Research Article

Biodiesel Extraction from *Jatropha curcas* L Using an innovative *Aspergillus niger* 10 enzyme cocktail

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ABSTRACT

The present article focused on the physiological production optimization of an enzyme cocktail (Cellulases, hemicellulases and pectinases) suitable for perfect biodiesel extraction process from *Jatropha curcus* L. by potent fungal producer utilizing some abundant Egyptian lignocellulosic waste. Among nine recommended local fungal strains, employing sugar beet pulp, jatropha cake, Egyptian orange pulp and peels or tomato cake residues remaining after industrial processing, as a sole carbon source, *Aspergillus niger 10* was the most potent producer in 4 days shaken culture (150 rpm) at 28 ºC and pH 5.0, affording an effective enzyme cocktail containing highly active cellulases, hemicellulases and pectinases as a cheap and powerful oil-extracting agent. The cocktail culture production medium was formulated as follows (g/L): dry beet pulp residue, 70; KH_2PO_4, 0.5; $MgSO_4.7H_2O$, 0.5; $NaNO_3$, 2.5. The enzyme cocktail was optimally operated after 20 min at 40 ºC, pH 5.0 and substrate concentration (%, w/v) 0.8, 0.1 or 1.5 of Na-CMC, hemicellulose or citrus pectin, respectively, which denoted its completely suitability for oil extraction process.

Keywords: Biofuel, biotechnology, Jatropha, cellulose, hemicellulose, pectinase.

INTRODUCTION

Biodiesel production and enzymatic biodegradation of agricultural wastes such as sugar beet pulp, jatropha cake, orange pulp and peels or tomato cake residues remaining after industrial processing require multienzyme (cocktail) systems containing cellulases, pectinases and hemicellulases which act synergistically upon this waste. These enzymatic cocktails are currently required due to their high potential biotechnological applications in many fields, which include their use as a new eco-friendly technique in the biodegradation of agricultural wastes and very safe extracting agent of biodiesel, oils and starches from plant materials specially Jatropha curcus L seeds which represents an excellent source of biodiesel oil (Achten *et al*.,2008; Shivani1 *et. al.* 2011; Ofelia *et al*.,2013 and Kannahi, M. and Arulmozhi, R. 2013). The need for the production of these enzymatic cocktails is due to their low cost, high safety and the fact that it represents new clean technology. The key element in bioconversion process of lignocellulosics to these useful products is the hydrolytic enzymes mainly cellulases (Ojumu *et al,* 2003; Fan *et al*., 1987; Immanuel *et al.,* 2007). In the present work a screening of some locally isolated fungi was made to choose the potent fungus which has the prevalence to all the other isolates for its multienzyme system productivity, employing sugar beet pulp, jatropha cake as a sole carbon source. Also studying the effect of different substrate concentrations on enzymes activities.

MATERIALS AND METHODS

MATERIALS

The fungal strains *Aspergillus niger 10, Penicillium* chrysogenuml, *P. chyysogenum2, P. chrysogenum3, P.chrysogenum4, P. chrysogenum5, Penicillium funicullosum* 1, *P. funicullosum* 2 and *Trichoderma ressei* 34 were kindly obtained from AUMC (Assiut university Mycological Center). Jatropha seed cake samples were obtained from seeds extracted by hexane. Sugar beet pulp waste samples (dried containing 14.6% moisture) were obtained from

Delta Sugar Company.

MEDIA

Fungal inoculum preparation medium (g/L) Glucose, 16; peptone 1.25; yeast extract, 0.25; $MgSO_4$, $7H_20$ 0.125 and K_2HPO_4, 0.25. The culture was grown in 250 ml Erlenmeyer flask that contained 50 ml of medium, left on shaker 150rpm for 3 days at 28 °C. Fungal Multienzyme systems production medium was furnished according to (Ismail, 1996) but orange peel waste was replaced by Jatropha seed cake of the same pectin quantity. 3.6g/50 ml; K_2HPO_4, 0.5; $MgSO_4$. $7H_20$, 0.5; $NaNO_3$, 2.5. five ml of the prepared inoculum was added over 250 ml Erlenmeyer flask that contained 50 ml of multienzyme system production medium, left on shaker 150rpm for *4* days at 28°C. The crude enzyme was filtered and centrifuged at 8000 rpm for 15 min.

Enzymesassay

In case of cellulase, the reaction mixture containing 10m1 of 1% (w/v) sodium carboxy methylcellulose in 0.2 M acetate buffer pH 5 and 2ml of the enzyme solution was incubated in a water bath at 40°C and the flow time was recorded. The flow time of the inactive reaction mixture and of the distilled water were also recorded the reduction in viscosity was then calculated.

For pectinase, the reaction mixture containing l0ml of 0.5% (w/v) pectin in 0.2M acetate buffer, pH 5 and 2.5 ml of the enzyme solution was incubated in a water bath at 40°C for 20 min. Thereafter, 10ml of the reaction mixture were piptted into an Ostwald viscometer and incubated in water both at 40°C and the time flow was recorded. The flow time of the inactive reaction mixture and of the distilled water were also recorded. The % reduction in viscosity was then calculated.

For hemicellulase, The reaction medium consisted of 2.5ml containing 1.56 mg of corn-cobs hemicellulose suspension, 0.05 M acetate buffer pH 5 (2.5m1) and 10 ml of the enzyme solution. Similar reaction mixtures using heated inactive enzyme solutions were also prepared as control. Unless other wise specified, the reaction mixtures were incubated in a water bath at 45°C for 20 min. The released pentoses due to hemicellulytic activity were determined by the method reported by (Neish, 1952) and based on those described by Nelson (1944) and Somogyi (1945) as follows:

• **Arsenomolybdate reagent**

25g of ammonium molybdate were dissolved in 450 ml distilled water and then stirred in 21 ml of conc. H_2So_4. 3g of Na_2HSO_4. $7H_2O$ in 25m1 of water were added and the whole mixture was incubated at 37°C for 2 days

• **Somogyi copper reagent**

28 g of Na_2HPO_4 and 40 g of Rochille salt were dissolved in 700 ml water, 100 ml of 1N- NaoH and 80 ml of $CuSO_4$. $2H_2O$ solution were then added with stirring. This was followed by dissolving 180 g of anhydrous sodium sulphate and the solution made to liter. After allowing the solution to stand for two days, the supernatant was decanted, filtered and stored. To 5ml of the reaction mixture, 2.Oml of the alkaline copper reagent were added. The contents of the tube were thoroughly mixed and heated in boiling water bath for 10 min. After cooling the tube in cold- water bath, 2.Oml of the Arsenomolybdate reagent were mixed and the mixture diluted to 25m1. A control sample of the reaction mixture at zero time addition of the culture filtrates was also prepared in the same way. The color was read at 520 nm. A standard curve was constructed using different concentration of xylose one enzyme unit is defined as the amount of enzyme that release 1.0 µ xylose from hemicellulose per min under assay conditions.

Effect of different substrate concentrations

The effect of substrate concentration was studied using different concentrations of pectin ranging from 0.1-2% (w/v) in the reaction mixture. The reaction mixture consisting of 10ml buffered citrus pectin (0.2 M acetate buffer, pH5) of each of the above substrate concentrations and 2.5 ml of enzyme solution of 4 mg/ml protein concentration. The reaction mixture was performed at 40°C for 20 min incubation period and the pectinases activity was measured as described before. A control sample was prepared as described above. For cellulases, Na-CMC concentrations ranging from 0.5-1.5 % (w/v) in the reaction mixture the reaction mixture consisting of 10 ml buffered Na-CMC (0.2 M acetate buffer, pH 5) of each of the above substrate concentration and 2.0 ml of enzyme solution of 4 mg/ml protein concentration. The reaction mixture was performed at 40°C for 20 min incubation period and the cellulases activity

was measured. For hemicellulases, different xylan concentration ranging from (0.01- 0.5) % xylan in the reaction mixture was used. The reaction mixture consisting of 2.5 ml xylan solution each above substrate concentrations+ 2.5 ml 0.05 M acetate buffer, (pH 5) + 1 ml enzyme solution of 4 mg/ml protein the reaction was performed at 45°C for 20 min incubation period and the hemicelulases activity was measured.

RESULTS AND DISCUSSION

The data showed that most of the fungal isolates had the ability to produce the multienzyme complex systems with different activities. In case of the sugar beet pulp waste medium, *P. funiculosum 1 and P. funiculbszan 2* showed the highest percent pectinase activity of 69% after 4 days shaken cultures, Table (1) while *Aspergillus niger* 10 showed the highest activity of 64% after 6 days. In case of jatropha seed cake medium, P. *funiculosum 2* showed the highest percent pectinase activity of 68% after 4 and 60% after 6 days shaken cultures in comparaison with other fungi under study. According to cellulase activity, Table (2) the higher percent activity of 98% after 4 days of incubation was achieved by *P. chrysogenum 3* and *Aspergillus niger* 10 upon using sugar beet pulp medium while after 6 days only *Aspergillus niger 10* gave the highest percent cellulase activity 99%. On the other hand in case of jatropha seed cake medium the highest percent cellulase activity was recorded by *Penicillium funiculosum 2, Aspergillus niger* 10 and *Trichoderma ressei* 34 reached 98%, 98% and 99% respectively. The same results were recorded after 6 days of incubation. According to hemicellulase activity, Table (3) in the sugar beet pulp medium *Penicillium chrysogenum 5, P. funiculosum* 1, *Aspergillus niger* 10 and *Trichoderma ressei* 34 recorded highest percent hemicellulase activity after 4 days of incubation reached 100% while after 6 days of incubation only *P. chiysogenzan* 4 recorded the same percent activity. In case of jatropha seed cake medium the highest percent hemicellulase activity was recorded by *Trichoderma ressei* 34 after 4 and 6 days reached 79% and 87% respectively.

Table 1: Changes in pectinase activity (% reduction in 0.5%, w/v pectin solution viscosity) of different fungal cultures through different incubation periods using shaken technique

Incubation period(Days)/ Microorganisms	Waste in culture medium			
	Sugar Beet Pulp		Jatropha Seed Cake	
	4	6	4	6
***Penicillium chrysogenum* 1**	32	36	36	52
***P. chrysogenum* 2**	24	32	42	52
***P. chrysogenum* 3**	68	57	62	30
***P. chrysogenum* 4**	60	52	55	27
***P. chrysogenum* 5**	60	52	56	42
***P. funiculosum* 1**	69	57	66	57
***P. funiculosum* 2**	69	60	68	60
***Aspergillus niger* 10**	60	64	52	52
***Trichoderma ressei* 34**	28	28	10	42

Table 2: Changes in cellulase activity (% reduction in 1% w/v NaCMC viscosity) of different fungal cultures through different incubation periods using shaken technique

Incubation period (Days)/ Microorganisms	Waste in culture medium			
	Suger Beet Pulp		Jatropha Seed Cake	
	4	6	4	6
***Penicillium chrysogenum* 1**	96	93	90	93
***P. chrysogenum* 2**	96	97	93	93
***P. chrysogenum* 3**	98	97	84	82
***P. chrysogenum* 4**	97	96	82	71
***P. chrysogenum* 5**	97	96	77	75
***P. funiculosum* 1**	88	94	92	95
***P. funiculosum* 2**	96	96	98	98
***Aspergillus niger* 10**	98	99	98	98
***Trichoderma ressei* 34**	90	98	99	99

(Sapunova *et al.,* 1995). Shen *et al.,* (1996) applied 0.3% (w/v) pectic acid for the purified polygalcturonase(PG) from rice weevil *Sitophilus ofyzae* (Cleoptera Curculionidac). Also, highest pectin concentration (4.0%, w/v) was used for commercial pectinases from Novo-Nordisk Ferment. (Mutlu *et al.,* 1999; Demir *et al.,* 2001).

Concerning the effect of the substrate concentration on the different enzymes activities, upon using different pectin concentrations ranging from (0.1- 2), 1.5 % (w/v) of citrus pectin, Fig. (1) was the optimum substrate concentration affording the maximal percent of pectinase activity reached 63% on jatropha cocktail upon using 22.66 mg protein/reaction, above this percentage, activity remained constant, denoting that it was the saturation concentration of pectin in the enzyme active sites. Using different CMC concentration ranging from (0.5-1.5%, w/v) maximal percent of cellulase activity, Fig. (2) was recorded at substrate concentration of 0.8% (w/v) on. SBP upon using 39.21mg protein/reaction. On the other hand percent cellulase activity recorded its maximum value of 95% at concentration 0.8% (w/v) on jatropha cocktail of 11.33mg protein/reaction. In addition, using different xylan concentrations ranging from (0.01- 0.15%) optimum xylan concentration, Fig. (3) was 0.1% (w/v) using SBP cocktail enzyme of 26.14mg protein/reaction, while in case of applying jatropha cocktail enzyme of 16.99mg protein/reaction the optimum xylan concentration was 0.15% (w/v). In this respect, Abdel-Fattah *et at.,* 1981, recorded typical optimum Na-CMC concentration (1.0%, w/v) for the crude cellulases and hemicellulases mixture produced by *T. viride* 253 utilizing the cane-bagasse and the CMC-ase activity released was 97.5% reduction in viscosity of Na-CMC solution. Also, 1.0% (w/v) apple pectin was the optimum concentration for the purified pectinase produced by the fungal strain *Trichoderma lignorum* (Abdel-Fattah *et aL,* 1981) and for the partially purified preparations of pectin lyase GlOX from *Penicillium adametzii, P. citrinum* and *P. janthinellum*.

Table 3: Hemicellulase activity (% xylan hydrolysis) of different fungal cultures through different incubation periods using shaken technique

Incubation period (Days)/ Microorganisms	Waste in culture medium			
	Sugar Beet Pulp		Jatropha Seed Cake	
	4 days	6 days	4 days	6 days
Penicillium chrysogenum 1	87	28	17	24
P. chrysogenum 2	81	44	38	31
P. chrysogenum 3	54	83	25	44
P. chrysogenum 4	79	100	27	35
P. chrysogenum 5	100	25	34	36
P. funiculosum 1	100	34	20	78
P. funiculosum 2	70	32	21	30
Aspergillus niger 10	100	35	26	21
Trichoderma ressei 34	100	44	79	87

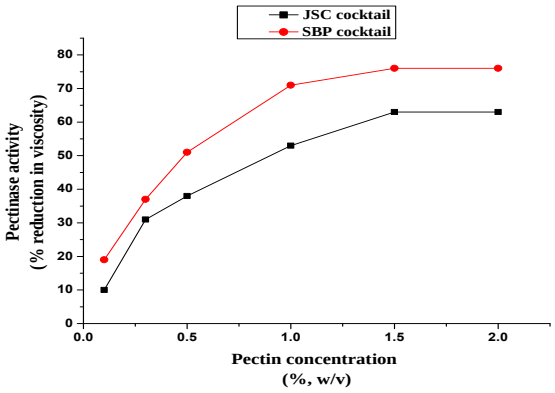

Fig. 1: Effect of different substrate concentrations on pectinase activity

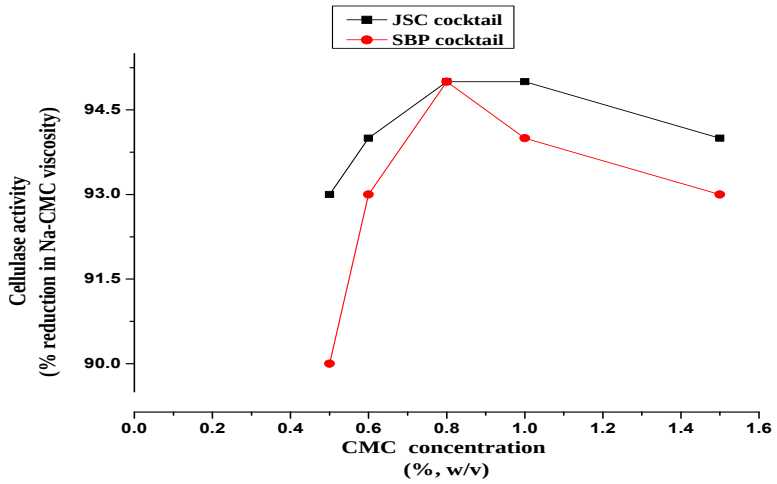

Fig. 2: Effect of different substrate concentrations on cellulase activity

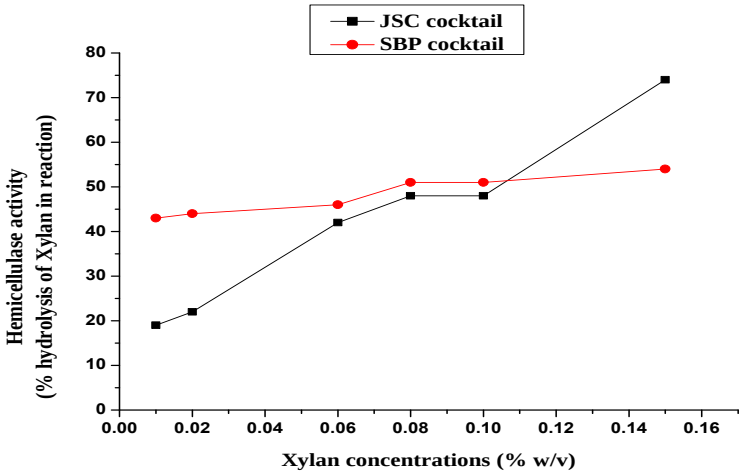

Fig. 3: Effect of different substrate concentrations on hemicellulase activity

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