African Journal of Biotechnology Vol. 9 (2), pp. 163-166, 11 January, 2010
Available online at http://www.academicjournals.org/AJB
ISSN 1684–5315 © 2010 Academic Journals

Full Length Research Paper

Cellulase production by *Trichoderma* sp. on apple pomace under solid state fermentation

Haiyan Sun[1], Xiangyang Ge[2], Zhikui Hao[2] and Ming Peng[1]*

[1]Key Laboratory of Tropical Crop Biotechnology, Ministry of Agriculture, Institute of Tropical Bioscience and Biotechnology, Chinese Academy of Tropical Agricultural Sciences, Haikou 571101, China.
[2]The Key Laboratory of Industrial Biotechnology, Ministry of Education, School of Biotechnology, Jiangnan University, China.

Accepted 4 June, 2009

The feasibility of using apple pomace for cellulase production by *Trichoderma* sp. under solid state fermentation was evaluated in this study. Our results indicated that initial moisture level of the medium, incubation temperature and inoculum size influenced the cellulase production greatly. The optimum initial moisture level, incubation temperature and inoculum size were 70%, 32 and 2×10^8 spores/flask, respectively. The supplement of lactose and corn-steep solid to the apple pomace favored the enzyme formation markedly.

Key words: Apple pomace, cellulase, *Trichoderma* sp.

INTRODUCTION

Apple pomace is the main solid waste generated in cider and apple juice making factories. It accounts for between 25 and 35% of the weight of the processed raw material. Worldwide, several million metric tonnes of this residue are produced annually (Beatriz et al., 2008). Apple pomace is a poor animal food because its protein content is extremely low.

Therefore, apple pomace does not find any significant commercial application till now and most of this by-product is generally disposed of in open areas, leading to potentially serious environmental problems. Given this situation, it is necessary to look for processes that allow the controlled elimination of this residue or, even better, its industrial reutilization. Recently much effort has been made to convert this waste into a variety of value-added products such as biofuels, citric acid, L-lactic acid, enzyme, edible fibers and others (Beatriz et al., 2008; Josh et al., 2008; Sudha et al., 2007; Silas et al., 2003; Shojaosadati and Babaeipour, 2002; Stredansky et al., 2000; Berovič and Ostroveršnik, 1997; Joshi and Sandhu, 1996; Rahmat et al., 1995; Bhalla and Joshi, 1994; Ngadi and Correia, 1992; Hours et al., 1988).

*Corresponding author. E-mail: mmpeng_2000@yahoo.com. Tel.: +86-898-66963161. Fax: +86-898-66890978.

Cellulases are industrially important enzymes having application in diverse industries such as textile, paper and pulp and food industry. Cellulases are relatively costly enzymes and a significant reduction in cost will be important for their commercial use. Production of cellulases using cheaper substrates is an effective strategy to reduce cost.

In recent years, much work has been carried out towards efficient utilization of agro-industrial residues to produce enzymes of commercial importance by microorganism (Shankar and Mulimani, 2007; Botella et al., 2007; Sun et al., 2007; Ramachandran et al., 2004). The objective of the present investigation was to examine the potential of using apple pomace as a substrate for the production of cellulase by *Trichoderma* sp. To our knowledge, this is the first report regarding the production of cellulase using apple pomace.

MATERIALS AND METHODS

Microorganisms

Trichoderma sp. GIM 3.0010, a newly isolated cellulase producer, was used in this study. It was identified and deposited in Guangdong microbial culture collection center in China as GIM 3.0010. It was preserved in potato dextrose agar at 4°C.

Substrate

Apple pomace was obtained from apple juice process. The residue was dried in an oven at 80℃, crushed and sieved to an average size of 300 - 500 µm.

Production of cellulase under solid state fermentation

Cellulase production experiments were carried out in 250 mL flasks containing 10 g apple pomace moistened with distilled water to a moisture level of 50%. All flasks were sterilized at 121℃ for 30 min, inoculated (10^8 spores/flask) and then incubated at 30℃ for 144 h. The samples were withdrawn at regular intervals to determine enzyme activities.

To investigate the effect of moisture level on cellulase production, different moisture levels (40, 45, 50, 55, 60, 65, 70, 75 and 80%) were used to compare the enzyme activity. Based on the optimum culture time and moisture level, different culture temperature (25, 28, 30, 32, 35 and 40℃) was compared to investigate the effect of temperature on cellulase production. Different inoculum sizes were also carried out to investigate its effect on cellulase production.

Based on the optimum culture time, moisture level, culture temperature and inoculum size, different carbon sources (glucose, fructose, maltose, starch, sucrose, lactose, avicel, carboxy methyl cellulose at 2% w/w) and nitrogen sources (peptone, yeast extract, corn-steep solid, sodium nitrate, ammonium sulphate, ammonium nitrate at 1% w/w) were added to the fermentation medium, respectively, to evaluate the influence of different carbon sources and nitrogen sources on cellulase production.

Analytical methods

During the process of enzyme production, 0.5 g sample was withdrawn, extracted with 10 ml distilled water (30 min) and filtered. The supernatant was used for enzyme assay. The activity of cellulase was assayed using 1% carboxy methyl cellulose, in 0.05 M citrate buffer (pH 4.8) as substrate. The reaction was carried out at 50℃ for 30 min. One unit (U) of enzyme activity was defined as the amount of enzyme, which liberates 1 µmol of glucose equivalent from carboxy methyl cellulose per min. Reducing sugars were estimated with 3,5-dinitrosalicylic acid (DNS), using glucose as standard (Miller, 1959). The residue was dried to constant mass at 80℃. The enzyme activity was expressed as U per g dried substrate (U/gds). All values given are means of three determinations.

RESULTS AND DISCUSSION

Time course of cellulase production by *Trichoderma* sp. on apple pomace

As indicated in Figure 1, the enzyme activity increased progressively with the incubation time from 0 - 120 h and reached the maximum (2.3 U/gds) at 120 h. After 120 h, the enzyme activity began to decrease.

Effect of initial moisture level of the medium on cellulase production

Moisture content is a critical factor for cell growth and enzyme production under SSF, which determines the outcome of the process. As shown in Table 1, the optimum initial moisture level was 70% for cellulase production by *Trichoderma* sp. GIM 3.0010 on apple pomace. Lower or higher than 70% both decreased the cellulase production. Lower moisture level gives a lower degree of swelling and higher water tension and then reduces the solubility of nutrients. Higher moisture level decreases porosity, changes particle structure, promotes development of stickiness, decreases diffusion, lowers oxygen transfer or increases formation of aerial hyphae.

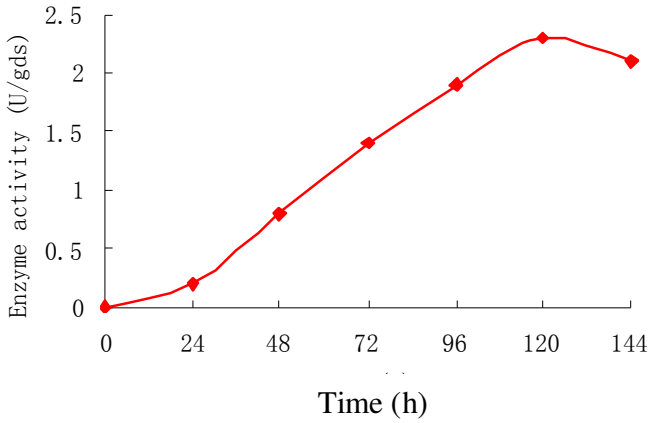

Figure 1. Time course of cellulase production by *Trichoderma* sp. on apple pomace.

Table 1. Effect of initial moisture level of the medium on cellulase production.

Initial moisture level	Enzyme activity (U/gds)
40	1.2
45	1.8
50	2.3
55	2.6
60	2.9
65	3.2
70	3.5
75	2.9
80	2.5

Effect of incubation temperature on cellulase production

As shown in Table 2, 32℃ proved to be the best temperature for the enzyme synthesis in the present study. Incubation at lower temperature resulted in longer time to the maximum enzyme activity. Incubation at higher temperature affected the fungus harmfully, which reflected on the enzyme synthesis. Since enzyme is a secondary metabolite produced during exponential growth phase, the incubation at high temperature could lead to poor growth and thus a reduction in enzyme yield (Sabu et al., 2002).

Table 2. Effect of incubation temperature on cellulase production.

Incubation temp. (℃)	Enzyme activity (U/gds)
25	1.8
28	3.1
30	3.5
32	3.9
35	2.9
40	1.0

Table 3. Effect of particle size on cellulase production.

Inoculum size (10^8 spores/flask)	Enzyme activity (U/gds)
0.2	1.5
0.5	2.8
1	3.9
2	4.2
3	4.1
4	4.0

Effect of Effect of inoculum size on cellulase production

Lower inoculum size required longer time for the cells to multiply to sufficient number to utilize the substrate and produce enzyme. An increase in the number of spores in inoculum would ensure a rapid proliferation and biomass synthesis. After a certain limit, enzyme production could decrease because of depletion of nutrients due to the enhanced biomass, which would result in a decrease in metabolic activity (Kashyap et al., 2002). A balance between the proliferating biomass and available nutrient would yield an optimum at which the enzyme synthesis would be maximum (Ramachandran et al., 2004). As shown in Table 3, when the inoculum size was lower than 10^8 spores/flask, the enzyme activity is obviously low. When the inoculum size ranged from 1×10^8 to 4×10^8 spores/flask, the cellulase activity varied slightly. 2×10^8 spores/flask maximized the enzyme production.

Effect of supplementation of apple pomace with different carbon sources

Although apple pomace can support the growth of *Trichoderma* sp. GIM 3.0010 and cellulase production, it may not provide enough nutrients needed by the organism for maximum enzyme production. Hence, the exogenous addition of various nutrients to the medium may improve cell growth and enzyme production. As shown in Table 4, addition of monosaccharide (glucose and fructose) significantly inhibited the production of cellulase. Particularly for the supplement of glucose, only 0.8 U/gds was obtained. The possible reason is that glucose usually acted as a catabolite repressor and repressed the enzyme formation (Rajoka and Yasmeen, 2005). The supplement of maltose, sucrose and starch had little effect on cellulase production, while lactose, avicel and carboxy methyl cellulose all enhanced enzyme production. Among them, lactose improved the cellulase production the most, which increased from 3.9 U/gds to 5.8 U/gds, compared to the control. Lactose was considered as a good inducer for cellulase production (Seiboth et al., 2005).

Table 4. Effect of supplementation of apple pomace with different carbon sources.

Carbon source	Enzyme activity (U/gds)
Control	4.2
Glucose	0.8
Fructose	1.2
Maltose	3.8
Sucrose	4.1
Lactose	5.8
Starch	3.9
Avicel	4.5
Carboxy methyl cellulose	4.6

Effect of supplementation of apple pomace with different nitrogen sources

Different organic and inorganic nitrogen sources were tried to improve cellulase production. As shown in Table 5, the nitrogen sources tested in this study all enhanced cellulase production by *Trichoderma* sp. GIM 3.0010 to different levels. Among them, corn-steep solid was the most appropriate supplement with the enzyme activity of 7.6 U/gds. It could be due to the deficiency of nitrogen sources in natural apple pomace.

Conclusion

These studies showed that apple pomace could be a good substrate for cellulase synthesis by *Trichoderma* sp. GIM 3.0010. Initial moisture level of the medium, incubation temperature and inoculum size influenced the cellulase production greatly. The optimum initial moisture level, incubation temperature and inoculum size were 70%, 32℃ and 2×10^8 spores/flask, respectively. The supplement of lactose and corn-steep solid favored the enzyme formation markedly.

ACKNOWLEDGEMENTS

This research was supported by Chinese 863 project

Table 5. Effect of supplementation of apple pomace with different nitrogen sources.

Nitrogen source	Enzyme activity (U/gds)
Control	4.2
Peptone	6.7
Yeast extract	6.2
Corn-steep solid	7.6
Sodium nitrate	5.4
Ammonium sulphate	4.8
Ammonium nitrate	4.5

tropical bioscience and biotechnology in Chinese academy of tropical agricultural sciences (No. ITBBZD0951).

REFERENCES

Beatriz G, Remedios Y, Alonso JL, Parajo JC (2008). L-Lactic acid production from apple pomace by sequential hydrolysis and fermentation. Bioresource Technol. 99: 308–319.

Berovič M, Ostroveršnik H (1997). Production of *Aspergillus niger* pectolytic enzymes by solid state bioprocessing of apple pomace. J. Biotechol. 53: 47-53.

Bhalla TC, Joshi M (1994). Protein enrichment of apple pomace by co-culture of cellulolytic moulds and yeasts. World J. Microbiol. Biotechnol. 10: 116-117.

Botella C, Diaz A, Ory I, Webb C, Blandino A (2007). Xylanase and pectinase production by *Aspergillus awamori* on grape pomace in solid state fermentation. Process Biochem. 42: 98–101.

Hours RA, Voget CE, Ertola RJ (1988). Apple pomace as raw material for pectinases production in solid state culture. Biol. Waste 23: 221-228.

Josh VK, Parmar M, Rana N (2008). Purification, characterization of pectinase produced from apple pomace and its evaluation in the fruit juice extraction and clarification. J. Biotech. 136: 294.

Joshi VK, Sandhu DK (1996). Preparation and evaluation of an animal feed byproduct produced by solid-state fermentation of apple pomace. Bioresource Technol. 56: 251-255.

Kashyap P, Sabu A, Pandey A, Szakacs G (2002). Extra-cellular L-glutaminase production by *Zygosaccharomyces rouxii* under solid-state fermentation. Process Biochem. 38: 307–312

Miller GL (1959). Use of dinitrosalicylic acid reagent for determination of reducing sugar. Anal. Chem. 31: 426–427.

Ngadi MO, Correia LR (1992). Kinetics of solid-state ethanol fermentation from apple pomace. J. Food Eng. 17: 97-116.

Rahmat H, Hodge RA, Manderson GJ, Yu PL (1995). Solid-substrate fermentation of *Kloeckera apiculata* and *Candida utilis* on apple pomace to produce an improved stock-feed. World J. Microbiol. 11: 168-170.

Ramachandran S, Patel AK, Nampoothiri KM, Francis F , Nagy V, Szakacs G, Pandey A (2004). Coconut oil cake—a potential raw material for the production of α-amylase. Bioresource Technol. 93: 169–174.

Sabu A, Sarita S, Pandey A, Bogar B, Szakacs G, Soccol CR (2002). Solid-State Fermentation for Production of Phytase by *Rhizopus oligosporus.* Appl. Biochem. Biotechnol. 102-103: 251-260.

Seiboth B, Lukas H, Salovuori N, Karin L, Robson DG, Vehmaanperä J, Penttilä ME, Kubicek CP (2005). Role of the *bga1*-encoded extracellular β-galactosidase of *Hypocrea jecorina* in cellulase induction by lactose. Appl. Environ. Microbiol. 71:851–857.

Shankar SK, Mulimani VH (2007). α-Galactosidase production by *Aspergillus oryzae* in solid-state fermentation. Bioresource Technol. 98: 958–961.

Shojaosadati SA, Babaeipour V (2002). Citric acid production from apple pomace in multi-layer packed bed solid-state bioreactor. Process Biochem. 37: 909-914.

Silas GV, Elisa E, Margarida MM (2003). Bioconversion of apple pomace into a nutritionally enriched substrate by *Candida utilis* and *Pleurotus ostreatus*. World J. Microbiol. 19: 461-467.

Stredansky M, Conti E, Stredanska S, Zanetti F (2000). γ-Linolenic acid production with *Thamnidium elegans* by solid-state fermentation on apple pomace. Bioresource Technol. 73: 41-45.

Sudha ML, Baskaran V, Leelavathi K (2007). Apple pomace as a source of dietary fiber and polyphenols and its effect on the rheological characteristics and cake making. Food Chem. 104:686-692.

Sun HY, Ge XY, Zhang WG (2007). Production of a novel raw-starch-digesting glucoamylase by *Penicillium* sp. X-1 under solid state fermentation and its use in direct hydrolysis of raw starch. World J. Microbiol. Biotechnol. 23: 603–613.