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Production of Xylanase by Free and Immobilized Cells of *Cladosporium macrocarpum* NRC 15

[1]*Ahmed F. Abdel Fattah,* [1]*Doaa A.R. Mahmoud,*
[2]*Seham M. Shash,* [2]*Fardous M. Pasiony and* [1]*Sherien M.M. Atalla*

[1]Department of Chemistry of Natural and Microbial Products, National Research Centre, Giza, Egypt
[2]Department of Botany Faculty of Science, Benha University, Egypt

Abstract: The present work deals with physiological studies on fungal xylanase. It includes studies on the productivity of xylanase by different fungal isolates and cell immobilization. Twenty one fungal isolates under shaken condition were investigated for their abilities to produce xylanase enzyme from them. *Cladosporium macrocarpum* NRC 15 was selected because it produced xylanase activity (12 U/ml x 10^2) after 7 days incubation period. Xylan concentration of 20 g /l was the most suitable for the production of xylanase activity (32 U/ml x 10^2). Sodium nitrate was the most favourable as nitrogen source and 2 g /L concentration, produce maximum xylanase activity (32 U/ml x 10^2). Potassium dihydrogen phosphate of 1 g/l was the most suitable and produced (33 U/ml x 10^2) of xylanase activity. Magnesium sulphate of 0.5 g/ l was also the most favourable. The maximum xylanase activity (33U/ml x 10^2) was obtained when cells of *C. macrocarpum* NRC 15 were grown on the following medium (g/l): xylan 20, $NaNO_3$2, $KH_2PO_4$1, $MgSO_4$. $7H_2$o 0.5 and H_2O 1. Cell immobilization was recorded by physical immobilization and sawdust was the most favourable for the production of the highest xylanase activity (28 U/ml x 10^2). Entrapment immobilization was the most suitable with sodium alginate by which xylanase activity reached 28 U/ml x 10^2. Wood chippes as carriers showed maximum xylanase activity at 190 x 10^3 spores / ml and efficiency loading of 44 U/ml x 10^2. One gm of wood chippes produced (78.8 U/ml x 10^2) while reached its half life after 12 week of incubation.

Key words: Xylanase · Fungi · Physiology · Immobilization

INTRODUCTION

Xylan is one of the major hemicelluloses components of plant cell wall and consists of backbone of â-14 linked xylopyranose residues with branches containing arabinofuranosyl, acetyl and glucuronosyl residues. In the plant cell wall, hemicellulose is highly branched and tightly associated with other biopolymers. Fungi have been used widely used for manufacturing of enzyme products. These products are widely used in food, feed, pulp and paper, biomass conversion and textile process. Because of the long use in fermentation, some species of anaerobic fungi produce broad spectra of plant cell wall degrading enzymes much higher than aerobic fungi. Naota *et al*. [1] selected strains producing higher levels of cellulolytic enzymes from *Fusaruim sp*. and *F. oxysporum* SUF850. These strains were cultured using xylan as carbon source. Xylanase was obtained from submerged cultures under shaken conditions. Amita and Datta [2] used *Aspergillus foetidus* ATCC 4298 for xylanase production. The maximum xylanase yield (210 U/ ml) was obtained at 30°C using birch wood xylan as substrate in 3 days of cultivation. Seyis Isil and Aksog Nilylez [3] studied some physiological conditions affecting the activity of xylanase produced from *Trichoderma harzianum* $1073D_3$, using xylan as the carbon source in the culture medium. Sanjay *et al.* [4] isolated xylanase producing fungus from *Trichoderma reesei* SAF3. Maximum growth of the organism was found at 48 h. Highest enzyme production (4.75 U/ ml) was recorded at 72 h. Husin *et al*. [5] studied growth conditions including, incubation times, temperature, agitation rate and initial pH of medium affecting xylanase production by *Aspergillus carneus* M 34. Yin *et al*. [6] optimized the

Corresponding Author: Sherien M.M. Atalla, Department of Chemistry Natural and Microbial Products, National Research Centre, Giza, Egypt.

effect of substrate concentration, pH and cultivation time on the production of xylanase from *Penicillium oxalicum* ZH–30, while Zulfiqar *et al.* [7] tested wheat bran, an indigenous carbon source, as substrate for *Aspergillus niger* for optimum synthesis of xylanase. Maria *et al.* [8] investigated the production of xylanase by filamentous fungi (*Trichoderma viride*) to determine the best cultivation conditions in the process aiming toward optimization of enzyme production. Zhengqiang *et al.* [9] isolated a novel thermophilic xylanase by *Chaetomium* sp. CQ31 which produced 13 U/ml of xylanase when grown in medium containing corncobs (3.5 % w/v) at 37°C for 6 days. Yishan *et al.* [10]employed response surface methodology (RSM) to optimize medium composition for the production of a thermostable xylanase from *Trichoderma lanuginosus* SDYKY-1 using economical carbon and nitrogen sources (soybean meal and corncobs, respectively).

MATERIALS AND METHODS

Microorganisms: Twenty one fungal isolates were provided from the Department of chemistry of Natural and Microbial Products Chemistry at the National Research Center (NRC), Dokki, Cairo, Egypt and from NRRL.

The following list represents the fungal strains used throughout the work:

- *Aspergillus awamori NRRL 3112*
- *Aspergillus oryzae NRRL 3487*
- *Aspergillus penicillioides* NRC 8
- *Aspergillus tamarii* NRC 9
- *Aspergillus terreus* NRC 10
- *Cladosporium macrocarpum* NRC 15
- *Cladosporium herbarum* NRC14
- *Fusarium semitectum* NRC 24
- *Fusarium solani* NRC 20
- *Fusarium sporotricoides* NRC 22
- *Fusarium verticilliodes* NRC 23
- *Gliomastix gueg* NRC 1
- *Mycogone sp.*NRC 32
- *Monascus rubber* NRC 7
- *Penicillium funiculosum NRRL 13039*
- *Penicillium funiculosum NRRL 13033*
- *Penicillium funiculosum NRRL 13041*
- *Penicillium requifortii* NRC 35
- *Penicillium paraphergal* NRC 37
- *Trichoderma harzianum* NRC 42
- *Trichoderma viride* NRC 65

Media

Czapek-Dox agar (according to Haung and Ling [11]): $NaNO_3$ 2.0 g, K_2HPO_4 1.0 g, KCl 0.5 g, MgSO4.$7H_2O$ 0.5 g, FeSO4 . $7H_2O$ 0.001g, sucrose 30 g, agar 20 g, H_2O 1 L.

Fermentation Medium: $NaNO_3$ 2.0 g, K_2HPO_4 1.0 g, KCl 0.5 g, MgSO4.$7H_2O$ 0.5 g, $FeSO_4$. $7H_2O$ 0.001g: Sucrose was replaced by xylan 5 g, H_2O 1 L.

Xylan: It was a preparation of Sigma from birch wood

Methods

Production of Xylanase: Two disks (6 mm in diameter) from 7 days old cultures were inoculated in Erlenmeyer flasks 250 ml containing 50 ml of fermentation medium and incubated at 28-30°C for 7 days at 200 rpm, then centrifuged. The cell free supernatant was used in enzyme assay and protein determination.

Determination of Xylanase Activity

Procedure: This was done according to the method of Monreal and Reese [12] using 1.0 ml of 1% (w/v) xylan in acetate buffer (pH 4.6) to which 1.0 ml of the culture filtrate was added and mixed well by shaking. The mixture was incubated in a water bath at 50°C for 30 minutes, then cooled and centrifugated before assaying.

Unit of Enzyme Activity: One unit of enzyme activity was taken as the amount of the enzyme which converts one micromole of substrate in one minute under specific conditions.

Determination of Protein: Protein content of the culture filterate was determined according to the method of Lowry *et al*. [13]

RESULTS

Survey of Some Fungal Cultures for the Production of Xylanase: Twenty one fungal strains were examined for the production of xylanase. At the end of each fermentation process, the culture broth of each strain was filtered, the dry weight of mycelium as well as the pH value, protein content and xylanase activity were determined. Data present in Table 1 indicated that, pH values of all culture filtrates lied in the neutral or slightly alkaline range (6.77 to 8.65). In some cases, the protein content of the culture filtrate depended on the fungal strain.

Table 1: Survey of some fungal cultures for the production of xylanase

Fungal isolates	Final pH	Mycelium dry/ wt (mg/flask)	Protein content (mg/ml)	Xylanase activity (U/ml x 10^2)
Aspergillus awamori NRRL 3112	7.70	60	1.33	5.0
Aspergillus oryzae NRRL 3487	8.06	50	0.95	1.7
Aspergillus penicillioides NRC 8	8.21	10	0.38	0.1
Aspergillus tamarii NRC 9	8.38	50	0.53	11.0
Aspergillus terreus NRC 10	8.07	40	0.56	1.7
Cladosporium macrocarpum NRC15	8.49	50	0.87	12.0
Cladosporium herbarum NRC14	8.50	20	0.72	2.0
Fusarium semitectum NRC24	8.47	50	0.54	6.0
Fusarium solani NRC 20	8.40	40	0.13	7.0
Fusarium sporotricoides NRC 22	8.65	50	0.83	9.0
Fusarium verticilliodes NRC 23	8.27	10	0.19	2.0
Gliomastix gueg NRC 1	7.94	80	0.72	3.9
Mycogone NRC 32	8.26	20	0.33	0.1
Monoascus rubber NRC 7	7.34	50	0.54	2.6
Penicillium funiculosum NRRL 13039	6.77	40	1.08	2.8
Penicillium funiculosum NRRL 13033	5.82	50	1.13	3.9
Penicillium funiculosum NRRL 13041	5.67	70	1.02	2.9
Penicillium requifortii NRC 35	8.03	80	0.57	3.4
Penicillium paraphergal NRC 37	8.12	40	0.43	0.7
Trichoderma harzianum NRC 42	8.29	70	0.85	6.0
Trichoderma viride NRC 65	8.33	50	0.42	0.2

Some Physiological Factors Affecting the Production of Xylanase by the *Cladosporium macracarpum* NRC 15: The maximum activity was produced by *Cladosporium macracarpum* NRC 15.

Effect of Xylan Concentrations in Fermentation Medium: Xylan concentration in the culture medium was studied and maximal productivity of xylanase was reached on using 20 g /l in the culture medium, which was 32 U/ml x 10^2. Higher xylan concentration led to dramatic loss of xylanase activity.

Effect of Different Nitrogen Sources on the Production of Xylanase by *C. macrocarpum* NRC 15: In order to investigate the effect of different nitrogen sources on the production of xylanase enzyme, sodium nitrate (2 g /l) as nitrogen source in the culture medium was substituted, an equal nitrogen basis, by each of the following nitrogen sources, ammonium sulphate, soybean, corn steep- liquor and peptone. Cultivation was carried out at 30°C under shaking conditions (200 rpm) for 7 days incubation in a medium containing xylan as the sole carbon source. Among all the nitrogen sources, results showed that, sodium nitrate was the most proper nitrogen source for the production of maximal xylanase activity 32 U/ml x 10^2. On the other hand, it was found that ammonium sulphate, soybean, corn steep-Liquor and peptone had adverse effects on the productivity of xylanase.

Optimization of the Culture Medium: The concentrations of potassium dihydrogen phosphate and magnesium sulphate in the culture medium were also investigated. Maximal concentrations of 1.0 g/l and 0.5 g/l for KH_2PO_4 and magnesium sulphate affected xylanase activities of 33 U/ml x 10^2 and 35 U/ml x 10^2, respectively.

Effect of different additives on the production of xylanase by *C. macrocarpum* NRC 15: The effect of different additives to the culture medium on the production of xylanase by *C. macrocarpum* NRC 15 was investigated. These included wheat bran (2.0 %), lactose (1.0%), tween 80(0.1 %), urea (3.5 %), bakers yeast (4 %). All this additives had adverse effects on xylanase productivity. Urea effected the most adverse effect (3 U/ml x 10^2) followed by tween 80 (11 U/ml x 10^2), bakers yeast (18 U/ml x 10^2), wheat bran (23 U/ml x 10^2) and lactose (27 U/ml x $10^{2)}$ compared to control (32 U/mlx 10^2). Collectively, the most favourable culture medium for the productive of active xylanase by *C. macrocarpum* NRC 15 consists of the following (g/l) : xylan (20), sodium nitrate (2), KH_2PO_4 (1.0) and $MgSo_4$. $7H_2O$ (0.5).

Production of Xylanase by Immobilized Cells

Cell Immobilization by Adsorption Method: Adsorption of *C. macrocarpum* NRC 15 cells on different supports, namely pumice, natural sponge, synthetic sponge, luffa cylindrica, chitin, sawdust, foam and stainless steel were examined. The results in Table 2 showed

Table 2: Production of xylanase by immobilizated cell *C. macrocarpum* NRC 15 by adsorption

Carrier	Final pH	Protein content (mg/ml)	Xylanase activity (U/ml x 10^2)
Free cells	8.83	2.72	30.0
Pumice	8.63	2.04	18.0
Natural sponge	8.73	1.916	26.0
Synthetic sponge	8.52	1.55	20.0
Luffa cylindrical	8.21	1.01	10.0
Chitin	8.22	1.65	22.0
Sawdust	8.29	1.87	28.0
Foam	8.41	1.05	12.0
Stainless steel	8.52	1.404	14.0

Table 3: Production of xylanase by cell immobilization of *C. macrocarpum* NRC 15 by entrapment

Carrier	Final pH	Protein content (mg/ml)	Xylanase activity (U/ml x 10^2)
Free cells	8.83	2.72	30.0
Agar	8.2	1.88	19.0
Agarose	8.67	2.01	19.0
Alginate	7.75	1.9	20.0

Table 4: Immobilization of *C. macrocarpum* NRC 15 on different forms of wood wastes

Carrier	Final pH	Protein content (mg/ml)	Xylanase activity (U/ml x 10^2)
Sawdust	8.29	1.87	28.0
Wood Chippes	8.53	1.06	44.0
Wood Shaves	8.51	1.07	39.0

Table 5: Effect of biomass loading (efficiency loading) on wood chippes on *C. macrocarpum* NRC15 by cell immobilization

Efficiency loading (x 10^3 spores/ml)	Final pH	Protein content (mg/ml)	Xylanase activity (U/ml x 10^2)
95(control)	8.39	2.73	44.0
142.5	7.9	2.88	55.0
190	8.5	3.33	78.8
237.5	8.51	3.33	69.7
285	8.82	2.53	62.33
332.5	8.49	2.28	49.5

Table 6: Effect of different concentrations of wood chippes (matrix) on cell immobilization

Different weight Of wood chippes (matrix) (gm) Final pH		Protein content (mg/ml)	Xylanase activity (U/ml x 10^2)
0.5	8.35	2.41	66.0
1(control)	8.5	3.1	78.8
1.5	8.49	2.6	71.5
2	8.53	2.44	58.67
2.5	8.55	2.11	53.17

Table 7: Production of xylanase by immobilized cells of *C. macrocarpum* NRC15 on wood chippes in repeated batch process

Cycle number (weaks)	Xylanase activity (U /ml x 10^2 /g carrier)	Relative activity %
Control	78.80	100
1	77.00	98.0
2	77.00	98.0
3	77.00	98.0
4	75.16	95.3
5	73.32	93.1
6	69.60	88.4
7	69.60	88.4
8	66.00	83.8
9	60.50	76.8
10	53.16	67.5
11	45.80	58.1
12	36.60	46.5

that sawdust,chitin and natural sponge were the most suitable for cell immobilization and afforded highest enzyme productivity. The fungal cell immobilization on sawdust was the most efficient and yielded active xylanase (28.0 U/ml x 10^2) which was less active than that produced by free cells (32.0 U/ ml x 10^2).

Cell Immobilization by Entrapment: Entrapment was performed using 3% concentration of a gar, agarose or alginate.

The results in Table 3 showed that immobilization on alginate were the most favourable (20 U /ml x 10^2) which were less active than immobilized cells on sawdust.

Optimization of Some Conditions for immobilization of *C. macrocarpum* NRC 15 on Wood Waste.

Immobilization of *C. macrocarpum* NRC 15 on Different forms of wood Wastes: *C. macrocarpum* NRC 15 was immobilized on wood chippes, shavings and sawdust to investigate the influence of support size on the xylanase activity. From results in Table 4, it was observed that wood chippes adsorbed highest amount of fungal cells and produced maximum xylanase activity (44 U/ml x 10^2) compared to the other forms of wood waste.

Effect of Biomass Load (Efficiency Load): 1.0 g of wood chippes was mixed with different spore concentrations (95, 142.5, 190, 237.5, 285 and 232.5) x 10^3 spores / ml/g wood chips. The resulted matrix with different spore concentrations were used for the inoculation of 50 ml culture portions. The results in Table 5 indicted that there was an increase in the enzyme activity with increasing cells load up to (190 spores / ml x 10^3 / g carrier), whereby the maximal enzyme yield was attained (78.8U/ml x 10^2). Further increase in spores concentration led to decrease in enzyme production.

Effect of Matrix (Wood chippes) Concentration: In this experiment different concentrations of wood chippes (0.5, 1, 1.5, 2 and 2.5 g/ 50 ml culture medium) were used for the immobilization process. In all cases, equal amounts of spore suspensions were used (190 spores /ml x 10^3). The highest xylanase activity was obtained with matrix concentration of 1.0 g wood chippes / 50 ml (Table 6).

Production of Xylanase by Immobilized cells of *C. macrocarpum* NRC 15 on Wood Chippes in Repeated Batch Process: The present experiments were carried out to investigate the production of xylanase by the immobilized cells of *C. macrocarpum* NRC 15 in repeated batch process. The results in Table 7 indicated the durability of the immobilized cells in repeated use. The retained activity after being used for 12 cycles was 46.5% of the initial value of the immobilized cells.

DISCUSSION

The present work deals with physiological and biochemical studies on fungal xylanase. It includes studies on the productivity of xylanases by different fungal isolates and cell immobilization of the most potent strain. Twenty one fungal isolates under shaken condition were investigated for their abilities to produce xylanase. Xylanase production by a newly isolated *Aspergillus foetidus* MTCC 4898 was studied using submerged fermentation [2]. Some cultural conditions affecting the production of xylanase were studied using *A. tamari* NRC 9 and *C. macro carpum* NRC 15 after 7 days of incubation. Increase in xylan concentration leads to increase in xylanase activity. Xylan concentration of 20 g/l was the most suitable for the production of xylanase activity. Xylan concentration of 10 g /l in the culture medium affect xylanase production by *Aspergillus terreus* 603 [14]. Different nitrogen sources in the culture medium were investigated for the production of xylanase enzyme. These included ammonium sulphate, soybean, corn steep, peptone and sodium nitrate. Low xylanase activity was recorded when using ammonium sulphate, soy bean and corn steep. Sodium nitrate was the most favorable as nitrogen sources. Different concentrations of sodium nitrate were studied and 2 g /l of sodium nitrate produced maximum xylanase activity for both organisms, while higher concentration of sodium nitrate decreased xylanase activity. These results are in agreement with those reported by Marie *et al.* [8] for *Trichoderma viride* and Anita *et al*. [14] for *Aspergillus terreus* 603. Different concentrations of potassium dihydrogen phosphate were investigated and 1 g / l was the most suitable one. The same procedure adopted with magnesium sulphate which proved that 0.5 g /l was the most favorable, high or low concentration decreased xylanase activity. Wheat bran, lactose, tween 80, urea, baker's yeast and sodium nitrate were investigated as different additives in culture medium for xylanse production by *C. macrocarpum* NRC 15, all the additives used had adverse effects on the productivity of xylanase. Of these urea had the most adverse effect. These results agreed with those reported by Zulfigar *et al*. [9] for the xylanase produced by *Aspergillus terreus* 603. Cell immobilization was preformed by physical immobilization using 8 carriers i.e. pumice,

natural sponge, synthetic sponge, lofa cylinderica, chitin, sawdust, foam and stainless steel. From these carries sawdust was the most suitable for the produced the highest xylanase activity. Entrapment immobilization was done using sodium alginate, agar and agarose of these sodium alginate produced maximal xylanase activity while agarose produce high protein content. This was accorded with the reported by Sunder *et al*. [15]. Different sizes of sawdust were investigated as carriers. Wood chippes produced maximum xylanase activity at 190 x 10^3 spores /ml. One g of wood chippes, at 190 x 10^3 spores / ml reached its half life after 12 week of incubation [16].

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