

Utilization of *Opuntia ficus indica* waste for production of *Phanerochaete chrysosporium* bioprotein

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Abstract: The highest *Opuntia ficus indica* waste saccharification was 75.6 % obtained with 1% (w/v) NaOH treatment. *Phanerochaete chrysosporium* was the most potential fungus among the tested microorganisms : *A. terreus* and *R. oryzae* for bioprotein recovery of 0.073/100 g waste. *Opuntia ficus indica* peels proved to be a suitable substrate among the other agricultural wastes, corn cob shred , and sugar cane bagasse which were used as carbon sources for *Phanerochaete chrysosporium* bioprotein production. Also ,The most optimum fermentation conditions were : 10(g /L)*Opuntia* waste as carbon source ; phosphate buffer for bioprotein extraction ; 3% (v/v) inoculum size; supplementation of Modified Czapek Dox medium (MCD) with 0.3 % (w/v) CSL; the initial PH , 4 at 150 rpm., and 75 ml (MCD)medium was suitable volume resulted in 0.123 /100 g bioprotein after 7 days of fermentation at 30°C. [Journal of American Science 2010;6(8):208-216]. (ISSN: 1545-1003).

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Introduction

Opuntia ficus indica is one of the most promissory by presenting the largest part of alimentation furnished to the animals during the drought. This increases the availability of fodder and alleviates the problems of the water supplement to these animals. It is also rich in sugars,minerals, calcium, iron and vitamin A. It presents an elevated texture of soluble carbohydrates besides presenting a high coefficient of digestibility of the dry matter and high productivity Lúcia de Fátima *et al.*, (2005). Besides, improper handling of solid waste is a health hazard and causes damage to the environment. The main risks to human health arise from the breeding of disease vectors, like flies and rats. Healthy life and cleaner environment is the end result of solving these problems in such a way by utilizing the waste into valuable by-products ; Nigam *et al.*, (2009). Mycelium biomass from *Rhizopus oryzae*, can partly substitute high-quality fishmeal in diets to rainbow trout without causing any major short-term adverse effects on growth, nitrogen and amino acids digestibility. Nutrient digestibility of diets containing mycelium biomass of *R. oryzae* are in carnivorous fish and larvae ; Uysal *et al.* ,(2002) and Olsen *et al.*,(2006)). For humans, it is also considered as food additive to improve flavor, fat binding and more recently as a replacement for animal protein in the diet(Jamal *et al.*, 2007). Bioprotein can also be used as additives in certain chemical and pharmaceutical industries. Bioprotein is the protein extracted from cultivated microbial biomass that can be produced using a number of different microorganisms and such

as low carbon cost, high energy sources agricultural wastes .Agro- wastes can be regarded as new sources for bioprotein production, which have a high nutritional value, do not compete with food for human consumption, economically feasible and locally available ;(Anupama and Ravindra, 2000, Uysal *et al.*, 2002).

Lignocellulosic waste is a complex mixture of cellulose, hemicellulose, lignin along with extractives,wastes pretreatment is therefore a necessary process in order to achieve high yield. Grinding and milling are the primary physical accomplished by using acids or bases . The objectives of this study were beneficial in production of nutritional bioprotein from a cheaper carbon source.

Materials and method

Microorganisms:

Three different fungi were used ; culture of *Phanerochaete chrysosporium* ATCC 28236 obtained from Cairo MERCIN , Ein Shams University; culture of *Rhizopus oryzae* obtained from Dr.M. U. Noaman, Biochemistry Dept. and *Aspergillus terreus* obtained from Dr.A.F. Salah, Plant Pathology Dept. NRC.,Egypt.

Maintenance medium:

All strains were maintained by subculturing on PDA slants for 7 days then stored at 4°C .

Inoculum preparation:

Spores were harvested from a week old slants in five ml of sterile distilled water. 0.5 ml of spore suspension was added to fifty ml broth with pretreated substrate based medium in 250 ml conical flasks. The inoculated flasks were incubated at rotary shaker 100 rpm for 30°C for 7 days. (Anupama and Ravindra, 2000).

Preparation of lignocellulosic substrates

-Fresh *Opuntia* peels were collected from local markets during summer season stored in a refrigerator until use. The fresh *Opuntia* skins were washed and cut into smaller pieces (1-2 cm) dried, grind to a very small pieces, before use; Olivera *et al.*, (2001).

-Corn cob shred: Obtained from Sugar and Starch plant, Mustard, Egypt, washed, dried in an oven at 60-100°C to a constant weight. The dried substrate was ground, sieved, before use.

-The sugar cane bagasse: The sugarcane dust was obtained from a market. The sugarcane dust was washed, dried at a temperature of 60 °C for 96 h, was grind and sieved before use.

lignocellulosic waste acid treatment:

Five grams of fine carbon sources; corn cob, *Opuntia* and bagasse were suspended in 50ml of 1%(v/v) HCL for 1 hr in a boiling water bath, reducing sugars were estimated. The treated materials were washed until the wash was neutral; (Gupta *et al.*, (1972).

lignocellulosic waste alkali treatment:

25 g of different substrates were suspended in 500 of 1 % NaOH solution in 1 liter conical flask boiling for 1 h in a water bath; Choudhury *et al.* (1980). reducing sugars were estimated. The treated material was washed until the supernatant was alkali free as checked by pH meter

Fermentation medium:

0.25 g of an accurately weighed treated lignocellulosic waste (corn cob, sugar cane bagasse and *Opuntia*) were placed in a 250 ml conical flask containing fifty mls of modified Czapek Dox medium (MCD medium) in order to obtain the biomass. MCD medium contained the following ingredients (g/100 ml): K₂HPO₄, 0.12; MgSO₄, 0.06; FeSO₄, 0.05; KCl, 0.02; NaNO₃, 0.3 and sucrose, 3; Anupama and Ravindra (2001), adjusted to pH 4, autoclaved at 121°C for 15 min. Inoculated with 1 %(v/v) from 7 days old slant, then incubated on a rotary shaker at 100 rpm and 30°C for seven days fermentation.

Culture Harvesting and biomass concentration :

The cultures were harvested by filtering the biomass through a weighed Whatman no. 1 filter paper, the fungal biomass washed with distilled water to remove medium adhered to the mycelium. The filter papers along with the biomass were dried at 60°C for 48 h to constant weight, APHA (1989), left in desiccators (Cardoso, 1981), ground in a mortar to a very fine powder and kept desiccated.

The biomass means the mycelium together with unfermented substrate residue and then analyzed for their protein content.

Chemical analysis:

The amount of total sugar in the homogenized *Opuntia* samples was determined. For estimation of **total sugars**, one g of the dried substrate was suspended in 60 ml of distilled water. This was kept at an ambient temperature for 12 h for the extraction of sugars that are measured by the phenol-sulfuric acid method with glucose as a standard (Dupois *et al.*, 1965). The moisture content of the sample was determined according to methods A.O.A.C. (1980) and total protein by Lowry *et al.* (1951).

Extraction buffer of microbial protein:

0.25 g of fermented moldy biomass was soaked with 50 mL of **phosphate buffer**, pH (6.9) and stirred for 30 min. The extract was collected by filtration. The temperature during the course of extraction was maintained at 4°C. The supernatant obtained was used for estimating protein content. The extraction process was repeated five times, all the extractants were transferred to a flask, and the final volume was made up to 100 mL with distilled water; Oliver *et al.*, (2001).

Total protein : Protein determination was measured according to Lowry *et al.* (1951) method using folin-phenol reagent and bovine serum albumin standard. All spectrophotometer readings were recorded at 660 after 20 minutes.

Determination of total reducing sugar:

Reducing sugars were determined according to Nelson (1944).

Culture conditions

Effect of carbon source

Different carbon sources were used; *Opuntia* waste, bagasses and corn cob in concentration of 0.5% (w/v).

Substrate concentration

Varying concentration of alkali treated *Opuntia ficus indica* substrate 0.5-1.5 % (w/v) were investigated.

Effect of initial pH: (MCD) medium was adjusted to different pH values before autoclaving.

Effect of agitation speed:

The fermentation medium were shaken at different speed ranging from 50 – 200 rpm at 30°C.

Effect of nitrogen supplementation:

0.3 % (w/v) ammonium sulphate ,peptone, yeast extract and CSL were supplemented instead of sodium nitrate in the(MCD) broth.

CSL(corn steep liquor) obtained from Sugar and Starch company, Mustrud, Egypt .

Effect of aeration:

To study the effect of air /medium ratio on bioprotein production ,different volumes of MCD cultivation medium in 250 ml Erlenmyer flask were used.

Calculations:

-(%)_saccharification =Amount of reducing sugars(mg/ml)/Amount of substrate(mg/ml) x 0.9 x100; Dey *et al.*, (1992).

Protein recovery(g/l) = protein content(%) x biomass (g/l);Ibrahim(1998)-

Protein recovery (g/100g) = protein recovery (g/l) / substrate used (g) / L.(Ebrahim,1998)

Results and Discussion

Lignocellulosic waste Treatment with 1% NaOH :

On using NaOH pretreated,corn cob, sugar cane bagasse and *Opuntia* wastes saccharification reached 75.6

% from *Opuntia* skin waste as a potential, low cost and high content of carbohydrate make it more suitable compared to other wastes ; 63.9 and 56.16 % for sugar cane bagasse and corn cob waste ,respectively . Corn cob is a very hard lignocellulosic waste of corn industry that must be delignify as it cannot be utilized efficiently by microbes without pretreatment for single cell protein with *P. chrysosporium*;Asad *et al.*,(2000) .

Table(1). % Saccharification of carbon sources after treatment with 1% Na OH .

Agro-waste	Total reducing sugar concn(mg/ml)	(%) Saccharification
<i>Opuntia ficus indica</i>	4.2	75.6
Sugar cane bagasse	3.55	63.9
Corn cob	3.12	56.16

Lignocellulosic waste treatment with 1% HCL:

As it could be seen from Table 2. 64.8 % saccharification could be obtained with *Opuntia* skin with HCL treatment compared to 45.9 % and 44.1 % for sugar cane bagasse and corn cob wastes .Such low cost agro-cultural lignocellulosic wastes must be treated by physical/chemical methods to liberate cellulose from lignins. Since cellulose in lignin-hemicellulose-cellulose complex is not accessible to enzymatic hydrolysis ;Asad *et al.* (2000. Table (1,2) showed that alkali treatment is more effective in preparing *Opuntia* skin for fermentation, so, this pretreatment will be applied in the next experiments. Many pre-treatment methods have been reported which vary from alkali or acid treatment, steam explosion or even x-ray radiation; Rodriguez-Vazquez *et al.*(1992). Table(2). % Saccharification of carbon sources after treatment with 1% HCL.

Agro-waste	Total reducing sugar concn(mg/ml)	(%) Saccharification
<i>Opuntia ficus indica</i>	3.60	64.8
Sugar cane bagasse	2.55	45.90
Corn cob	2.45	44.1

Evaluation of potential microorganism:

As could be indicated from table 3, the total protein % production by each strain differ from the other. As many fungal species are used as a protein-rich food. They provide the B-complex group of vitamins and they also show a low level of nucleic acid content. Biomass obtained from *P. chrysosporium* has been found to contain most of the essential amino acids (Balagopalan and Padmaja, 2000). However, as stated by Jamal (2007),the fermentation time for maximum production of bioprotein was different for every microorganism, e.g. *A. niger* and *P. chrysosporium* showed highest concentration on sixth day.Also, As it could be seen from table 3, *P. chrysosporium* is of importance due to the highest bioprotein production 6.90 /100g substrate together with the highest biomass 5.95 g/l compared to the other microorganisms. . Other strains gives 3.374, 1.016 /100g *Opuntia* for *A.terreus* and *R.oryzae*, respectively. The protein from microorganisms is cheap and competitive with other protein sources. It may have good nutritive value depending, however upon their amino acid composition. It is necessary to use microorganisms which is non toxic to the animal. Most organisms used in direct single cell protein are fungi as *Aspergillus* , *Fusarium* and *Trichoderma*

Table (3). Screening on microbial bioprotein production using *Opuntia ficus indica* peels waste.

Microorganism	Biomass (g/l)	Total protein (%)	Total protein recovery (g/l)	Protein recovery (g/100g substrate)
<i>P. chrysosporium</i>	5.95	5.8	0.345	0.069
<i>A. Terreus</i>	4.82	3.5	0.169	0.034
<i>R.oryzae</i>	4.62	2.2	0.101	0.20

Initial substrate concentration is 5 g/l broth.

Production of *P. chrysosporium* bioprotein using different carbon sources :

The proximate composition of *Opuntia* waste used was as follows : 0.05% total protein, moisture 85%, and 55.25% total carbohydrates of its weight.

The term single cell protein (SCP) refers to dead, dry cells of micro-organisms such as yeast, bacteria, fungi and algae which grow on different carbon sources. As it could be seen from table 4, *P.chrysosporium* protein recovery reached 0.073 /100g. Hence, *Opuntia* waste could be used as one of less expensive means of increasing the protein quality such as cassava and wheat flour ;Ghaly *et al.*, (2004) than sugar cane bagasse and corn cob which produce 0.051 and 0.046/100 g substrate ,respectively . Other potential substrates for SCP include citrus wastes, sulphite waste liquor, sewage ,molasses, animal manure, whey, starch and wheat flour, Jamal *et al.*, (2008).

Table (4). *P.chrysosporium* bioprotein on using different carbon sources .

Waste	Biomass (g/l)	Total protein (%)	Protein recovery (g/l)	Protein recovery (g/100g substrate)
<i>Opuntia</i>	5.6	6.5	0.364	0.073
Sugar cane bagasse	4.59	5.6	0.257	0.051
Corn cob shred	4.61	5.1	0.234	0.046

Initial substrate concentration is 5 g/l broth.

Effect of carbon source concentration on *P.chrysosporium* bioprotein production:

The biomass values on using different concentrations of *Opuntia* skin is shown in table (5) . It could be stated that reduced substrate, result in greater protein yield as more oxygen required for the oxidation of the substrate. 1%(w/v) substrate concentration result in 0.086 /100 g *Opuntia* waste so, it will be used in the next experiments . 2%(w/v) substrate result in 0.049/100g waste, this may be due to exhaustion of nutrients required in fermentation medium other than the *Opuntia* waste.

One of the main advantages of SCP compared to other types of protein is the small doubling time of cells as in yeast. Due to this property, the productivity of protein from micro-organisms is greater than that of traditional proteins. For the assessment of the nutritional value of SCP, factors such as nutrient composition, amino acid profile, vitamin and nucleic acid content as well as palatability must be evaluated.

Table (5). Effect of varying concentrations of *Opuntia ficus indica* skin on *P. chrysosporium* protein production.

* (substrate concentration % (w/v))	Biomass (g/l)	Total protein (%)	Protein recovery (g/l).	Protein recovery (g/100g substrate).
0.5	6.6	5.5	0.363	0.073
1.0	8.15	10.5	0.856	0.086
1.5	14.4	5.6	0.806	0.054
2.0	19.1	5.1	0.974	0.049

Initial substrate concentration is 5,10,15,20 g/l broth.

Effect of *P. chrysosporium* inoculum size on bioprotein production :

As it could be seen from fig 1, the total protein content of the biomass was only 8.02 % with 1% (v/v) *P. chrysosporium* inoculum when *Opuntia* based medium incubated for 7 days at 30°C on 100

rpm. The total protein was found to be increased to 9.139 % with elevation of the inoculum size to 3 (v/v)%. However, no increase in total protein was noticed with 4 (v/v) %, as it gave 4.5% total protein. So, 3% (v/v) inoculum will be used in the next experiments.

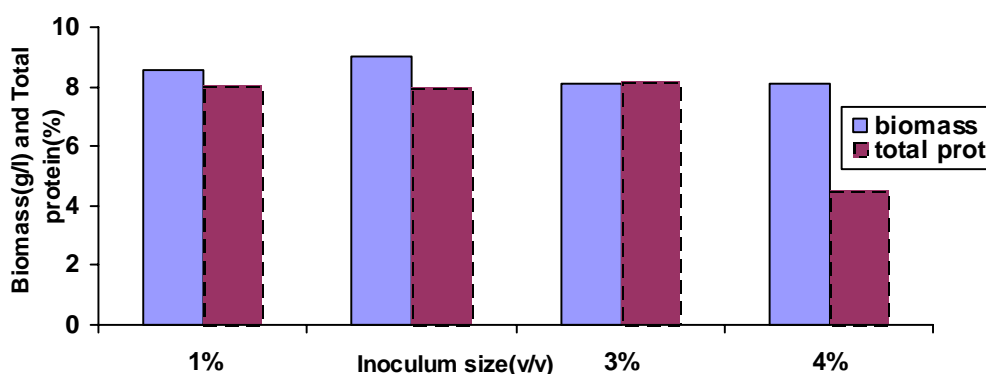


Fig1. Effect of inoculum size on *P. chrysosporium* bioprotein production.

Effect of (MCD) nitrogen source on *P. chrysosporium* bio protein production:

As it could be seen from Table (6), CSL resulted in 0.096 /100g *Opuntia cactus*. This was essential in supporting and enhancing the growth of *P. chrysosporium*, as it gave 8.10 g/l biomass as well as to promote the production of bioprotein, this comes in agreement with Chahal *et al.* (1987) who produced maximum biomass protei

n (40%) from CSL as additional nitrogen source is required to support both microbial and biomass production. These results are also in accordance with those by (Nacib *et al.*, 2001); who stated that the production of lactic acid by using date juice as fermentation medium could be increased after supplementing it with nitrogen sources, and also Rosma and Cheong (2007) who stated that addition of inorganic nitrogen source as the main components in the growth of biomass and building block of proteins is due to the lack of nitrogen sources in pineapple juice. The effect of inorganic

nitrogen source was studied by Chung and Muhammad (2000) to reach the highest yield of cellulose. The importance of using carbon and nitrogen source were reported to be essential in providing a suitable growth media for *P. chrysosporium* which was used in the molasses wastewater (MWW) (Ahmadi *et al.*, 2006), while there are also many reports on use of C and/or N sources to support of *P. chrysosporium* growth (Kirk *et al.*, 1978; Jansheker and Fiechter, 1983). Urea, and peptone when supplemented to the medium along with *Opuntia* peels waste gave higher protein yield compared to medium supplemented by $(\text{NH}_4)_2\text{SO}_4$ as they gave, 0.082, 0.066 and 0.061/100 *Opuntia* substrate, respectively. So, CSL will be used in the next experiments. CSL is an unexpensive source of nutrient in the fermentation medium, also used as the sole source of nitrogen, vitamin, growth stimulant and other nutritional requirement; Amarty and Jeffries (1994).

Table (6). Effect of nitrogen source for the production of *P. chrysosporium* bio protein using *Opuntia ficus indica* based medium.

Nitrogen source in MCD)	Biomass(g/l)	Total protein(%)	Total protein recovery (g/l)	Recovery protein (g/100g substrate)
CSL	8.10	11.9	0.964	0.096
Urea	7.12	11.6	0.825	0.082
Peptone	7.76	8.5	0.659	0.066
$(\text{NH}_4)_2\text{SO}_4$	7.82	7.8	0.611	0.061

Initial substrate concentration is 10 g/l broth.

Initial pH effect on *P. chrysosporium* bioprotein production.

On optimizing medium conditions for attaining higher production of bioprotein, as it could be seen from Table (8) pH 4 result in 0.092 g /100 g waste recovery protein, while higher pH values

reduce the bioprotein, this findings are in agreement with Jamal *et al.*, (2009) who stated that any increase in pH medium becomes inhibitory for the organism and induction of enzyme synthesis.

Table(7) . Effect of initial pH on bio protein production by *P. chrysosporium* grown on *Opuntia ficus indica* based medium .

Initial PH	Biomass (g/l);BM	Total protein(%)	Protein recovery g/l)(Recovery protein(g/100g substrate)
2	7.4	3.3	0.244	0.024
4	8.12	11.4	0.925	0.092
6	7.12	1.6	0.113	0.011
8	7.1	1.1	0.078	0.008

Initial substrate concentration is 10 g/ l broth.

Effect of agitation on *P. chrysosporium* bioprotein production using *Opuntia ficus indica* based medium.

As could be seen from fig (2), with 200 rpm, lower protein content 6.7 % protein on 7 th day of fermentation at 30°, this is in agreement with

Daugulis, (2004) who stated that, higher agitation rates results in reduced levels of microbial protein. While, with 150 rpm speed resulted in 10.85 % protein whereas at 200 rpm the biomass still growing 9.11 g/l. So, 150 rpm will be used in the next experiment.

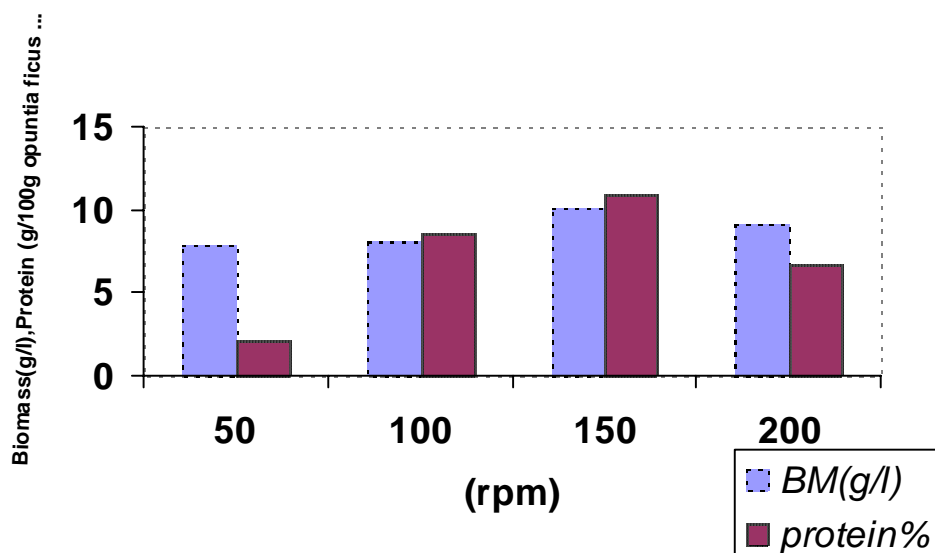


Fig.2. The effect of agitation on Phanerochaete chrysosporium bioprotein production.

Effect of aeration on *P. chrysosporium* bioprotein production using *Opuntia ficus indica* based medium :

As it could be seen from Table(8), with 50 ml (MCD) medium the biomass was 8.9 g/l while

protein was 0.105 g/100 g *Opuntia* substrate. With 75 ml broth volume /250 ml flask capacity at pH 4, when inoculated with 3% spore suspension and incubated for 7 days at 30°C on 150 rpm, resulted in 0.123g *P. chrysosporium* bioprotein /100 g *Opuntia*

waste, with 9.5 g biomass /l concentration This represents the highest biomass concentration because it reached maximum growth yet and were still in growth phase. The biomass should increase exponentially as the cell is growing and when only the cells enter the decline phase or death phase, biomass will decrease. With 125 ml culture volume , the reduction in protein content 0.032 g/100g

Opuntia waste because of the decreased aeration which may reduce the growth of the organism. It should be mentioned although there is an increase in the bioprotein on applying the optimal cultivation conditions, yet the amount is still small compared to the amount of protein initially present , this may be due to the protein extraction method, the buffer used or even the cultivation season of *Opuntia*.

Table(8). Effect of aeration on *P. chrysosporium* bio protein production by using *Opuntia ficus indica* based medium .

Culture volume(ml)	Biomass (g/l)	Total protein(%)	Total protein recovery (g/l)	recovery protein(g/100g substrate)
50	8.9	11.85	1.054	0.105
75	9.5	12.98	1.233	0.123
100	8.5	4.12	0.350	0.035
125	7.8	4.12	0.321	0.032

Initial substrate concentration is 10 g/l broth.

Hatem *et al.*, (2001) stated that the extract obtained from nopal peel at neutral pH of carbohydrate polymers from *Opuntia ficus-indica* and their physicochemical characterization indicated that they are polysaccharides .Their sugar composition indicates that all the polysaccharides obtained contain anionic moieties, galacturonic acid residues and are typical of pectin. It could be deduced the valuable reuse of this carbohydrate rich solid waste in minimization of costs associated with nutritional supplements in a fermentation medium ,it is also essential for economic large-scale production, which somehow contribute to the pollution problem in the environment, either for the production of useful bioprotein or other beneficial metabolites .

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