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PRODUCTION OF AMYLASE FROM *EICHHORNIA CRASSIPES* AND *PISTIA STRATIOTES* AS SUBSTRATES USING SOLID STATE FERMENTATION

#T. Godbole, *C. Velhal and C. S. Kulkarni
Mitcon Biotechnology and Pharmaceutical Center, Shivajinagar, Pune - 411005.

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ABSTRACT:

B. Subtilis (NCIM 2063) and *A. Niger* (NCIM 620) were used for amylase production from *E. Crassipes* and *P. Stratiotes*. The fermentation batches were set up in minimal basal media. Maximum amylase activity was seen by *Aspergillus niger* on *Pistia stratiotes* (Day 2) and *Eicchornia crassipes* (Day 4) at 32°C. *A. niger* with a spore count of $6.4x10^9$ spores/ml after partial purification produced 6.8 units/ml enzyme in *P. stratiotes* and 7.3units/ml enzyme in *E. crassipes* with a spore count of $3.2x10^9$ spores/ml. *B. subtilis* with CFU of $4.1x10^9$ after partial purification produced 6.239 units/ml enzyme in *P. stratiotes* and 6.08 units/ml enzyme in *E. crassipes* with CFU of $4.6x10^9$. The results obtained are significant as there have been no reports of production of amylase from these two plants using solid state fermentation (SSF).

Keywords: *Aspergillus niger, Bacillus subtilis, Eichhornia crassipes, Pistia stratiotes, Amylase* and Solid substrate fermentation.

***Corresponding Author:**
Mr. Chaitanya Velhal
Mitcon Biotechnology and Pharmaceutical Center
Agriculture College Campus, Near D.I.C.,
Shivajinagar, Pune - 411005.
Phone : 020 66289451 / 52/ 53 / 54
Fax : 020 - 25521607 / 25530307
Email: chaitanyavelhal@yahoo.com

INTRODUCTION

Amylases are a group of enzymes which consists of α-amylase, β-amylase and glucoamylase which hydrolyses starch and sugars. These are industrially important enzymes which are obtained from various microbial sources usually through submerged fermentation method. These enzymes capture about 25-30% of the worlds industrially important enzymes market[1]. Among these enzymes the most widely used and important enzyme is α-amylase also called as 1, 4-α-D-glucan glucanohydrolase ([EC 3.2.1.1]) which find a wide array of application in the food industry, fermentation industry paper industry and textile industry.

These enzymes have been produced traditionally by submerged fermentation method owing to its ease in controlling various environmental parameters and handling. Glancing at the history we see that the submerged fermentation had become a role model technique since the development of penicillin due to which the ancient technique of solid state fermentation had got neglected[2]. Solid state fermentation is a technique involving the absence of free water in the moist substrate[3]. This method of fermentation is an interesting alternative to the other techniques which eases the downstream processing of the metabolites[4]. The beginning of fermentation technique in nature is thought to begin on the moist surfaces of solids in ancient times (solid state fermentation). This method involves a number of advantages like it involves less energy requirements, less waste water as no free water is involved in it, less downstream processing, more concentrated products after downstream processing and utilisation of natural waste substrates thus taking care of the waste disposal making this method ecofriendly[5]. Also the environmental conditions that are provided to the organism are more similar to its habitat.

Thus there is a renewed interest in harnessing this method in the industrial or large scale production of the enzymes and has been often used for the same[3]. It has been reported that by using solid state fermentation the cost for the production media and downstream processing of the enzyme can be reduced by 50-60% as compared to the traditional submerge fermentation method thus making it a cost effective and cheaper method[5].

Eichhornia crassipes which belongs to the family Pontederiaceae, commonly known as water hyacinth is a perennial free floating aquatic plant bearing long, pendant roots, rhizomes, stolons, leaves, inflorescences and fruit clusters and usually 40 cm in height which may grow upto 1m also[6]. This plant is also considered as a noxious weed as it grows very fast and depletes the nutrients thereby lowering the BOD of the water body adversely affecting the flora and fauna. They can achieve a growth rate of 17.5 metric tons per hectare per day[7]. They reproduce sexually and asexually through seeds and stolons respectively. These seeds remain viable for up to 20 years making it difficult to control. If not controlled in time there have been instances when they have caused blockage of the water body making recreational activities like fishing very difficult also making them inhabitable and inaccessible. They also out-complete the other species in its vicinity outgrowing them and decreasing biodiversity. These plants also act as breeding grounds for insects and pests and also facilitate the water evaporation from the water bodies[7]. They also make the ecosystem less fertile by absorbing the nutrients in the water bodies surrounding it[6, 8].

Pistia stratiotes commonly called as water lettuce is a free floating aquatic weed which resembles an open head of lettuce. It forms dense mats restricting the water flow causing an increased evaporation in the water bodies, reduces infiltration of sunlight affecting the submerged aquatic plants and also acts as a breeding ground for mosquitoes[9]. Similar to *Eicchornia crassipes* this plant also acts as a noxious element and harms the environment adversely[9].

As both these plants have been causing a nuisance worldwide the need to control them is the necessity at the present date as they grow and spread at an alarming rate. These plants cause enormous blockages and facilitate the breeding and spread of various diseases, insects as well as they themselves grow and proliferate in such conditions[8]. A substantial amount is spent annually worldwide to control them by various physical, chemical, and biological means. These plants have been reported to act as substrates for the production of various industrially important enzymes. Here in this study we are going to use these nuisance causing plants as substrates for the production of amylase and check for its feasibility for the same. These plants will be used as carbon sources for *Aspergillus niger*, which is known to produce amylase in large quantities with other substrates[10].

MATERIALS AND METHODS

Standard cultures of *B. subtilis* (NCIM2063) and *A. niger* (NCIM 620) were used for this experiment.

1) **Substrate preparation:** Whole plants of *Eichhornia crassipes* and *Pistia stratiotes* were first collected washed and the roots were excised. These were further washed and cut into small pieces which were used as the raw substrate for the enzyme production.
2) **Amylase production medium:** Using *P. stratiotes* and *E. crassipes* as the sole source of carbon and nitrogen; *B. subtilis* was inoculated in basal media containing 0.1% KH_2PO_4, 0.025% $MgSO_4$, 0.01% $CaCl_2$, 0.2% $NaNO_3$, 0.05% K_2HPO_4 and 0.05% KCl whereas, *A. niger* was inoculated in basal media containing 0.6% K_2HPO_4, 0.20% KH_2PO_4 and 0.01% $MgSO_4.7H_2O$ (11 ,12). 300 ml of basal media was supplemented with 50 grams of each *E. crassipes* and *P. stratiotes*, which was inoculated with 1 ml of 3.6×10^8 bacterial cells and 1 ml of 10^9 fungal spores at room temperature for 3 days.

Same cultures of the two organisms were also inoculated in a media containing only 50 grams of *P. Stratiotes* and *E. crassipes* and 300 ml distilled water where the plants acted as the sole source of all nutrients needed for the organisms to grow and produce enzymes. The prepared flasks were incubated at room temperature for 72 hours.

3) **Partial purification:** 10 ml aliquot of culture filtrate was cooled to 4°C for 30 min. This was treated with an equal volume of pre-chilled acetone and centrifuged at 2500 rpm for 15 min. The supernatant was discarded and the pellet collected and micro-centrifuged at 10,000 rpm for 5 min to remove residual supernatant. The pellet was resuspended in 1 ml distilled water and used for further analysis.
4) **Enzyme assay:** The standard DNSA method was used to quantify the amylase enzyme activity with 2% starch as the substrate.

RESULTS AND DISCUSSION

Both the organisms *Aspergillus niger* and *Bacillus subtilis* were grown on both the substrates *Eicchornia crassipes* and *Pistia Stratiotes* and observed for the amylase activity daily.

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For *Eicchornia crassipes,* on day 1, BS showed zero activity whereas *A. Niger* showed an activity of 1.134. Following this there is a rise seen in the activity of *A.Niger* to 1.7015 and also a steep rise in the activity by BS to 3.970 on day 2. The activity by AN was observed to remain constant on day 3 and that of BS increased to 5.672. on day 4, there is a steep rise in the activity by AN (7.373) accompanied by BS to 6.806 after which a decrease in the activities was seen in both the organisms BS (3.402) and AN (3.402) on the following 5th day.

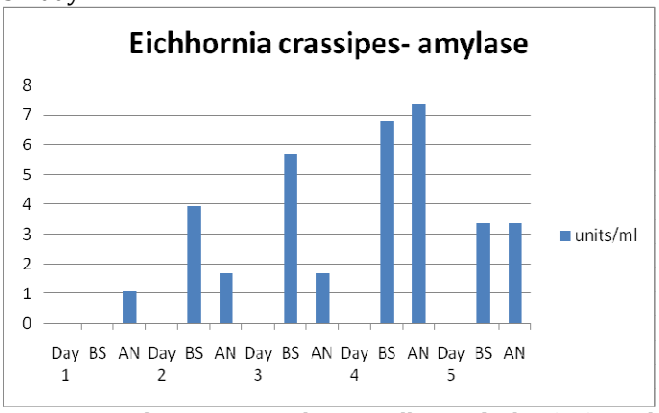

Fig. 1: Amylase activity by *Bacillus subtilis* (BS) and *Aspergillus niger* (AN) using *Eichhornia crassipes* as a substrate.

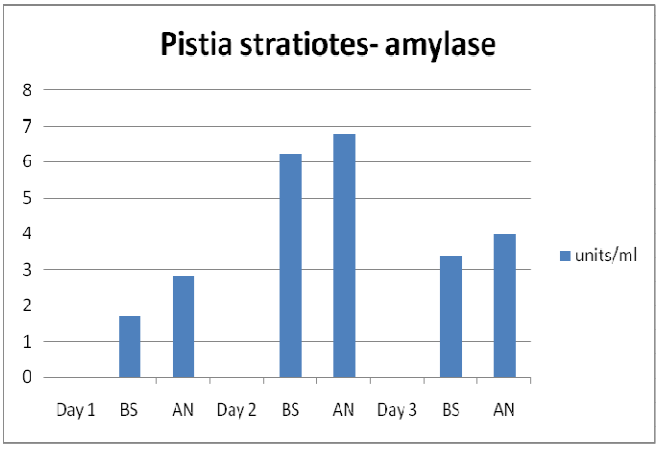

Fig. 2: Amylase activity by *Bacillus subtilis* (BS) and *Aspergillus niger* (AN) using *Pistia stratiotes* as a substrate.

As we can see from the fig. 1, the amylase activity expressed by BS increases gradually and reaches its peak point on day 4 after which there is a decrease seen in its activity by considerable amount. However in AN, initially there is a low expression of amylase till day 3 which is followed by a drastic increase in the amylase activity on day 4 following which there is again a reduction observed. Thus, both the organisms show maximum amylase activity on day 4 using *Eichhornia crassipes* as a substrate. However the activity exhibited by AN (7.373 U/ml) is greater than that shown by BS (6.086 U/ml) on day 4.

For *Pistia stratiotes,* BS and AN showed an activity of 1.702 and 2.836 respectively on day 1. This was then followed by a steep rise in the activity in both the organisms BA ans AN to 6.239 and 6.806, respectively which was then followed by a drop in the activity to 3.403 and 3.970 respectively.

This particular graph represents the amylase activity that is shown by both the organisms; AN and BS, using *Pistia stratiotes* as a substrate. As shown in the graph the activity expressed by both the organisms gradually increases, reaching its maximum on day 2 followed by a reduction. On day 2, the amylase activity shown by AN (6.086U/ml) is more than BS (6.239 U/ml).

Comparing both the graphs we see that the maxiumum activity shown is by AN on day 4 (7.373 U/ml) and day 2 (6.086U/ml) using *Eichhornia crassipes* and *Pistia stratiotes* as a subsrate respectively. More untis of enzyme is expressed on day 4 when grown on *Eichhornia crassipes* than on day 2 when grown on *Pistia stratiotes* by 1.287 units suggesting that *Eichhornia* is a more suitable substrate than *Pistia stratiotes*.

The starch content in *Pistia stratiotes* is reported to be more than that in *Eichhornia crassipes* thus explaining the early activity of *Pistia* (day 2)[13]. However *Eichhornia* exhibits more activity on day 4 as compared to *Pistia* on day 2. This may be attributed to the fact that lignin which is a type of acid detergent fiber (ADF) has been reported to be an activator of α-amylase[14] and *water lettuce* is lower in ADF content than *water hyacinth.* Thus the ADF in water hyacinth must have acted to be an activator and thus the higher activity.

CONCLUSION

Aspergillus niger and *Bacillus subtilis* organisms were successfully grown on both the substrates with the expression of amylase. Maximum amylase activity was seen by *Aspergillus niger* on *Pistia stratiotes* (Day 2) and *Eicchornia crassipes* (Day 4). Currently the commercially available amylase costs around 1-10 USD. In our study, we are using an environmental weed as a substrate which has a negligible value facilitating its degradation and thus taking care of its disposal. Thus in this the cost involved for substrate will be next to zero also adding to environmental value. Certainly, further studies are needed in the improvisation of the current strains for the production of amylase along with its optimisation, downstream processing, purification and its scale up for the industrial level production of amylase.

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