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Optimization and kinectics of pectinase enzyme using *Aspergillus niger* by solid-state fermentation

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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 wheat bran and sugarcane bagasse as substrates utilizing *Aspergillus niger*. Optimization of media and fermentation conditions for maximum production of pectinase was carried out by one at a time procedure. Various combination of substrates were tried to achieve maximum pectinase production. The mixed substrates consisting of 90% of wheat bran and 10% of sugarcane bagasse gives maximum pectinase yield during the fermentation period of 96 h. The optimum temperature was found to be 40°C and optimum pH was found to be 5.The kinetics of pectinase production by solid state fermentation using *Aspergillus niger* was studied. The kinetic parameters value were found to be K_m = 294.12 and V_{max} = 2.33 h ml/U.

Keywords: Pectinase, solid-state fermentation, sugarcane baggase, *Aspergillus niger*.

Introduction

Pectinolytic enzymes catalyzing the degradation of pectic substances are of great industrial importance (Alkorta *et al.,* 1998). Pectinases have extensive applications in fruit juice industries in order to improve fruit juice yield and clarity (Alkorta *et al.,* 1982). 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 (Charley,1969). At the same time pectin is broken down into such an extent that the viscosity of mash is reduced (Mutlu *et al.,* 1991). Other areas of applications include the pulp and paper industry (Reid & Ricard, 2000), animal feed (Barreto *et al.,*1989), retting of flax and other vegetable fibers (Hoondal *et al.,* 2000), haze removal from wines (Revilla *et al.,* 2003), coffee and tea fermentation (Gar,1985), oil extraction (Scott,1978), purification of plant viruses (Salazar,1999), bio-scouring of cotton fibers (Ranveer Singh, 2005), degumming of plant bast fibers (Kapoor *et al*., 2001), protoplast fusion technology (Kashyap *et al.,* 2001), textile industry (Revilla *et al.,* 2003) and waste management (Kashyap *et al.,* 2001).

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) (Acuna-Arguelles *et al.,* 1995). 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 (Spagnuolo *et al.,*1998). On the other those enzymes less affected by catabolic repression are more thermostable and their optimum temperature values are higher than those obtained by SmF (Lozano *et al.,* 1988). 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 as carbon sources in enzyme production reduces the cost of production and also helps in solving disposal problems (Pandey *et al.,* 2000).

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 fungi in the enzyme engineering, the present research reported the kinetics and optimization of media and process parameters by classical method in solid state fermentation for maximum production of pectinase under optimum conditions.

Materials and methods

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

Aspergillus niger (MTCC-281) was obtained from microbial culture collection and GenBank (MTCC), Institute of microbial technology (IMTECH), Chandigarh, India. Culture was maintained on Czapek's agar medium.

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After three days incubation at 30°C the agar slants were stored at 4°C.

Czapek concentrate

Dissolve $NaNO_3$-30 g, KCl-5 g, $MgSO_4.7H_2O$-5 g and $FeSO_4.7H_2O$-0.1 g in 1 l distilled water and store in refrigerator. Dissolve Czapek concentrate- 10 ml, K_2HPO_4-1 g, yeast extract - 5 g and sucrose-30 g in 1 l distilled water and store in a refrigerator.

Enzyme extraction

The crude pectinase was extracted by mixing 10 g of fermented materials with distilled water, stirred for 20 min. in the shaker, filtered and then centrifuged for 20 min. The supernatant was used as the crude enzyme and then studied for enzymatic measurements by DNS method (Miller, 1959).

Total pectinase assay

A suitably diluted sample of 0.5 ml 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 min. 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.

Solid state fermentation (SSF)

The powdered wheat bran 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), followed by sterilization for 15 min. at 15 psi (121°C) in an autoclave. To the production medium 10^8 spores of *Aspergillus niger* were inoculated aseptically and the flasks were then covered with cotton to allow CO_2 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 (24 h interval) and were analyzed for total pectinase enzyme activity.

Table 1. Media composition.

Media composition	M_1	M_2	M_3
Wheat bran (g)	10	9	8
Sugar cane bagasse (g)	0	1	2
Nutrient solution (ml)	30	30	30

Results and discussion

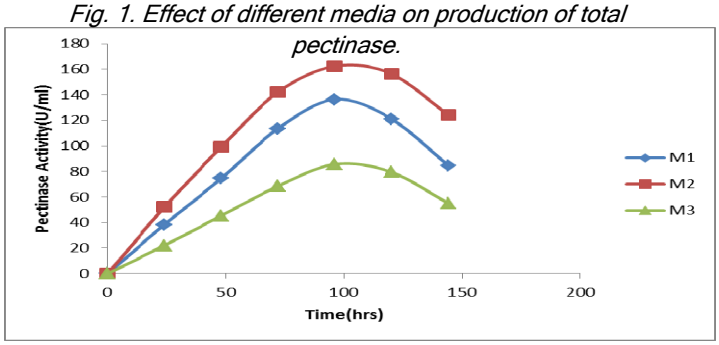
Pectinase production using different media

The effect of substrate concentration on enzyme activity in solid-state fermentation using *A. niger* was studied. Experiments were conducted with three different medium M_1, M_2 and M_3 whose composition was described in Table 1. The samples were drawn at regular intervals of 24 h and analyzed for pectinase activity. The results are given in Table 2 and Fig. 1. The pectinase enzyme activity was found to increase exponentially reached a maximum 164.15 U/ml of pectinase activity at the end of 96 h was obtained with mixed substrate of 90% of wheat bran and 10% of sugarcane bagasse and later on it was decrease gradually till the end of fermentation. Similar trends were observed for other mediums M_1 and M_3. The medium M_2 gave a maximum total pectinase activity of 164.15 U/ml and was chosen as the best medium for maximum pectinase enzyme production.

Table 2. Effect of different media on production of total pectinase.

Time (h)	Enzyme activity U/ml		
	M_1	M_2	M_3
24	38.45	52.12	22.12
48	74.50	99.05	45.36
72	113.50	142.12	68.45
96	136.50	162.50	85.66
120	121.12	156.45	79.50
144	84.50	124.05	54.90

Fig. 1. Effect of different media on production of total pectinase.



The total pectinase activity was more for medium M_2 throughout the fermentation when compared to media M_1 and M_3. This may be due to the addition of fibrous material (sugarcane bagasse by 10%) increased the interparticle spacing, possibly increasing the aeration and diffusion of nutrient and enzyme; thereby resulting in the higher yield of pectinase (Mitchell *et al.,* 1991). The

Table 3. Production of total pectinase.

Time (h)	Enzyme activity (U/ml)		
	35°C	40°C	45°C
24	12.12	23.12	22.12
48	24.32	51.25	49.056
72	64.50	121.50	94.36
96	107.54	169.50	141.50
120	102.24	161.12	134.12
144	84.90	146.326	126.326

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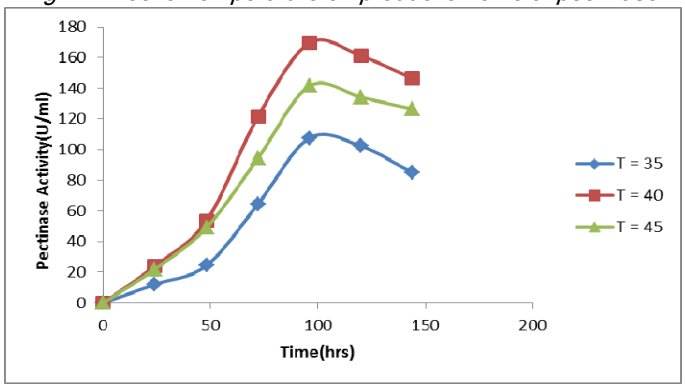
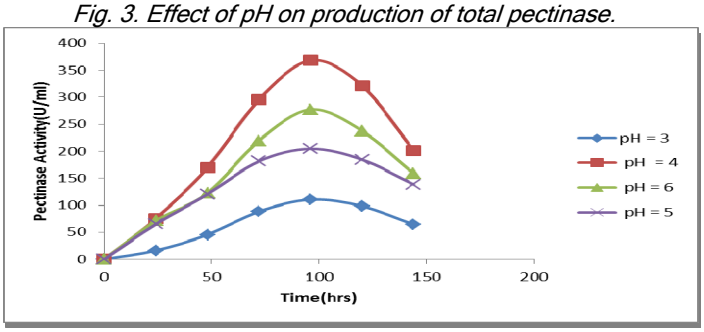
Fig. 2. Effect of temperature on production of total pectinase.



addition of sugarcane bagasse by 20% decreased the yield indicating that the microorganism was not able to hydrolyze enough cellulose and hemicellulose fibers to support mycelium formation. The sugarcane bagasse appeared to be only inert support while growth of the microorganism and enzyme production stimulated by added wastes. Thus the use of sugarcane bagasse as support is suitable for the growth of filamentous fungi and it allowed the utilization of high concentration of substrate solutions (Ooijkaas *et al.,* 2000).

Fig. 3. Effect of pH on production of total pectinase.



Effect of temperature on production of total pectinase

The optimum temperature on the pectinase enzyme production was studied using M_2 medium (mixed substrate with 90% wheat bran & 10% sugarcane bagasse). The experiments were conducted at different temperatures namely 35°C, 40°C and 45°C by keeping all other conditions constant for the fermentation period of 144 h. The results are given in Table 3 and Fig. 2. As temperature increases from 30°C to 40°C, the pectinase enzyme activity was found to increase and maximum pectinase activity of 141.50 U/ml was found at 40°C. Further increase in temperature, decreased the pectinase activity till the end of fermentation. Hence, optimum temperature was chosen as 40°C and was used for further studies. The decrease in enzyme activity at higher temperature may be due to enzyme denaturation.

Effect of initial pH on production of total pectinase

The effect of initial pH on pectinase production was studied by conducting experiments at different pH namely 3, 4, 5 and 6 by keeping temperature at 40°C and using mixed substrate with 90% wheat bran and 10% sugarcane bagasse (M_2 medium). The results are given in Table 4 and Fig. 3. As initial pH was increased from 4 to 5, the pectinase activity also increased. Further increase in initial pH beyond 5 reduced the pectinase activity. It reflects the preference of fungi *A. niger* to lower pH for its growth and metabolism. A maximum pectinase activity of 367.92 U/ml was obtained with mixed substrate for a fermentation period of 144 h at temperature 40°C and at pH value of 5. Hence optimum pH value was chosen as 5.

Table 4. Effect of pH on production of total pectinase.

Time (h)	Enzyme activity (U/ml)			
	pH 4	pH 5	pH 6	pH 7
24	16.34	75.35	71.22	65.12
48	45.47	169.81	122.36	120.32
72	88.50	295.22	219.50	181.25
96	110.377	367.92	277.32	204.50
120	98.360	321.12	237.50	184.42
144	64.321	201.36	158.12	138.50

Table 5. Effect of fermentation time on pectinase production.

Time (h)	Pectinase activity (U/ml)	Rate of product formation (U/ml h)
0	0	0
24	75.35	1.70
48	169.81	2.30
72	295.22	2.85
96	367.92	3.50
120	321.12	1.91
144	201.36	1.60

Kinetics of pectinase production

The kinetics of pectinase production using *A. niger* with M_2 medium (90% wheat bran & 10% rice bran) under optimum conditions of temperature and pH was studied. The results are given in Table 5 and Fig. 4. The pectinase activity was found to increase with respect to fermentation time as shown in Fig. 4 and reaches a maximum of 367.92 U/ml at the end of 96 h and thereafter it decreased. The rate of product formation (dp/dt) was found to increase gradually and was maximum at the end of 96 h and later on it decreased due to non-availability of substrates (Table 5). The results showed that the maximum rate of 3.5 was obtained at the end of the 96 h 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} = 2.33 h ml/U and k_m = 294.12.

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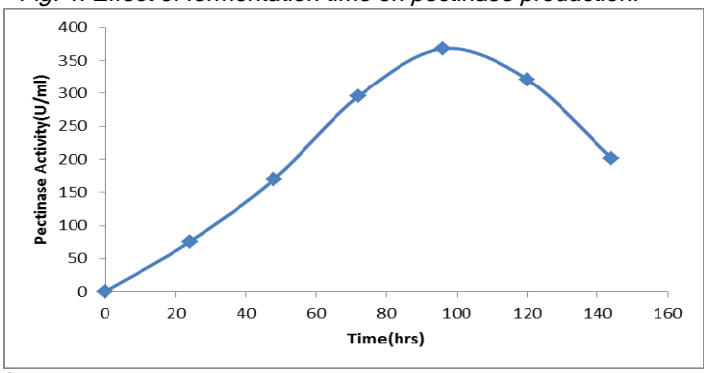
Fig. 4. Effect of fermentation time on pectinase production.



Conclusion

Optimization of media and process parameters namely temperature and pH were carried out using *Aspergillus niger* microorganism in solid state fermentation. The maximum total pectinase activity of 367.92 U/ml was achieved using the media M_2 with 90% (by wt.) wheat bran and 10% (by wt.) sugarcane bagasse. The optimum temperature was found to be 40^0C and optimum pH was found to be 5 for maximum production of pectinase. The kinetics of pectinase production was studied using medium M_2 under optimum condition of temperature and pH. The kinetic parameters were determined using Lineweaver-Burk plot and are given below k_m = 294.12 and V_{max} = 2.33 h ml/U.

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