



Isolation, characterisation and optimization parameters of pullulanase [Pullulan6-glucohydrolase-code no: 120160-1] on solid substrate fermentation

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ABSTRACT

Pullulanase has great significance due to its wide range of potential applications in pharmaceutical and food industries. Pullulanase is used for the saccharification of starch in various industries and it is also a good amyolytic agent. The enzyme hydrolyzes the α -1, 6-glycosidic linkages of pullulan, amylopectin, glycogen, etc. The enzyme is used for manufacture of glucose, maltose, etc. In present study Pullulanase enzyme collected from *Aspergillus Niger* [ATTC16404] strain of onion peels. Optimum parameters were studied for the maximum enzyme activity at different physical conditions such as temperature 50°C, Time 120hrs, pH 6 and in different culture medias. After the detail analysis the enzyme activity was found to be stable at different optimum parameters. It was found to be 130.66 IU at 540nm in U.V.spectroscopy.

Keywords: *Aspergillus Niger*, Onion peels, Temperature, PH, Incubation period, Nutrient Medias.



INTRODUCTION

The roles of enzymes in different fields have been known for long time. Microbial enzymes were used in baking, brewing, alcohol production and in cheese making etc. Pullulanase is industrially useful enzyme in the starch processing industries. Pullulanase belongs to α -amylase class of enzymes which is an extracellular carbohydrase and which was first discovered by Bender and Wallenfels in 1961 from mesophilic organism *Klebsiella pneumoniae* formerly known as *Aerobacter aerogenes* or *Klebsiella aerogenes* [1,2,3]. Pullulanase are also called debranching enzyme which hydrolyze the extracellular yeast, polysaccharide and pullulan into the trisaccharides, maltotriose. It consists of repeating units of α -maltotriose joined "head to tail" by 1, 6-bonds [4]. Pullulanase specifically attack on α -1, 6-glycosidic linkage and also on α -1, 4- glycosidic linkage to hydrolyse the carbohydrates. This enzyme also belonging to group of glycosylhydrolases which are widely distributed in nature and are produced extremely by wide variety of microbial species. Among them thermophilic and mesophilic bacteria are rich sources of these enzymes. It is produced as an extracellular, cell surface-anchored lipoprotein by Gram-negative bacteria of the genus *Klebsiella*. Type I pullulanases specifically attack α -1,6 linkages, while type II pullulanases are

also able to hydrolyze α -1,4 linkages. It is also produced by some other bacteria and archaea. Starches of different origins have different amylose and amylopectin moieties in different ratios which differ significantly in many physical properties [5,6]. Starch is major industrial raw material and it is enzymatically processed into different commercial products for its subsequent use in various industries, ranging from food (especially high-fructose and glucose syrups) to detergent industrie[7]. Pullulanase is used as a processing aid in grain processing biotechnology (production of ethanol and sweeteners). Pullulanase type I (pullulanase) was first isolated from a culture of *Aerobacter aerogenes* (*Klebsiella pneumonia*). It is widely used in food processing such as in the manufacturing of high-quality candy and ice cream [8]. Pullulanase has also been used to prepare high-amylose starches, which have huge market demand [9]. High-amylose starches are of great interest and can be processed into "resistant starch" which has nutritional benefits [10]. Pullulanase also finds some application in the manufacturing of low-calorie beer [11] and in baking industry as the ant staling agent to improve texture, volume and flavor of bakery products [12]. It is also possible to use pullulanase as a dental plaque control agent [13]. Some alkaline pullulanases have been used as effective additives in dishwashing and laundry

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detergents for the removal of starches under alkaline conditions [14].

- Mineral rich medium
- Dextrose rich medium

Pullulanase type I has been characterised from mesophilic bacteria such as *Aerobacter aerogenes* [15], *Bacillus acidopullulyticus* [16], *Klebsiella pneumonia* [17,18] and *Streptomyces sp.* [19]. Moderate thermophilic gram positive bacteria such as *Bacillus flavocaldarius* [20], *Bacillus thermoleovorans* [21], *Clostridium sp.* [22], and *Thermos caldophilus* [23] also have ability to secrete pullulanase type I while pullulanase type I from hyperthermophilic bacterium, *Fervidobacterium pennavorans*, pullulanase type III has been isolated and characterized from hyperthermophilic archaeon, *Thermococcus aggregans* [24]. This is the only enzyme that attacks α -1,4- as well as α -1,6-glucosidic linkages in pullulan and is active above 100°C. Pullulanase has greater potential to reduce photosensitizer (PS) phototoxicity in normal tissue and to enhance the efficacy of tumor treatment. In the present study we are optimizing the physical parameters for the maximum yield of this commercially useful pullulanase enzyme.

EXPERIMENTAL WORK

Materials and methods:

Chemicals: Citrate buffer (PH-5) and Ammonium sulphate (80percent)

Preparation of inoculums

Microorganism: in the present study we used the fungal strain *Aspergillus Niger* (ATCC16404) which was collected from NCIM Pune. The strain was grown on onion rich potato dextrose agar medium. Its composition is Agar[5gm], Dextrose [5gms], Potatoes[25gms], Yeast extract[0.25gms], Onion peels[25gms]and water[250ml].*The isolates were maintained on potato onion dextrose agar slants for 46hrs. The strain was propagated in OPDA agar slants and maintained at 30⁰ for 120hrs at acidic pH .The well grown cultures were used for the production of pullulanase enzyme.*

Preparation for 80%ammonium sulphate solution: The 8gms of ammonium sulphate was dissolved in 800ml of distilled water

Preparation for citrate buffer at pH-5: It is prepared by using citric acid (48.5ml) and disodium hydrogen phosphate (51.5ml) and made at PH-5

Experimental procedure:

Optimization of media components

- PDA(potato dextrose agar medium)
- OPDA(onion rich potato dextrose agar medium)
- Maltose rich medium

Different enrichment medias were prepared and grown *Aspergillus Niger* (ATCC16404) inoculums were inoculated and incubated for different time intervals such as 24hrs, 48hrs, 72hrs, 96hrs, 120hrs, 144hrs, and 168hrs at different temperatures such as 20°C, 28°C, 30°C, 35°C, 45°C and at different pH conditions such as acidic(5.5),basic(9-10),neutral(7.2).Among all the Medias OPDA (Onion Potato Dextrose Agar Medium) optimum growth at 540nm by using U.V. spectroscopy.

Enzyme recovery: The grown culture was collected by using citrate phosphate buffer at pH -5 in a centrifuge at 1200rpm for 20minutes. Separate the supernatant layer and add ammonium sulphate to half of the volume of the liquid. And then the solution was incubated for overnight at 4°C.The enzymatic activity is measured at 540nm by U.V. spectroscopy.

Test for pullulanase: The culture plates were flooded with iodine and observe cleared colonies of *Aspergillus Niger*.

RESULTS AND DISCUSSION

In the present study we studied and optimize the various parameters on pullulanase enzyme by

Aspergillus Niger (ATCC-16404)

- Effect of nutrient media components
- Effect of pH
- Effect of incubation period
- Effect of temperature

Effect of nutrient media on pullulanase enzyme:

It was found that media components plays an important role in pullulanase enzyme.The effect of media components on pullulanase enzyme was carried out and results were tabulated in table4, Graph 4.A. Maximum pullulanase enzyme growth was observed in onion peels of potato dextrose agar medium due to maximum availability of nutrients. In the present study we used one mold fungal culture i.e. *Aspergillus Niger* (ATCC-16404) will utilize the more carbon source and less nitrogen source and again it gave the high enzyme yield on utilizing the onion peels of potato dextrose agar medium which is having the maximum carbohydrate source.

Effect of pH: It was found that pH plays an important role in pullulanase enzyme .The effect of pH on pullulanase enzyme was carried out and results were tabulated in table3,Graph in 3.A.The results shows that there was maximum pullulanase

enzyme occurred at basic PH(7-10) due to maximum utilization of H⁺ ions and the substrate. Microorganisms need some hydrogen ion concentration for the cell transportation and for its growth.in the present study the inoculated culture utilize the basic H⁺ ions concentration for its growth.

Effect of incubation period: Effect of different incubation periods were studied and results were tabulated in table.2.and in Graph.2.A.The optimum enzyme activity was observed at 120hrs, because of maximum utilisation of carbon sources, beyond

that it was unable to utilize the substrate due to saturation of the active sites of enzyme. Beyond that the enzyme yield and growth rate was decreased.

Effect of temperature: Effect of temperature on pullulanase enzyme was carried out at various temperatures and results were tabulated in table.1, Graph.1.A.Optimum pullulanase enzyme activity was observed at 28°C and on further increase in temperature causes reduction of pullulanase enzyme activity because of decrease in catalytic activity of that enzyme beyond that temperature.

Table 1: Effect of Nutrient medium components on pullulanase enzyme

S.No	Culture Medium	Absorbance (540nm)
1	Maltose	0.033
2	Onion	0.093
3	NaCl	0.112
4	potassium	0.278
5	Onion potato dextrose	0.583

Table 2: Effect of pH on pullulanase enzyme

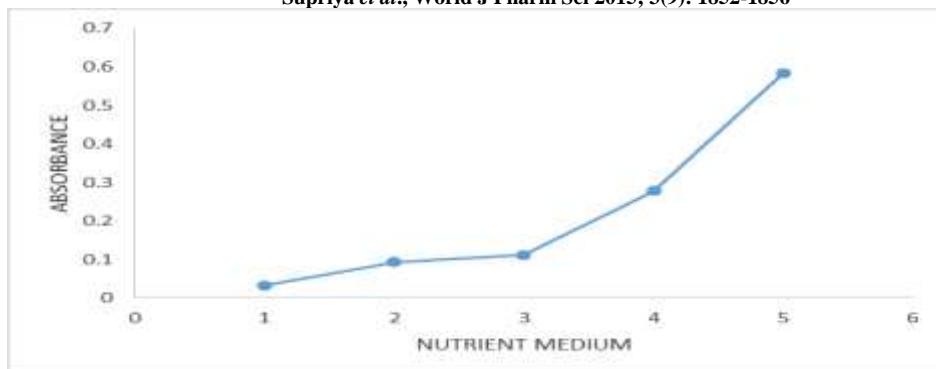
S. NO	pH	Absorbance (540nm)
1	Acidic(2.5-5)	0.246
2	Basic(7.2-12)	0.255
3	Neutral(7)	0.150

Table 3: Effect of incubation periods on pullulanase enzyme

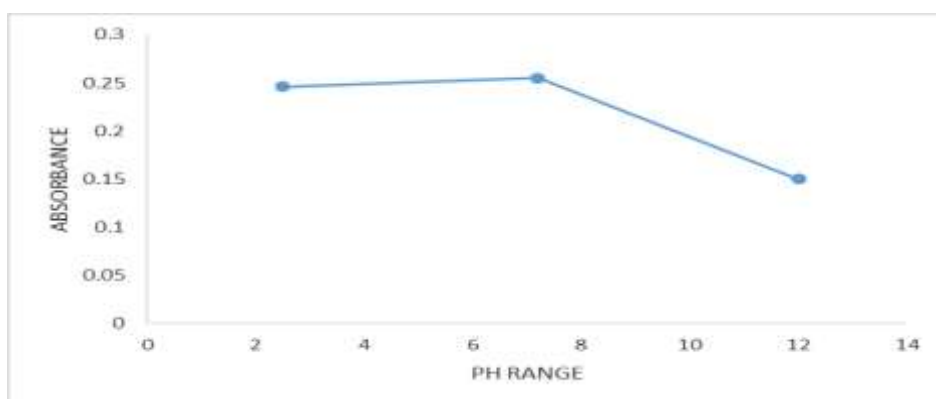
S.No	Time (hrs)	Absorbance (540nm)
1	24hrs	0.117
2	48hrs	0.198
3	72hrs	0.259
4	96hrs	0.398
5	120hrs	0.512
6	144hrs	0.421
7	168hrs	0.408
8	192hrs	0.448

Table 4: Effect of temperature on pullulanase enzyme

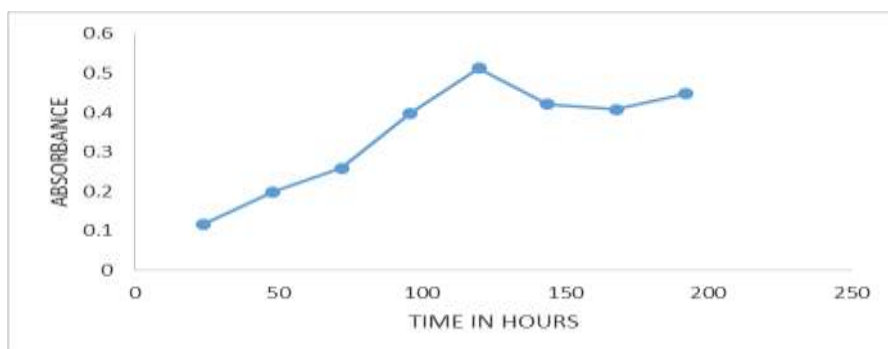
S.No	Temperature	Absorbance (540nm)
1	20°C	0.299
2	28°C	0.542
3	30°C	0.374
4	35°C	0.464
5	45°C	0.347



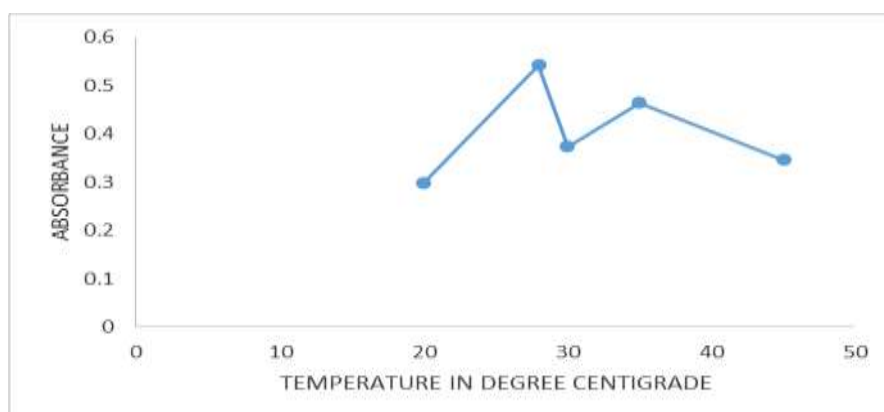
Graph 1: EFFECT OF NUTRIENT MEDIA ON PULLULNASE PRODUCTION



Graph 2: EFFECT OF pH ON PULLULANASE PRODUCTION



Graph 3: EFFECT OF INCUBATION PERIOD ON PULLULANASE



Graph 4: EFFECT OF TEMPERATURE ON PULLULANASE ENZYME

CONCLUSIONS

Microbial enzymes are having lots of commercial, medicinal applications, so, that enzyme yield can be increased on varying the different media components and physical parameters. In the present study we have found the optimum parameters for the maximum yield of pullulanase which have more commercial and medicinal values. The pullulanase enzyme by *Aspergillus niger* (ATCC16404) was maximum at temperature 28°C. The pullulanase enzyme by *Aspergillus niger* (ATCC16404) was maximum in onion peels of potato dextrose agar medium compared to maltose media, minerals media, potato media, onion media,

dextrose media. The pullulanase enzyme by *Aspergillus niger* (ATCC16404) was maximum incubation period 120hrs compared to other incubation period like 24hrs, 48hrs, 72hrs, 96hrs, 144hrs, 168hrs, 192hrs. The pullulanase enzyme by *Aspergillus niger* (atcc16404) maximum at basic PH 7.

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