Production Of Pullulanase Using Novel Organic Substrates

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Abstract: Production of pullulanase was carried out from I. batatas, P. vulgaris, E. crassipes and P. stratiotes using A. pullulans. The fermentation reactions were carried out in minimal basal media at 28℃. E. crassipes and P. stratiotes as substrates produced more pullulanase than I. batatas and P. vulgaris. The highest enzyme production from Pistia stratiotes was of 9.3 U/ml after 72 hours of incubation at 28℃ and Eichhornia crassipes showed most enzyme production of 4.65 U/ml after 48 hours of incubation at 28℃. The two novel substrates, E. crassipes and P. stratiotes, could successfully be used for the production of pullulanase and are a promising resource for large-scale production of the same.

Keywords: Eichhornia crassipes, Pistia stratiotes, Ipomoea batatas, Phaseolus vulgaris, pullulanase, Aureobasidium, Solid state fermentation

1 INTRODUCTION

The roles of enzymes in many processes is no new to us as their use can been dated back to the history of ancient Greece when the Greeks used enzymes from microorganisms for applications such as in baking, brewing, alcohol production and cheese making [1]. In modern age they often find themselves useful in food, beverage and pharmaceutical and chemical industries [2].The growing demand for industrially important enzymes is demanding the usage of cheap resources to fulfil these needs. One such industrially important enzyme is pullulanase also known as*α*-dextrin 6-glucanohydrolase [3] that is responsible for the hydrolysis of pullulan and amylopectin to produce maltosetriose units [4], [5] Various types of pullulanases are found in nature. Pullulanase type I hydrolyses α-(1,6) glycosidic linkages in pullulan and branched polysaccharides to give maltosetriose units [6], [7]. Pullulanase type II is used extensively because of its ability to hydrolyse both α-(1,6) and α-(1,4) glucosidic linkages in amylopectin [3] which is one of the main components of starch[8], [9], [10]. Pullulanase hydrolase type 1 acts on the α-(1,4)glycosidic linkages in pullulan to give panose [11], [12], [13]. Pullulanase hydrolase type II hydrolyses α-(1,4) bonds in pullulan to give isopanose and pullulanase hydrolase type III acts on both α-(1,4) and α-(1,6) glycosidic linkages of pullulan and starch to give out various end products such as panose, maltose and maltotriose[14], [15]. Pullulanases have a wide variety of applications as it acts as a starch de-branching enzyme [5]. Some of its applications include the production of high-glucose syrup, high-maltose corn syrup that is mainly used in the food processing industry and high-fructose corn syrup that is used as a carbon source in production media. Pullulanases are also used for the production of 'resistant starch' in starch processing industries, detergent industry, baking industry and for the production of cyclodextrins and low-calorie beer. It can also be used as a dental plaque control agent [16]. The use of this enzyme for a variety of applications has been inhibited due to the high cost of production and low yield [5]. This limitation required the use of cheap substrates that are high in starch for the potential large-scale production of this enzyme. The substrates considered are two commonly grown plants in India i.e. *Ipomoea batatas*(sweet potato) and *Phaseolus vulgaris* (Local red kidney beans). The other two novel substrates are pest plants i.e. *Pistia stratiotes*(water cabbage) and *Eichhornia crassipes*(water hyacinth). *Ipomoea batatas*(sweet potato) is a dicotyledonous plant that belongs to the family Convolvuceae[17]. It has a tuberous growth that is a rich source of starch [17]. Another common source of starch and one of the most commonly consumed legumes is *Phaseolus vulgaris* (Local red kidney beans) [18], [19]. It mainly consists of carbohydrates (50%-70%) in its dry state [18]. *Eichhornia crassipes*(water hyacinth) and *Pistia stratiotes*(water cabbage) are classified as pest plants worldwide [20], [21]. They form dense matt growth over the surface of the water bodies often covering them. These plants are free floating plants with leaves growing above the water. Water hyacinth grows tall leaves with a single lavender colored flower whereas; water cabbage leaves grow in the form of a rosette. Both have a high reproduction rate as the seeds of water hyacinth may remain viable for over 28 years[22]. This profound growth causes numerous problems such as eutrophication, blockage of rivers, hampers fishing and endangers the existing flora and fauna by blocking the penetration of sunlight. One of the major rivers in Pune, India i.e. the Mulariver is also facing a problem due to the weed infestation and a huge amount is being spent for the control of these weeds that are causing problem environmentally, economically and socially [23]. Despite the attempts, they still continue to be a hazard. The use of these plants as a suitable substrate is being considered as they do not compete for land, have a negligible cost and grow at a fast rate[20]. This study attempts to test the use of cheap substrates that are well known to contain a high level of starch i.e. sweet potato and local red kidney beans. It also tests the use of novel substrates i.e. water hyacinth and water cabbage as a carbon source for pullulanase production.

2 METHODS AND MATERIALS

Standard culture of *Auerobasidium pullulans* was used for this experiment.

1. Substrate preparation

Sweet potato *(I.batatas)*, water hyacinth *(E. crassipes)* and water cabbage *(P. stratiotes)* were first cut into small pieces and kept in the hot-oven at 80℃. After drying, they were powdered and used as substrates for pullulanase production. Local red kidney beans *(P. vulgaris)* was grounded and used as a substrate for enzyme production.

2. Pullulanase production medium

The production medium consisted of (g/L): $(NH_4)_2SO_4$ (0.6), yeast extract (2.0), K_2HPO_4 (5.0), $MgSO_4.7H_2O$ (0.2) and NaCl(1.0) (pH 7.5). 15g of powdered substrate was supplemented with 300ml of production medium. [24]

3. Protein content (Folin-Lowry method)

The total protein content was determined by the method of Lowry *et al.* with bovine serum albumin as the standard. The protein content was calculated by checking the absorbance at 620nm. [25]

4. Enzyme assay

Enzyme activity was determined by measuring the enzymatic release of reducing sugar from 1%pullulan solution. The amount of reducing sugar released at the end of the reaction was determined by Dinitrosalisyilic Acid (DNSA) method. Sample blank was used to correct for the non-enzymatic release of reducing sugar. One unit of pullulanase activity is defined as the amount of enzyme required to produce 1 µmol reducing sugar (equivalent to glucose) min-1 under the assay conditions[26].Standard pullulan reagent was ordered from Sigma Aldrich Company.

3 Results And Discussion

Starch is a polysaccharide found abundantly in nature. This starch is mainly contained in plants as their source of energy. This study required the use of substrates that are not only a rich source of starch but should also be available cheaply. As stated, sweet potato and local red kidney beans are consumed daily and hence, are grown abundantly in India [18]. They are not only available easily but are also cheap in cost. Looking at the starch levels, a study conducted by Wani*et al.* in 2010for various legume starches concluded that local red kidney beans contain the most amount of amylopectin, which is the primary source for pullulanase production. The content ranges from 59-64% and the rest is made up of amylose [17]. On the other hand, sweet potato is one of the richest sources of starch. The amylopectin level ranges from 73-87% [17], [27]. The significantly high starch levels and their easy availability led to the use of these agriculture products as potential substrates. Water hyacinth and water cabbage are both known to contain a high level of carbohydrates but sufficient study has not been conducted on them [20]. They are both abundantly available and have a very low commercial value that led to the use of these weeds as a potential substrate for pullulanase production.

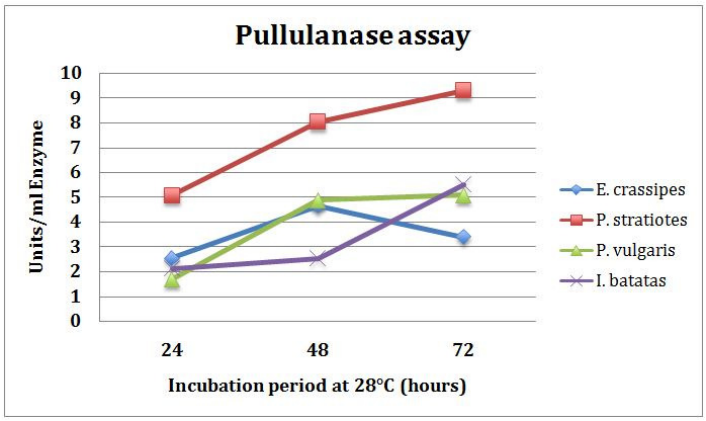

Fig 1: Pullulanase units produced by *A. pullulans* at three time intervals (24, 48 and 72 hours at 28℃)

Comparison between *P. vulgaris* and *I. batatas*:

According to chart 1, both *P. vulgaris* and *I. batatas* showed the highest enzyme production after 72 hours of incubation of 5.0792 U/ml and 5.5026 U/ml respectively. Although the enzyme production is approximately the same after 24 hours and 72 hours of incubation, there is a discrepancy after 48 hours. *A. pullulans* showed a slow increase in the beginning in enzyme production using *I. batatas*as a substrate as there was only a slight increase after 48 hours and increased dramatically at 72 hours whereas, the enzyme units using *P. vulgaris* as a substrate increased greatly after 48 hours of incubation and steadied after 72 hours of incubation. This may be due to the complexity of the starch components.

Comparison between *E. crassipes*and *P. stratiotes:*

According to chart 1, *Pistia stratiotes* as a substrate showed more enzyme production on all the three time intervals as compared to *Eichhornia crassipes. Pistia stratiotes* showed its lowest enzyme production of 5.07 U/ml after 24 hours of incubation that is still more than the highest level of enzyme production using *Eichhornia crassipes. Pistia stratiotes* showed highest enzyme production of 9.3 U/ml after 72 hours of incubation at 28℃ and *Eichhornia crassipes* showed most enzyme production of 4.65 U/ml after 48 hours of incubation at 28℃. This differential production of enzyme can be attributed to various reasons. Firstly, it may be due to the varying nutritional content of the plants. Cellulose and hemicellulose are polysaccharides that help in maintaining the cell integrity of plant cells and according to the paper by Mishima *et al.* (2007); cellulose is more abundant in water hyacinth as compared to water cabbage. The higher content of cellulose could have rendered water hyacinth to be less accessible to *A. pullulans* due to its more complex structure as compared to water cabbage. Secondly, water cabbage has higher starch content as compared to water hyacinth [28], [29]. This explains the higher enzyme production by *A. pullulans* using water cabbage as a substrate than water hyacinth. It can then be concluded that the starch content is directly proportional to the amount of enzymes produced by *A. pullulans*.

4 Conclusion

Overall, it can be concluded that the pest plants, *Eichhornia crassipes* and *Pistia stratiotes* are a better source of substrate for pullulanase production using *Auerobasidium pullulans* than *Ipomoea batatas* and *Phaseolus vulgaris*. Although *E. crassipes* showed approximately the same enzyme production as *I. batatas* and *P. vulgaris,* it is much cheaper source of substrate with negligible commercial value. The highest enzyme production was obtained using *P. stratiotes* as a substrate after comparing all the four substrates. The significance of this study lies in the fact that the substrates that showed the highest enzyme production are not only found abundantly in nature but have commercially negligible value. They are classified pest plants worldwide that have become a nuisance due to the difficulty in controlling their growth [30]. The use of these plants as a resource would not only help in the reduction of the production cost of the enzyme but will also help the environment by their elimination.

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