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Optimization and Kinetics of Solid-State Fermentative Production of Pectinase by Aspergillus awamori

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Abstract: Pectinase enzyme finds extensive application in food and beverage industries. Pectinase production was studied in solid-state fermentation process using rice bran and sugarcane bagasse as substrates. Optimization of media and fermentation conditions for maximum production of pectinase was carried out by one at the time procedure. Various combination of substrates were tried to achieve maximum pectinase production. The mixed substrates consisting of 85% rice bran and 15% sugarcane bagasse gave maximum pectinase yield of 103.33U/ml during the fermentation period of 72 hours. The optimum temperature was found to be 35^{o}C and optimum pH was found to be 5.The kinetics of pectinase production by solid state fermentation using *Aspergillus awamori* was studied. The kinetic parameters value were found to be K_m = 79.3 and V_{max} = 1.724 hr.ml/U .
Key words : Pectinase, diffusion, interparticle spacing, aeration ,catabolic repression.

1. Introduction

Pectinolytic enzymes catalyzing the degradation of pectic substances are of great industrial importance(1).Its finds extensive applications in fruit juice industries in order to improve fruit juice yield and clarity(2).The use of liquefying enzymes for mash treatment results in improvement of juice flow ,leading to a shorter press-time, without the necessity for pressing aids(3).At the same time, pectin is broken down into such an extent that the viscosity of mash is reduced(4).Other areas of applications include the pulp and paper industry(5), animal feed(6), retting of flax and other vegetable fibers(7), haze removal from wines(8), coffee and tea fermentation(9), oil extraction(10), purification of plant viruses(11), bio scouring of cotton fibers(12), degumming of plant bast fibers(13) protoplast fusion technology(14) , textile industry(8) and waste management(14).

Among processes used for enzyme production, solid-state fermentation (SSF) is an attractive one because it presents higher productivity per reactor volume, lower capital and operating costs, lower space requirements, simpler equipment and easier downstream processing compared to that of submerged fermentation (SmF) [15]. In addition, it permits the use of agricultural and agro-industrial residues as substrates which are converted into bulk chemicals and fine products with high commercial value such as alcohol, organic acids, fats, proteins, enzymes, etc. [16–17]. On the other hand, there is evidence that some enzymes are less affected by catabolic repression, are more thermo stable and their optimum temperature values are higher than those obtained by SmF [18].

Major problems in the exploitation of commercial enzymes are their yield, stability, specificity and the cost of production. New enzymes for use in commercial applications with desirable biochemical and physio-chemical characteristics and a low cost of production have been the focus of research. Application of agro-industrial wastes which are largely available in India as carbon sources in enzyme

production reduces the cost of production and also helps in solving disposal problems [19-20]. Literature highlighting the optimization, biochemical, characterization, genetics and strain improvement for pectinase production from fungi are available. However, kinetics studies on solid-state fermentative production of pectinase are lacking. Considering the biotechnological importance of kinetic model for cost effective production of pectinase enzyme, the present research reported the kinetics and optimization of media and process parameters by classical method in solid state fermentation for maximum production.

2. Materials and Methods

2.1. Substrate Preparation:

Rice bran and Sugarcane bagasse samples were obtained from the agricultural field, Salem District, Tamilnadu. The sample was made into 100 mesh (0.15 mm) fine powder by the use of laboratory grinder at 3000 rpm and was preserved in a sealed plastic bag at 4°C to prevent any possible degradation or spoilage.

2.2. Microorganism and culture conditions

Fungal strain *Aspergillus awamori* MTCC 548 was obtained from Microbial Type Culture Collection (MTCC), Institute of Microbial Technology (IMTECH), Chandigarh, India. Culture was maintained on Czapek's Agar medium. After three days incubation at 30°C the agar slants were stored at 4°C. The liquid medium for the growth of inoculum for fungi was yeast extract – malt extract – peptone – glucose medium (YMP) composed of 3 g/l of yeast extract, 3 g/l of malt extract, 5 g/l of peptone and 10 g/l of glucose.

Inocula were grown aerobically in 250 ml Erlenmeyer flasks containing the above mentioned medium at 30°C in an Environmental Shaker (Remi Scientific) at 200 rpm for 24 h. Active cells were centrifuged in a clinical centrifuge (1200 rpm), washed with sterile water and were used as inoculum. Fermentations for pectinase production were conducted on a shaker at 200 rpm in 250 ml flasks and the samples were withdrawn periodically for the analysis of enzyme concentration.

2.3. Enzyme extraction

The crude pectinase was extracted by mixing 10g of fermented materials with distilled water, stirred for 20 minutes in the shaker, filtered and then centrifuged for 20 minutes. The supernatant was used as the crude enzyme and then studied for enzymatic measurements by DNS method [21].

2.4. Total pectinase assay

A suitably diluted sample of 0.5ml was added to a solution containing 2 ml of 1% citrus pectin in acetate buffer (pH 4.8) in a test tube. Samples are kept at 45°C for 30 minutes in a water bath, cooled, added with 2.5 ml of DNS reagent, seethed for 5 min. Finally the contents were cooled and 10 ml of distilled water was added to it and was measured at 540 nm using UV/Vis Biospectrophotometer (ELICO BL 198). The concentration of β-galacturonic acid was determined from the standard β-galacturonic calibration curve.

2.5. Solid State Fermentation (SSF)

The powdered rice bran and sugarcane bagasse samples of different compositions were weighed (10 g/flask) and distributed into 250 ml Erlenmeyer flasks with the addition of Czapek's nutrient medium (without carbon source) to a desired solid-liquid ratio (up to 20% solid) and 0.1 M Potassium phosphate buffer (pH = 6.0), followed by sterilization for 15 min at 15 psi (121°C) in an autoc1ave. To the production medium 10^8 spores of *A. awamori* were inoculated aseptically and the flasks were then covered with cotton to allow CO2 produced during fermentation to escape. The flasks were incubated in a rotary shaker (200 rpm) at 30°C for 144 h. Samples were withdrawn periodically (24h interval) and were analyzed for total pectinase enzyme activity.

3. RESULTS AND DISCUSSION

3.1. Pectinase Production using Different Media:

The effect of substrate concentration on enzyme activity in solid-state fermentation using *Aspergillus awamori* was studied. Experiments were conducted with five different medium M_1, M_2, M_3, M_4 and M_5 whose composition were described in Table 3.1.The samples were drawn at regular intervals of 24 hours and analyzed for pectinase activity. The results are given in **Table 3.2 and Fig. 3. 1**.

The pectinase enzyme activity was found to increase exponentially and reached a maximum pectinase activity at the end of 72 hours. A maximum of 88.65 U/ml of pectinase activity was obtained with mixed substrate of 85% of rice bran and 15% of sugarcane bagasse (Medium M_4) and later on it was decreased gradually till the end of fermentation. Similar trends were observed for other mediums M_1, M_2, M_3 and M_5.

The medium M_4 gave a maximum total pectinase activity of 88.65 U/ml and was chosen as the best medium for maximum pectinase enzyme production. The total pectinase activity for medium M_4 was found to be more throughout the fermentation

when compared to all other medium M_1, M_2, M_3 and M_5. This may be due to the addition of fibrous material as sugarcane bagasse by 15% (w/w) increased the interparticle spacing, possibly increasing the aeration and diffusion of nutrient and enzyme and hence resulted in the higher yield of pectinase enzyme (22).The addition of sugarcane bagasse by 20% (w/w) decreased the yield of pectinase enzyme indicating that the microorganism was not able to hydrolyze enough cellulose and hemicellulose fibers to support mycelium formation. The use of sugarcane bagasse as support was suitable for the growth of filamentous fungi and it allowed the utilization of high concentration of substrate solutions (23).

3.2. Effect of Temperature on Production of Total Pectinase

The effect temperature on the pectinase enzyme production was studied using M_4 medium (mixed substrate with 85% rice bran and 15% sugarcane bagasse) by conducting experiments at different temperatures namely 30°C, 35°C and 40°C by keeping all other conditions constant for the fermentation period of 120 hours. The results are given in **Table 3.3 and Fig. 3.2**. As temperature increases from 30°C to 35°C the pectinase enzyme activity was found to increase and maximum pectinase activity of 91.67 U/ml was found at 35°C .Further increase in temperature beyond 35°C decreased the pectinase activity till the end of fermentation. Hence optimum temperature was chosen as 35°C and was used for further studies. The decrease in enzyme activity at higher temperature may be due to enzyme denaturation.

Table 3.1. Media Composition

Media Composition	Medium **M_1**	Medium **M_2**	Medium **M_3**	Medium **M_4**	Medium **M_5**
Rice Bran (g)	10	9.5	9	8.5	8
Sugar cane bagasse (g)	0	0.5	1	1.5	2
Nutrient solution (ml)	30	30	30	30	30

Table 3. 2. Effect of Substrate concentration on Production of Total Pectinase

Time h	**Pectinase Activity (U/ml)**				
	Medium **M1**	Medium **M2**	Medium **M3**	Medium **M4**	Medium **M5**
24	30.33	33.45	35.67	38.75	25.33
48	58.33	63.36	69.25	74.25	38.37
72	67.37	73.86	82.33	88.65	50.25
96	36.33	43.26	54.25	58.75	30.85
120	30	34.22	39.45	45.25	23.75

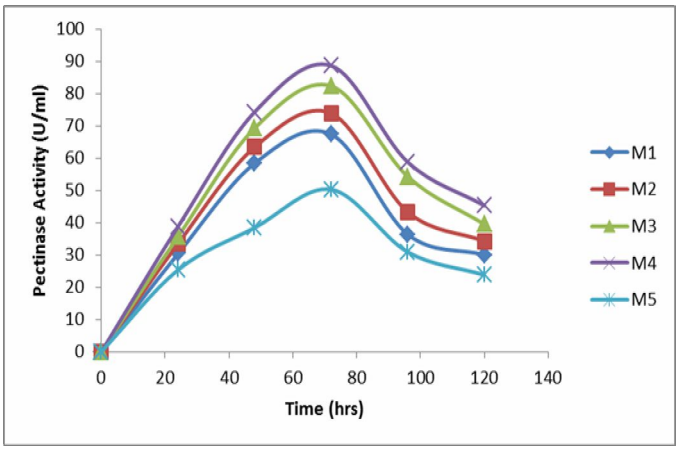

Fig.3.1. Effect of Substrate concentration on Production of Total Pectinase.

Table 3. 3. Effect of Temperature on Pectinase Activity

Time h	Pectinase Activity (U/ml)		
	30°C	35°C	40°C
24	36.67	58.33	30.50
48	70.12	81.67	55.25
72	83.33	91.67	70
96	55.75	63.33	43.50
120	40.50	50.12	30.33

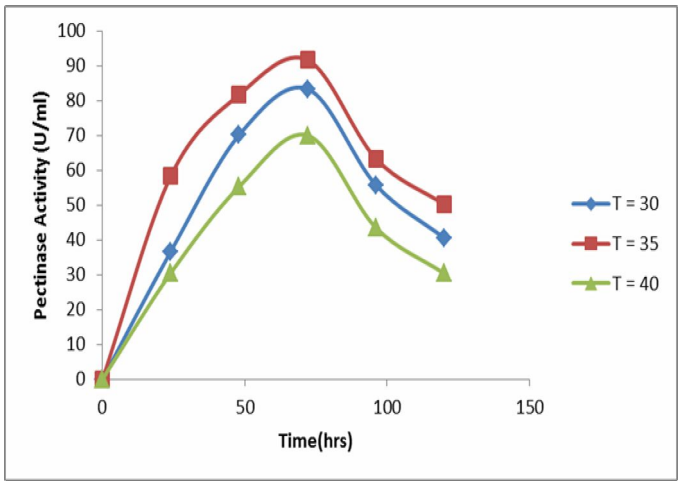

Fig.3. 2 Effect of Temperature on Pectinase Activity.

3.3. Effect of Initial pH on production of Total pectinase

The effect of initial pH on the pectinase production using was studied by conducting experiments at different pH namely pH 3, pH 4, pH 5, and pH 6 by keeping temperature at 35°C and using mixed substrate with 85% rice bran and 15% sugarcane bagasse (M_4 medium). The results are given in **Table 3.4 and Fig. 3.3**. As initial pH was increased from pH 3 to pH 5, the pectinase activity was found to increase. Further increase in initial pH beyond pH 5, the pectinase activity was found to decrease. The decrease in enzyme activity at higher pH may be due to preference of fungi *A.awamori* to lower pH for its growth and metabolism. A maximum pectinase activity of 103.33 U/ml was obtained with mixed substrate for a fermentation period of 72 hours at temperature 35°C and at pH value of 5. Hence optimum pH value was chosen as pH 5.

Table 3.4. Effect of pH on Pectinase Activity.

Time h	Enzyme Activity U/ml			
	pH 3	pH 4	pH5	pH 6
24	58.33	61.67	65.25	58.33
48	81.67	83.33	86.67	75.12
72	91.67	95.50	103.33	90.25
96	63.33	65.12	76.67	55.50
120	50	56.67	63.33	38.33

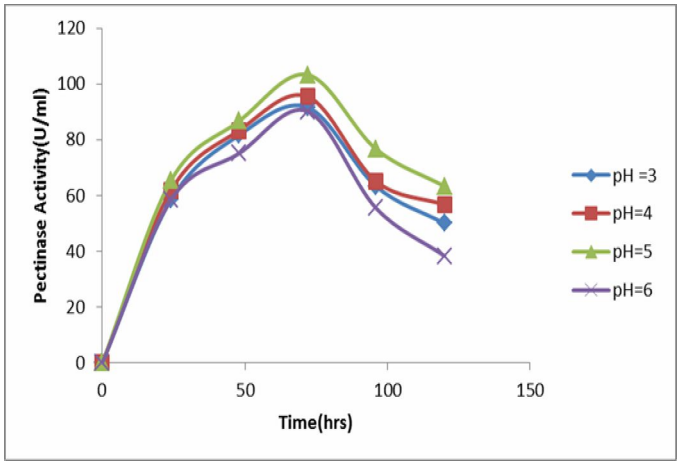

Fig.3.3. Effect of pH on Pectinase Activity.

3.4. Kinetics of Pectinase Production:

The kinetics of pectinase production using *A.awamori* with M_4 medium (85 % rice bran and 15 % sugarcane bagasse) under optimum conditions of temperature and pH was studied. The results are given in Table 3.5 and Fig. 3.4.The pectinase activity was found to increase with respect to fermentation time as shown in Fig.3.4 and reaches a maximum total pectinase activity of 103.33U/ml at the end of 72 hours and later on it was found to decrease till the end of the fermentation. The rate of product formation (dp/dt) was found to increase gradually and was maximum at the end of 72 hours and later on it was found to decrease due to non-availability of substrates (as indicated in Table 3.5.).The results showed that the maximum rate of 4.57 was obtained at the end of the 72 hours and was found to be optimum fermentation period.

The kinetic parameters were evaluated using Lineweaver-Burk plot. The kinetic parameters were found to be V_{max} = 1.724 hr.ml/U and k_m = 79.3 Therefore Michaelis-Menten Kinetic model was found to be

$$V = \frac{1.724 \times S}{79.3 + S.}$$

The Fig.3.5 showed that our kinetic model was well fitted with the experimental values.

.Table 3.5.Effect of fermentation time on pectinase production.

Time (hrs)	Pectinase Activity(U/ml)	Rate of product formation(U/ml.hr)
0	0	**0**
24	65.25	1.63
48	86.67	2.95
72	103.33	4.57
96	76.67	2.45
120	63.33	1.45

Table 3.6. Comparison between Experimental and Predicted values.

Substrate (rice bran) concentration (%)	Experimental values	Predicted values
100	0.924	0.961
95	0.930	0.939
90	0.937	0.916
85	0.947	0.891
80	0.883	0.865

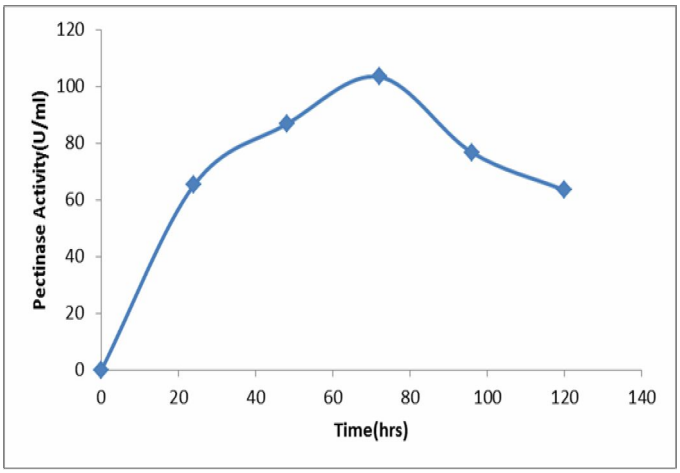

Fig.3.4. Effect of fermentation time on pectinase production.

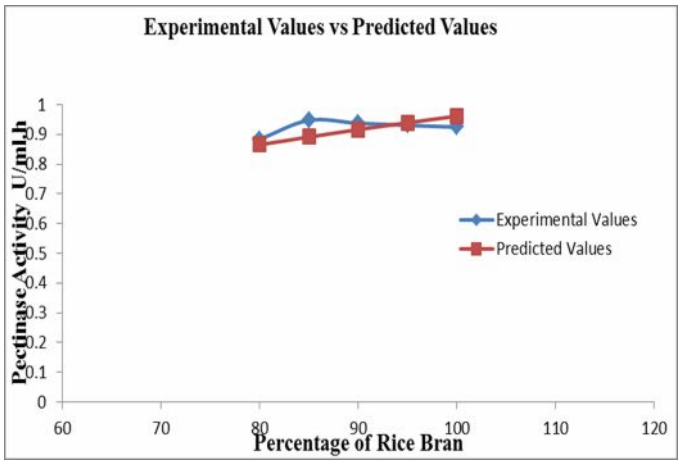

Fig.3.5. Comparison between experimental and predicted values.

Conclusion

Optimization of media and process parameters namely temperature and pH were carried out by classical method using *A.awamori* microorganism in solid state fermentation. The maximum total pectinase activity of 103.33 U/ml which was achieved using the medium M_4 with 85% (by weight) rice bran and 15% (by weight) sugarcane bagasse .The optimum temperature was found to be 35°C and optimum pH was found to be pH 5 for maximum production of pectinase. The kinetics of pectinase production was studied using medium M_4 under optimum condition of temperature and pH. The kinetic parameters were determined using Lineweaver –Burk Plot and their values are given below: k_m = 79.3 and V_{max} = 1.724 hr.ml/U.

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