity of sample extract	0.839(p= 0.01)	0.283(p= NS)
α-amylase activity of crude enzyme extract in 30 minutes of incubation period	-0.571(p= NS)	0.475(p= NS)
Protease activity		-0.253(p= NS)
*N.S. = Not Significant		

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References

[1] R. Prakasham, C. Subba Rao, R. Sreenivas Rao, and P. Sarma, "Enhancement of acid amylase production by an isolated aspergillus awamori," *Journal of applied microbiology*, vol. 102, no. 1, pp. 204–211, 2007.

[2] R. Gupta, P. Gigras, H. Mohapatra, V. K. Goswami, and B. Chauhan, "Microbial α-amylases: a biotechnological perspective," *Process biochemistry*, vol. 38, no. 11, pp. 1599–1616, 2003.

[3] S. Becks, C. Bielawski, D. Henton, R. Padala, K. Burrows, and R. Slaby, "The application of a liquid stable amylase reagent on the ciba corning express clinical-chemistry system," in *Clinical Chemistry*, vol. 41, no. 6. Amer Assoc Clinical Chemistry 2101 L Street Nw, Suite 202, Washington, DC , 1995, pp. S186–S186.

[4] R. S. Mishra and R. Maheshwari, "Amylases of the thermophilic fungus thermomyces lanuginosus: their purification, properties, action on starch and response to heat," *Journal of Biosciences*, vol. 21, pp. 653–672, 1996.

[5] W. Gams and T.-H. Anderson, *Compendium of soil fungi*. Academic press, 1980.

[6] G. Mamo and A. Gessesse, "Thermostable amylase production by immobilized thermophilic bacillus sp." *Biotechnology Techniques*, vol. 11, pp. 447–450, 1997.

[7] O. H. Lowry, N. J. Rosebrough, A. L. Farr, R. J. Randall *et al.*, "Protein measurement with the folin phenol reagent," *J biol Chem*, vol. 193, no. 1, pp. 265–275, 1951.

[8] J. Schellart, F. Visser, T. Zandstra, and W. Middelhoven, "Starch degradation by the mould trichoderma viride i. the mechanism of starch degradation," *Antonie van Leeuwenhoek*, vol. 42, pp. 229–238, 1976.

[9] J. L. D. Marco, M. C. Valadares-Inglis, and C. R. Felix, "Production of hydrolytic enzymes by trichoderma isolates with antagonistic activity against crinipellis perniciosa, the causal agent of witches' broom of cocoa," *Brazilian journal of microbiology*, vol. 34, pp. 33–38, 2003.

[10] Y. J. Yoo, J. Hong, and R. T. Hatch, "Comparison of α-amylase activities from different assay methods," *Biotechnology and bioengineering*, vol. 30, no. 1, pp. 147–151, 1987.

[11] S. Mahmood and S. R. Rahman, "Production and partial characterization of extracellular α-amylase by trichoderma viride," *Bangladesh J Microbiol*, vol. 25, no. 2, pp. 99–103, 2008.