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Experimental investigations of submerged fermentation and synthesis of pectinolytic enzymes by *Aspergillus Niger*: Effect of inoculum size and age of spores

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The paper investigates effects of the different concentration of inoculums and age of spores in the synthesis of pectinolytic enzymes with the aim of their optimizing for maximal enzyme production and growth of the microorganism. The microorganism used in this work was the fungus Aspergillus niger MK-15, which was isolated from soil as a highly active producer of pectinolytic enzymes.

The results showed that the concentration of the inoculum and age of spores have a pronounced effect on the pectinolytic activity and dry weight. Obrained results sugest that for submerged fermentation and synthesis of pectinolytic enzymes by Aspergillus niger MK-15, the optimal inoculum concentration is 3% or 6x106 spores and optimal age of spores is 3 days old.

Keywords: Keywords: Pectinolytic enzymes, apple pulp, inoculum size, age of spores, Aspergillus niger, optimization.

Introduction

Pectinolytic enzymes from *Aspergillus niger* are pectin-degrading enzymes which are used extensively in the food industry as processing aids for extraction, clarification, and maceration. These enzymes are usually produced in solid or submerged fermentation (Friedrich et al., 1989; Bailey and Pessa, 1990; Schmidth et al., 1995; Naidu and Panda, 1998). Submerged fermentation generally produces smaller quantities of secretory enzymes and solid fermentations is not susceptible to automation.

For the industrial production of pectinolytic enzymes it is important to improve fermentation conditions, for better production of extracellular enzymes in liquid culture on inexpensive carbon sources such as apple pomace, citric peels, pectin or other agricultural wastes (Leuchtenberger et al., 1989; Aguilar and Huitron, 1986; Larios et al., 1989). The most authors describe the use of an optimized medium composition to increase the enzyme content (Pericin et al., 1992; Acuna-Arguelles et al., 1995; Berovic and Ostroversnik, 1997).

The objective of the present study was to investigate effects of the different concentration of inoculums and age of spores on the synthesis of pectinolytic enzymes with the aim of their optimizing for maximal enzyme production and growth of the microorganism.

Materials and methods

Micro-organism

The microorganism used in this work was the fungus *Aspergillus niger MK-15*, which was isolated from soil as a highly active producer of pectinolytic enzymes and was maintained on slant agar according to Czapek with 2% pectin (Table 1). Spores from 2, 3, 4, and 5 days old agar slants were collected by adding sterile distilled water to each slant. The spore suspension was adjusted in the culture medium different of $4x10^6$ spores (2%) to $1x10^7$ spores (5%). The spores' suspension was counted and diluted to final concentration of $2\mathbf{x}10^6$ spores ml^{-1}.

TABLE 1. CONTENT OF THE BASE AFTER CZAPEK WITH 2% PECTIN

Pectin	20 g.
NaNO3	2 g.
KH2PO4	1 g.
MgSO4.7H2O	0.5 g.
KCl	0.5 g.
FeSO4	0.01 g.
Agar-agar	15-20 g.
Distilled water	1 l.

Media and fermentation procedure

The growth of the microorganism and synthesis of pectinolytic enzymes were performed in 500 ml flasks (100 ml base) with rotational shaking (200 min^{-1}) on a rotational laboratory shaker, at 30 ^{0}C within 120 h. Natural base with 1% refuse apple pulp was used as the nourishing base (Table 2): refuse apple pulp-1% ; corn flour - 0.5% ; $(NH_4)_2HPO_4$ - 0.7% ; KH_2PO_4 - 0.1% ; $MgSO_4.7H_2O$ - 0,05% ; and KCl - 0.05%. The initial *pH* on the base - 4. The base was previously sterilized by autoclaving at 121 ^{0}C for 30 min.

TABLE 2. CONTENT OF THE NATURAL BASE WITH 1% REFUSE APPLE PULP, %(WEIGHT/VOLUME)

Apple pulp	1
Corn flour	0.5
(NH4)2HPO4	0.7
KH2PO4	0.1
MgSO4.7H2O	0.05
KCl	0.05

TABLE 3. EFFECT OF INOCULUM SIZE ON SYNTHESIS OF ENDO -PECTINOLYTIC ENZYMES AND GROWTH BY ASPERGILLUS NIGER

Fermentation time, (h)	Concentration of inoculum, (%)	pH	Experimentally founda Endo-PG, (U l-1)	Experimentally founda Dry wight, (g l-1)
0	2	4.00	0	9.9 ± 0.1
24	2	3.12	40 ± 5.3	12.5 ± 0.2
48	2	3.05	170 ± 7.6	15.6 ± 0.3
72	2	2.99	329 ± 2.1	13.5 ± 0.2
96	2	3.23	312 ± 2.1	12.6 ± 0.1
120	2	3.43	306 ± 2.1	11.9 ± 0.1
0	3	4.00	0	9.9 ± 0.1
24	3	3.60	43 ± 4.0	14.3 ± 0.2
48	3	3.20	185 ± 4.0	18.2 ± 0.2
72	3	3.20	345 ± 2.1	15.3 ± 0.3
96	3	3.66	335 ± 2.1	14.4 ± 0.2
120	3	4.58	314 ± 2.1	15.2 ± 0.2
0	4	4.00	0	9.9 ± 0.1
24	4	3.32	41 ± 2.1	11.4 ± 0.2
48	4	3.12	179 ± 4.0	13.7 ± 0.3
72	4	3.02	323 ± 2.1	12.5 ± 0.2
96	4	3.23	299 ± 2.1	11.8 ± 0.2
120	4	3.45	306 ± 2.1	12.7 ± 0.2
0	5	4.00	0	9.9 ± 0.1
24	5	2.96	38 ± 2.1	10.5 ± 0.2
48	5	2.98	160 ± 5.3	12.3 ± 0.2
72	5	3.15	297 ± 2.1	11.3 ± 0.3
96	5	3.39	290 ± 2.1	10.8 ± 0.2
120	5	3.60	285 ± 2.1	11.9 ± 0.2

Note: Values are the average from 3 replicates ± SD

Enzyme assay

Endo-pectinolytic activity, based on change in the viscosity of the reaction mixture (0.35% pectin solution, buffered at *pH* 4.5 in 0.1 mol L^{-1} citrate) at 30 ^{0}C, was determined using Ostwald viscometer. The degree of degraded pectin (A) under known amount of filtrate (enzyme) was calculated with the formula: 100 * (Ts-Tt)/(Ts-Tw) where: Ts is the flow time of the substrate control; Tt - the flow time of the test; and Tw - the flow time of water. 1 U is defined as the amount of enzyme which catalyses hydrolyse of 1 g pectin per 1 h at 40 ^{0}C.

Biomass production measurements

Biomass production was measured as dry weight. After filtering, the retained cell mass was dried at 100 ^{0}C to constant weight.

TABLE 4. EFFECT ON OLD OF SPORES ON SYNTHESIS OF ENDO-PECTINOLYTIC ENZYMES BY ASPERGILLUS NIGER

Fermentation time, (h)	Age culture, (d)	pH	Experimentally founda Endo-PG, (U l-1)
0	2	4.00	0
24	2	3.45	27 ± 2.1
48	2	3.12	132 ± 7.6
72	2	2.99	233 ± 2.1
96	2	3.13	230 ± 2.1
120	2	3.23	225 ± 2.1
0	3	4.00	0
24	3	3.60	43 ± 4.0
48	3	3.20	185 ± 7.8
72	3	3.20	345 ± 2.1
96	3	3.60	335 ± 2.1
120	3	4.50	314 ± 2.1
0	4	4.00	0
24	4	3.42	40 ± 4.0
48	4	3.22	174 ± 7.6
72	4	3.02	323 ± 2.1
96	4	3.27	299 ± 2.1
120	4	3.35	294 ± 2.1
0	5	4.00	0
24	5	3.10	38 ± 4.0
48	5	2.95	167 ± 7.8
72	5	2.87	307 ± 2.1
96	5	3.14	297 ± 2.1
120	5	3.35	285 ± 2.1

Note: Values are the average from 3 replicates ± SD

Results and discussion

Effect of inoculums size and old of spores

During these experiments results displayed that the concentration of the inoculum and age of spores had a pronounced effect on the pectinolytic activity and dry weight. The pectinolytic enzymes delivered maximum endo-pectinolytic activity (345 U l^{-1}) during the third day of growth with 3% inoculum ($6x10^6$ spores) (Table 3) and 3 days old spores (Table 4). The growth of the microorganism (dry weight) (Table 3) showed maximum dry wight (18.2 g l^{-1}) during the second day of fermentation with 3% inoculum and 3 days old of spores.

The results showed that the concentration of the inoculum and age of spores have a pronounced effect on the pectinolytic activity and dry weight.

Obrained results sugest that for submerged fermentation and synthesis of pectinolityc enzymes by *Aspergillus niger MK-15,* the optimal inoculum concentration is 3% or $6x10^6$ spores and optimal age of spores is 3 days old.

Similar results were obtained from the other authors who described effects of the inoculum concentration and age of spores for better production of enzymes (Sikyta, 1983; Kuhad et al., 1998; Shah and Madamwar, 2005; Smith and Wood, 1991; Qinnghe et al., 2004). The inoculum concentration of $1x10^7$ spores/ml contributed to the maximum xylanase activity (177.9 U ml^{-1}) relative to the other concentrations. The lowest activity was observed when using a concentration of $1x10^6$ spores ml^{-1} (78.3 U ml^{-1}) (Maria Lucia Garcia Simoes and Samia Maria Tauk-Tornisielo, 2006). In order to verify the enzyme activity, the spore concentration in fungi cultivation must be high enough to colonize substrate particles (Sikyta, 1983).

Many studies, however, indicate that there can be a decline in this activity over a determined spore concentration. Kuhad et al. (1998) obtained maximum xylanase activity by *Fusarium oxysporium* using $1x10^7$ spores ml^{-1}; on the other hand, using $2x10^7$ spores ml^{-1}, they achieved the same level of activity, and the one containing higher concentrations of spores led to a decrease in activity. Shah and Madamwar (2005) observed that, during the cultivation of Aspergillus foetidus, maximum xylanase activity (210.0 U ml^{-1}) occurred when the used inoculum had a concentration of $1.5x10^8$ spores ml^{-1}, two times higher than the one obtained using $1.5x10^4$ spores ml^{-1}. However, the increase in the inoculum concentration was not beneficial for xylanase activity, verifying that over $10^{8,}$ a drastic decrease occurred in activity. As general mean, the optimal spore concentration is between 10^6 and 10^7 spores ml^{-1}: outside this range, a decrease in xylanase activity occurres (Smith and Wood, 1991; Qinnghe et al., 2004).

Conclusion

Significance and impact of the study is that the concentration of the inoculum and age of spores had a pronounced effect on submerged fermentation and synthesis of pectinolityc enzymes by *Aspergillus niger MK-15*.

Obrained results sugest that for submerged fermentation and synthesis of pectinolytic enzymes by *Aspergillus niger* MK-15, the optimal inoculum concentration is 3% or 6x106 spores and optimal age of spores is 3 days old.

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