CELLULASE AND HEMICELLULASE PRODUCTION BY MICROORGANISMS ISOLATED FROM PLANT WASTES COMPOSTING PILES

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ABSTRACT

Bacteria and fungi with cellulolytic and hemicellulolytic activity were isolated from horticultural plant wastes composting piles. A group of 13 fungi and 2 bacteria were selected because of its ability to hydrolyze cellulose and hemicellulose in agar plate assays. These microorganisms also showed high enzymatic activity. A simple biphasic culture medium was designed for enzyme production composed by milled pepper plant residues as the sole carbon source amended with a mineral solution and yeast extract. Optimal conditions allowed for the simultaneous production of high levels of endoglucanase (0.086 U/ml) and endoxylanase (2.45 U/ml). According to findings of this research, horticultural plant residues may serve as a low-cost substrate for cellulase and hemicellulase production by selected microorganisms.
Keywords: *Agricultural wastes, Cellulolytic, Hemicellulolytic.*

INTRODUCTION

Lignocellulolytic microorganisms produce cellulases, hemicellulases, pectinases and/or ligninases able to hydrolyze plant cellular walls. These microorganisms and their enzymes are being used for valorisation of plant residues together with many other industrial and environmental applications (Bhat, 2000). The increasing demand of these enzymes has intensified the search for microorganisms producing high levels of enzyme activity and for improved fermentation processes for their production.

Plant residues are very abundant in the Southeast of Spain (Almeria), as a result of greenhouse intensive horticulture. They are low-cost and highly available substrates that, on the other hand, need to be disposed off.

In the present study, cellulolytic and hemicellulolytic activities were screened on microorganisms isolated from horticultural plant wastes composting piles. Culture conditions for simultaneous production of the extracellular enzymes from plant residues were also investigated.

MATERIALS AND METHODS

Cellulolytic and hemicellulolytic microorganisms were obtained by enrichment of samples taken from aerated composting piles mainly composed of pepper plant wastes. Samples of 5 g (fresh weight) were added to 250 ml Erlenmeyer flasks containing 95 ml of mineral basal medium (MBM) (Janshekar et al., 1981) supplemented with microcrystalline cellulose (0.5 %, w/v) (MBM-C) or xylan (0.5 %, w/v) (MBM-X). Enrichment cultures were incubated at 120 rpm and 30ºC for 7 days. A 5 ml aliquot was taken from the culture and transferred into fresh medium. This process was repeated four times and isolates were obtained in plates with specific media. Cellulolytic microorganisms were isolated on plates with MBM-agar, microcrystalline cellulose (0.5 %, w/v) and aniline blue-black (0.005 %, w/v). Decolorization around growth zones indicated cellulolytic activity (Kauri and Kushner, 1988). Hemicellulolytic microorga-

nisms were isolated on plates with MBM-agar and xylan (0.5 % w/v). A halo around colonies indicated hemicellulolytic activity (He et al., 1993).

Cellulases and hemicellulases production assays were performed in 250 ml Erlenmeyer flasks with 25 ml MBM added of 0.2 % (w/v) yeast extract and supplemented with 1% (w/v) carbon source. These cultures were inoculated with a 1cm^2 block of a 5-days-old culture of fungus on nutrient agar or 0.25 ml of 24-days-old culture of bacteria on nutrient broth. Oat spelt xylan (XYL, Sigma), carboxymethylcellulose sodium salt (CMC; Fluka), and milled and sieved to 2 mm particle size dry melon plants (MEL) or pepper plants (PEP) were used as carbon sources. Otherwise indicated, cultures were incubated at 120 rpm and 30ºC for 7 days. Cultures were centrifuged at 10000 x g for 10 min at 4ºC and supernatants were used for enzyme assays.

The enzymes were determined by measuring release of reducing sugars from appropriate substrate. For endoxylanase activity (XYLase) the reaction mixture contained 500 µl of supernatant with enzyme and 500µl of 1% (w/v) XYL on 50 mM citrate-phosphate buffer (pH 6.5). One unit of enzyme activity was defined as the amount of enzyme that released 1 µmol/ml of xylose equivalents per min at 30ºC and pH 6.5. Endoglucanase (CMCase) activity was measured by replacing xylan with 1% (w/v) of CMC in 50mM sodium acetate buffer (pH 5). One unit of CMCase activity was defined as the amount of enzyme that released 1 µmol/ml of glucose equivalents per min at 37ºC and pH 5. All reactions were stopped by boiling for 5 min. The amount of reducing sugar levels in the supernatant obtained after centrifugation at 10000 x g for 15 min at 4ºC were determined by the dinitrosalicylic acid (DNS) method (Miller, 1959). Enzyme and substrate controls were routinely included. All assays were performed in triplicate.

RESULTS AND DISCUSSION

Enrichments of cellulolytic and hemicellulolytic microorganisms were successfully obtained from composting material. From these enrichment cultures, a total of 75 isolates showed either cellulolytic or hemicellulolytic activity indicated by clearing zones in specific solid medium (MBM-C and MBM-X). These isolates were screened for endoglucanase and endoxylanase production in MBM medium with 1% (w/v) CEL or XYL as the sole carbon source. Only 51 strains (19 bacteria and 32 fungi) gave significant amounts of endoglucanase or endoxylanase activities. Some of these isolates showed both enzymatic activities simultaneously and were selected along with other with high rates of at least one of tested activities. As a result, 15 microorganisms (13 fungi and 2 bacteria) were used for further studies. These included the analysis of culture conditions for enzymes production.

The production of enzymes by 15 strains was investigated on several carbon sources. All selected strains were capable of growth in MBM-yeast extract medium containing either 1% (w/v) CEL, XYL, CMC, MEL or PEP. However, production of extracellular endoxylanase and endoglucanase was found to vary between the different carbon sources (Table 1). Most strains produced endoglucanase and endoxylanase from all substrates analysed. A comparison of enzyme activities revealed that the activity of endoxylanase (maximum of 2.49 U/ml) was greater than those of the endoglucanase with a maximum activity of 0.32 U/ml. Two fungi, HC5 and HC7, exhibited high enzymes activities, ranging from 0.05 to 0.08 U/ml for endoglucanase, and 0.06 to 2.4 U/ml for endoxylanase. Moreover, these strains produced enzymes with high activity from all carbon sources tested. Complex carbon sources as Melon plant residues (HW-Melon) and Pepper plant residues (HW-Pepper) were as good inducer substrates as cellulose, xylan or CMC; in adition, the use of these pure substrates is not economic. HW-pepper was slightly better substrate than HW-melon, and consequently was used as the only carbon source for enzymes production.

***Table 1**. Endoxylanase (XYLase) and endoglucanase (CMCase) activities (U/ml) of selected microorganisms from different carbon sources*.*

	Carbon Source									
	Cellulose		CMC		Xylan		HW-Melon		HW-Pepper	
Strains	XYLase	CMCase	XYLase	CMCase	XYLase	CMCase	XYLase	CMCase	XYLase	CMCase
HC-1	1.20	0.10	0.06	0.06	1.72	0.01	1.37	0.04	1.61	0.09
HC-2	0.47	0.00	0.27	0.01	2.39	0.01	2.26	0.05	2.08	0.07
HC-3	0.05	0.00	0.06	0.00	2.37	0.00	1.33	0.04	1.10	0.04
HC-4	0.17	0.02	0.11	0.02	2.04	0.03	1.90	0.03	1.91	0.02
HC-5	1.56	0.05	1.97	0.09	2.45	0.06	2.10	0.05	2.31	0.06
HC-6	0.18	0.00	0.07	0.05	2.33	0.00	0.75	0.04	1.07	0.04
HC-7	0.43	0.08	0.06	0.08	2.38	0.07	1.88	0.07	2.25	0.07
HC-8	0.35	0.02	0.06	0.02	2.13	0.02	1.89	0.07	1.93	0.06
HC-9	0.01	0.00	0.04	0.00	1.69	0.01	0.08	0.01	0.05	0.01
HC-10	0.90	0.13	0.34	0.06	2.49	0.02	1.03	0.10	1.68	0.10
HC-11	1.04	0.00	0.02	0.00	2.00	0.32	0.19	0.00	1.17	0.00
HC-12	0.15	0.00	0.23	0.00	1.53	0.05	0.16	0.00	0.05	0.00
HC-13	0.02	0.00	0.22	0.00	1.64	0.08	0.21	0.00	0.04	0.00
BC-1	0.45	0.00	0.33	0.01	2.38	0.04	2.39	0.02	2.02	0.00
BC-2	0.19	0.00	0.09	0.01	1.99	0.06	1.36	0.01	0.63	0.00

**Carbon source: Cellulose, microcrystalline cellulose; CMC, carboxymethylcellulose; Xylan, oat spelt xylan; HW-Melon, milled and 2mm sieved dry melon plant; HW-Pepper, milled and 2 mm sieved dry pepper plant. All carbon sources at 1% (w/v) in medium with MBM and 0.2% (w/v) yeast extract. Incubation at 120rpm, 30ºC for 7 days. Strains: HC refers to fungi and BC to bacteria.*

Further studies using the two selected fungi (HC5 and HC7) included the analysis of environmental factors on enzyme production and activity (data not shown). The effects analysed were pH (5-7), temperature (30-50ºC) and agitation (0, 80 and 150 rpm). Among those factors, only pH showed to have any effect on enzymatic activity. The other factors did not lead to variation on enzyme production or even decreased it within the range analysed. As for pH, a value of

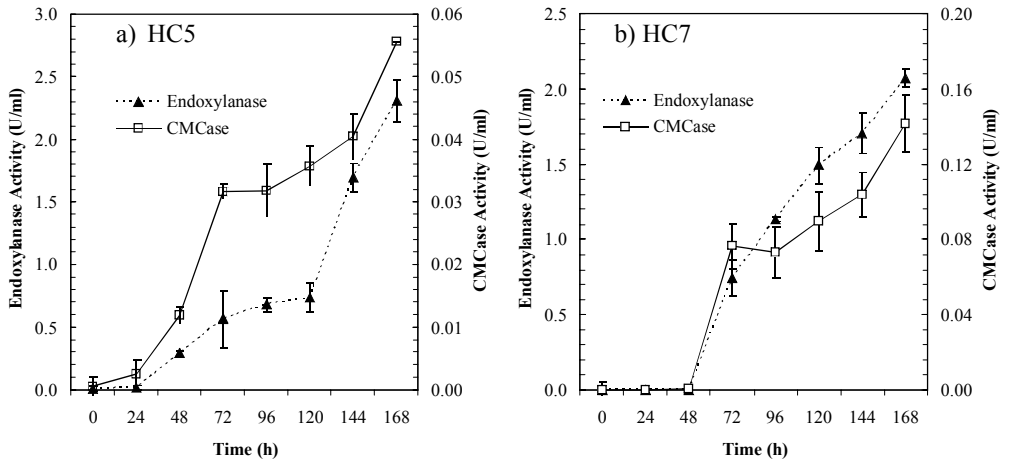

***Figure 1**. Production patterns of endoxylanase and endoglucanase (CMCase) in MBM-yeast extract supplemented with 1% (w/v) pepper plant wastes as sole carbon source by two selected fungi isolated from compost HC5, and HC 7. Conditions: medium pH 5, culture at 120 rpm and 30ºC. Values are mean of three determinations and vertical error bars represent standard deviation.*

5 showed to be slightly better than pH of 6. The adjustment of the growth medium to 5 caused an increase of 5% of endoxylanase activity by the two strains. From the analysis of the results, it was evident that the optimum conditions for the production of extracellular endoxylanase and endoglucanase occurred by the use of a medium with HW-pepper as the sole carbon source with pH adjusted to 5 and culture incubated at 120 rpm and 30ºC.

The time course of enzymes production by HC5 and HC7 under selected conditions is shown in Figure 1. Both microorganisms exhibited similar levels of enzyme activities and pattern of production. A typical growth-associated enzyme production time course occurred for the two fungi. At 24 h, very little activity was detected. For HC5 production of endoxylanase and endoglucanase began after 24 h incubation. At 72h, the secretion of both enzymes increased and continued to increase progressively up to 168 h. The behaviour of HC7 was similar but this fungus produced enzyme after 48 hours culture. Maximum activity for the two enzymes and fungi occurred at 168 h.

CONCLUSIONS

It is possible the simultaneous production of high levels of cellulolytic and xylanolytic enzymes from simple medium by newly microorganisms isolated from compost. Two fungal strains demonstrated that can grow and produce enzymes on simplified low-cost waste, such as agricultural pepper plant as the sole carbon source.

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REFERENCES

Bhat, M.K. 2000. Cellulases and related enzymes in biotechnology. Biotechnol. Adv., 18: 355-383.

He, L., Bickerstaff, G.F., Paterson, A., Buswell, J.A. 1993. Purification and partial characterization of two xylanases that differ in hydrolysis of soluble and insoluble xylan fractions. Enzyme Microb. Technol., 15: 13-18.

Janshekar, H., Haltmeier, T., Brown, C. 1982. Fungal degradation of pine and straw alkali lignins. Europ. J. Appl. Microbiol. Biotechnol., 14: 174-181.

Karui, T., Kushner, D.J. 1988. Detection of cellulolytic activity of bacteria and fungi growing on agar surfaces. Biotechnol. Tech., 2: 149-152.

Libmond, S., Savoie, J. 1993. Degradation of wheat straw by a microbial community-stimulation by a polysaccharidase complex. Appl. Microbiol. Biotechnol., 40: 567-574.

Miller, G.L. 1959. Use of dinitrosalicylic acid reagent for determination of reducing sugars. Anal. Chem., 31: 426-428.