Research Article

A Powerful Enzymatic Cocktail Agreeable for Perfect Jatropha curcus L. Oil Extraction

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ABSTRACT

Jatropha seeds oil (biodiesel) actually represents the highly safe and renewable alternative to the constantly decreased petroleum diesel. The enzymatic oil extraction instead of the organic solvent n- hexane is of great interest all over the world. Jatropha seeds oil extraction with enzymes cocktail containing active cellulases, hemicellulases and pectinases is an eco-friendly process saving human from n-hexane toxicity and carcinogenicity. The enzymatic method affords high quality and rancidity-resistant oil, according to its high antioxidants content. *Aspergillus niger* 10 enzymes cocktail is prepared in low costs through very simple fermentation process, utilizing some available Egyptian agricultural wastes. As this cocktail preparation is entirely free of mycotoxins and completely soluble in water and buffer solutions with good heat stability, it could be applied in edible oil and starch extraction. The prepared enzyme cocktail proved to be compatible with the other high-priced enzyme cocktail preparations as Viscozyme L of Novo-Nordisk Corporation.

Keywords: Biodiesel, Jatropha seed, Multienzymes cocktail, oil extraction.

1. INTRODUCTION

Recently, biodiesel had gained high importance for its ability to replace fossil fuels, which are likely to run out within a century. The need of energy is increasing continuously due to rapid increase in the number of industries and vehicles owing to population explosion[1]. The environmental issues concerned with the exhaust gases emission by the usage of fossil fuels, also encourage the usage of biodiesel which has proved to be eco-friendly more than fossil fuels. Biodiesel is a mixture of mono-alkyl esters obtained from vegetable oils like jatropha oil, soya bean oil, etc[2]. According to all the dangers of oil extraction with the commonly used n-hexane on account of its high toxicity and powerful carcinogenicity as extensively reported[3-4], beside the undesirable effects on the extracted oil quality due to oxidative deterioration of polyunsaturated fatty acids and development of rancidity[5], in addition, during the successive steps of extraction with n-hexane, oils exposed to high temperature and metallic catalysts leading to losses of valuable compounds, especially vitamin E and polyphenolics, it seemed that the only and perfect alternative to n-hexane is the multienzyme coktails involving cellulases, hemicellulases and pectinases. The application of enzymes in plant oil extraction is obligatory needed to the pretreatment of the oil-containing cells before enzymatic application due to the structural tissue surrounding the cell wall and composed of cellulose, hemicellulose, pectin and lignin[6]. These enzymatic cocktails (multienzyme system complexes, MESC) are currently known in the world market, due to their high potential biotechnological applications in many fields, which include their use as very safe extracting agents of oils and starches from plant materials instead of the carcinogenic solvents and chemicals. The fact that the oil of *Jatropha curcas* cannot be used for nutritional purposes without detoxification makes its use as an energy source for fuel production very attractive[7], that's why Jatropha was selected to be used in this study.

2. EXPERIMENTAL / MATERIAL AND METHODS

2.1 Chemicals

All the following chemicals used in this study were of analytical grade.

Glucose, dipotassium hydrogen phosphate (K_2HPO_4), hydrous magnesium sulphate ($MgSO_4$ $7H_2O$), sodium nitrate ($NaNO_3$), yeast extract, peptone, hydrogen peroxide solution (30 %, w/v), ammonium sulphate, potassium chloride (KCl), sodium hydroxide (NaOH), ethanol, n-hexane. Viscozyme L cocktail was purchased from Novo Nordisk Corporation as enzymatic concentrate and kept at 4° C. This preparation was used as standard for comparison.

2.2 Microorganism

The fungal strain *Aspergillus niger* 10 was provided from AUMC (Assiut University Mycological Center), Assiut, Egypt.

2.3 Sugar beet pulp (SBP) waste

Dried sugar beet pulp (SBP) waste (containing 14.6% moisture) was obtained from Delta Sugar Company, Kafr El-Sheikh, Egypt and chosen for its suitability as lignocellulosic waste[8] to be utilized for microbial multienzyme system production in the present study.

2.4 Media

The following media were used and composed of (g/L)

2.4.1 Maintenance and sub-culturing medium (medium 1)

Potato dextrose agar (PDA) medium, 39. It was purchased from Merck Corporation, Germany[9].

2.4.2 Fungal inoculum preparation medium (medium 2)

Glucose, 16; peptone 1.25; yeast extract, 0.25; $MgSO_4$, $7H_2O$, 0.125 and K_2HPO_4, 0.25.

2.4.3 Multienzymes cocktail production medium (medium 3)

This medium was investigated for the production of the fungal multienzyme cocktail preparation. This was furnished according to **Ismail,**[10] but orange peels waste was replaced by SBP. SBP, 72; K_2HPO_4, 0.5; $MgSO_4$. $7H_2O$, 0.5; $NaNO_3$, 2.5.

2.5 Subculturing and maintenance of fungal stock culture

The original stock culture was subcultured on PDA slopes, which were then incubated at 30°C for 5 days and stored in a refrigerator at 4°C[10].

2.6 Preparation of fungal inocula and cultivation

Aspergillus niger 10 spore suspension was prepared in PDA slopes, which transferred to 50 ml sterilized inoculum medium (No.2) and incubated in a thermostatic incubating shaker (100 rpm) for 4 days at 30°C[10]. For cultivation, five ml of growth pellets inoculum were used for inoculating 250 ml Erlenmeyer flask containing 50 ml of sterile enzyme production medium. After incubation in a thermostatic incubating shaker (150 rpm) for 4 days at 30°C, the culture was filtered off to separate the mycelium, using a refrigerated centrifuge at 3980g for 15 minutes. The clear supernatant represents the crude multienzyme system preparation[10].

2.7 Batch production of the crude multienzymes cocktail by *Aspergillus niger* 10

After inoculation, the incubation lasted for 4 days in thermostatic shaker (150 rpm) at 29-30°C. At the end of fermentation process, the culture was collected; mixed, filtered, centrifuged then the clear filtrate was frozen and lyophilized. Repeated batches of the lyophilized filtrates were collected, mixed and homogenized to represent the crude multienzyme systems preparation[11].

2.8 Preparation of 90% bulk partially purified enzymatic cocktail fraction with ethanol

The centrifuged enzyme solution was kept in an ice-salt bath. This was followed by adding ethanol slowly while stirring until the required concentration of ethanol (90%), was obtained. The solution was centrifuged at 3980xg for 11 min. in a cooling centrifuge. The precipitated fraction was collected and applied for oil extraction[11].

2.9 Application of the crude multienzymes cocktail and the bulk partially purified enzyme cocktail fraction in jatropha oil extraction

In this series of experiments the crude cocktail enzyme as well as its 90% bulk ethanol fraction was applied separately on the crushed jatropha seeds, then the oil extraction was proceeded in the hydrolytic press HP63. Many different physical, chemical or physico-chemical pretreatments of the crushed jatropha seeds were separately carried out before enzyme treatments, followed by cold pressing of the enzyme - treated seeds. The yield oil was determined in gram/kg seeds.
The different processes carried out could be summarized as follows:
It is noteworthy that the cold press of seeds without any treatment represents control, while solvent (n-hexane) extraction represents the industrial control experiment.

1. Cold press of the crushed seeds as a control (without any treatment).
2. Solvent extraction of the crushed seeds by n-hexane as an industrial control.
3. Freezing and thawing of the crushed seeds followed by cold press.
4. Soaking of the crushed seeds in H_2O (1:2, w/v) overnight followed by cold press.
5. Crushed seeds soaking in 30% (w/v) H_2O_2 (1:2, w/v) overnight followed by cold press.
6. Crushed seeds boiling in distilled H_2O (1:2, w/v) for 10, 15 min followed by cold press.
7. Crushed seeds soaking in acetate buffer (0.02 and 0.2M, 1:2, w/v) overnight followed by cold press.
8. Crushed seeds soaking in 0.1M- NaOH (1:2, 1:4, w/v) overnight.
9. Crushed seeds soaking in 0.1M-NaOH (1:4, w/v) overnight then soaking in crude cocktail enzyme solution (1:2, w/v) overnight followed by cold press.
10. Crushed seeds soaking in crude cocktail enzyme solution overnight (1:2, w/v) followed by cold press.
11. Crushed seeds boiling in distilled H_2O (1:2, w/v) for 10 min then soaking in crude cocktail enzyme solution (1:2, w/v) overnight followed by cold press.
12. Crushed seeds boiling in distilled H_2O (1:2, w/v) for 10 min then soaking in the partially purified cocktail fraction solution (1:2, w/v)) overnight followed by cold press.

3. RESULTS AND DISCUSSION

The large scale application of Multienzymes cocktail for different oils extraction represents an excellent replacement for the carcinogenic n hexane solvent. In addition, the high potential biotechnological applications of those Multienzymes cocktail in many fields, which include their uses as very safe extracting agents of oils and starches from plant materials instead of the carcinogenic solvents and chemicals. In previous work[11], also the fungal isolate *Aspergillus niger* 10 among nine local strains was the most potent and was able to produce an effective enzyme cocktail possessing highly active pectinases,cellulases and hemicellulase in shaken culture containing either sugar beet pulp (SBP) or jatropha seed cake (JSC) waste after 4 days incubation at 40° C. An enough quantity of the SBP enzymatic cocktail was prepared, lyophilized and kept at 4° C. The plant seeds are characterized by highly ordered crystalline cellulose fibers, low accessible area, protection of cellulose by lignin and the heterogeneous character of biomass particles and cellulose sheathing by hemicellulose and pectin, which contribute to the recalcitrance of agriculture biomass materials to enzymatic hydrolysis. Accordingly, the pretreatment of the biomass material before enzymatic hydrolysis is required to alter the structure of cellulosic fibers and make it more accessible to the enzymes that degrade the carbohydrate polymers[12-15]. In the present study, an effective and simple pretreatment, which causes disruption of the aforementioned barriers so that the hydrolytic enzymes can penetrate and cause hydrolysis to seed fibers is required. The different methods known for pretreatment of cellulosic materials to facilitate the enzymatic oil extraction include: physical (ball-milling, hammer-milling, weathering, boiling, high pressure steam, electron irradiation, photooxidation, wetting gamma irradiation), chemical (NaOH, NH_4OH, HCl, CH_3COOH, H_2SO_4, Na_2SO_4, H_2O_2, NaOCl, SO_2 gas), biological (use of cellulase & laccase microorganisms) and miscellaneous as hot ball-milling, NaOH-ball-milling, NO_2-irradiation[16], so before extraction of biodiesel from the jatropha seeds, some of the foregoing pretreatments either chemically, physically or physicochemically were applied on the crushed seeds before enzymatic degradation by each of the two enzyme forms, i.e. the crude enzyme cocktail and its partial purified cocktail fraction to study their influence on oil extraction improvement. Two control experiments were performed for comparison; the first is oil extraction by cold press only and the second is the extraction with n-hexane (industrial control). Six pretreatments on the crushed jatropha seeds were proceeded as shown in Table 1, these included: freezing of crushed seeds followed by thawing, soaking in dist. water overnight at room temperature, soaking in 30% (w/v) H_2O_2 overnight at room temperature, boiling in water for 10 & 15 min, soaking in

acetate buffer (0.02-0.2M), pH 5.0 overnight at room temperature, soaking in 0.1M-NaOH (1:2 & 1:4, w/v) overnight. All chemical-pretreated crushed seeds were well washed before treatment with the buffered enzyme preparation also the heat-treated seeds were left to cool then submitted to the cold press step.

The data asserted that the freezing of seeds followed by thawing in room temperature to rupture the seed cell walls had no effect on the oil extraction after cold press. Nevertheless, more than 23% oil yield increase was brought about by any of the following pretreatments at room temperature overnight before cold press: soaking in dist. water, soaking in 30% (w/v) H_2O_2, soaking in acetate buffer (0.02 & 0.2M, pH 5.0) or soaking in 0.1M-NaOH (1:2, w/v). It is worthy to note that acetate buffer concentration had no effect on oil extraction yield. The oil yield increase (2.1-fold) achieved after boiling in dist. H_2O for short period (10 & 15min) and more boiling time extension led to no further oil extraction. On the other hand, the solid: liquid ratio, applying 0.1M-NaOH in seed soaking at room temperature overnight had a distinct effect on seed oil extraction, where the S:L ratio 1:4 (w:v) led to oil yield exceeded than the double of yield with 1:2 ratio and amounted to 102 g/kg seeds compared with 42g/kg of the control.

Although the good efficacy of 0.1M-NaOH (1:4, S:L) pretreatment in oil extraction yield, the addition of crude SBP enzyme cocktail, applying all the specified conditions after this pretreatment had insignificant effect on oil extraction and the oil yield not exceeded than 3% that without the enzyme and this was coincided with the earlier properties shown in Table 1. This may be due to the sensitivity of the crude enzyme to the alkalinity as earlier discussed on the crude enzyme cocktail properties in previous studies[11]. The dramatic increase in the oil extraction was accomplished with seeds soaking in the crude SBP enzyme cocktail solution (4 mg /ml) with the S:L ratio of 1:1 overnight at room temperature achieving 184g/kg seeds, which exceeds 4.38-fold that of the control. Another excellent yields were attained either by soaking of the physically pretreated jatropha seeds (boiling in dist. water for 10min) in the crude enzyme cocktail (4 mg/ml) with S:L ratio of 1:1 overnight at room temperature or in the partially purified enzyme cocktail (1 mg/ml) with S:L ratio of 1:1, where each of the foregoing treatments afforded equal oil yields, which approach the industrial control yield with n-hexane. The crude enzyme cocktail of *A. nigher* 10 and its partially purified fraction is superior to the other enzyme cocktail preparations applied in oil extraction, where Winkler[17] could maximally extract 86% of *Jatropha curcas* oil applying a mixture of enzyme cocktails containing Alcalase, Neutrase and Viscozyme from Novo-Nordisk (Denmark), also, Beatriz[18] applied Viscozyme L in enzymatic aqueous technology for coconut protein and oil extraction with maximum yield of 83%. In addition, Kapchie[19] applying enzyme – assisted aqueous extraction of oleosomes from soybeans (Glycine max), through three successive extractions by a mixture of three cocktail preparations, namely Multifect pectinase, Cellulase A and Multifect CX from Rochester, NY could extract 84.7% of the original soybeans oil.

Table 1: Large scale application* of the crude SBP cocktail enzyme and 90% bulk ethanol fraction on the pretreated jatropha seeds oil extraction

Jatropha seeds pretreatment before oil cold press		Oil yield (g/kg)**
None (control)		42
Solvent extraction (Industrial control)		200
Freezing overnight followed by thawing		42
Soaking in H_2O (overnight at room temperature)		52
Soaking in 30%, w/v H_2O_2 (overnight at room temperature)		52
Boiling in dist. H_2O	(10 min)	86
	(15 min)	86
Soaking in acetate buffer ,pH 5.0 (overnight at room temperature) then cold press	(0.02M)	52
	(0.2M)	52

Soaking in 0.1M NaOH (overnight at room temperature)	(1:2, w/v)	52
	(1:4, w/v)	102
Overnight soaking in 0.1M-NaOH (1:4, w/v) followed by addition of the crude SBP cocktail enzyme (at room temperature)		105
Overnight soaking in the crude SBP cocktail enzyme (at room temperature)		184
Boiling for 10 min in dist. H_2O, cooling followed by soaking in crude cocktail enzyme overnight (at room temperature)		186.4
Boiling for 10 min in dist. H_2O, cooling followed by soaking in 90% bulk ethanol fraction (at room temperature)		186.4

*** Before any enzyme addition the treated seeds were well washed with dist. H_2O**
**** In all experiments, jatropha seeds oil was extracted from one kg seeds.**

CONCLUSION

Application of either the crude or the partially purified enzyme cocktail had an excellent efficacy in jatropha seeds oil extraction, which amounted to 93 & 91.13% that of n-hexane oil extraction and the original jatropha seed oil, respectively. It is worthy to mention that the typical oil yield by any of the two enzyme cocktail forms (crude & partially) required four-times of the crude cocktail protein as that of the partially purified, pointing out the higher efficacy of the latter than the crude form, which amounted to 400% under the same specified conditions at the large- scale application experiment. Moreover, the crude enzyme cocktail of *A. nigher* 10 and its partially purified fraction proved to be superior to the other familiar enzyme cocktail preparations applied in oil extraction.

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