REDUCING AND RECYCLING AGRICULTURAL RESIDUES BY USING OLEAGINOUS YEASTS IN ORDER TO PRODUCE HIGH VALUE PRODUCTS

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ABSTRACT

Micro-organisms such as bacteria, yeasts, molds and algae that have the ability to accumulate lipid more than 20% of their biomass are oleaginous. Microbial lipid has many similarities with plant's oil and this similarity is valuable because it can be use as the substrate for biodiesel production and also many high valuable products such as polyunsaturated fatty acids with pharmaceutical application. Production of microbial lipid is valuable when we decrease it's cost by optimization of cultivation condition and using low cost materials as the substrate. In this investigation we converted agricultural residues in to Single Cell Oil (SCO), which is the substrate for biodiesel production. At first an oleaginous yeast with high potential of lipid production and with ability of assimilating xylose was selected. Then culture condition was optimized and Corn stalk and rice bran that are agricultural residues were used as the substrate for lipid production. The maximum lipid yields were 46%, 44% and 39% in xylose, rice bran and corn stalk respectively.

1. INTRODUCTION

Lipid production by oleaginous yeasts accrues in a medium rich in carbon and limited in another substrate, especially nitrogen. This process contains two stages. In the first stage cell grows and increase their number. This step finishes by eliminating some nutrition except carbon. During the next stage the excess amount of carbon store as lipid particles in the cells (2).

The most important parameters which determine the price of microbial oil are substrate cost, production rate and final lipid concentration (12). Using the yeasts among other microorganisms have some advantages such as their high growth rate, accumulating lipid in separate lipid bodies and the most important matter is that they can use low cost fermentation medium such as waste agricultural materials and also some industrial byproducts(3). Also the effect of season and climate on them is less than plants so they are good oil producers (3, 10).

The lipid obtained from oleaginous yeasts has potential of being substitute instead of plant's oil (17, 2). The important lipids that produce by these microorganisms are triacylglycerol which is comparable with conventional plant's oil (7, 13). Some yeasts which are valuable from

economical point of view can metabolize pentoses. It shows the ability of them for using lignocellulosic agricultural residues and other low cost materials (15, 9).

The economic value of this process becomes more favorable when zero or negative waste materials utilize as carbon source (5, 13). With this bioprocess we can solve the problem of lacking energy source and also air pollution caused by fossil fuels. Energy sources such as biodiesel which are fatty acid methyl esters have a lot of advantages. They are renewable, biodegradable and nontoxic (4, 6, 11). The only problem for using this source of energy is the cost of biodiesel. With using microbial oil and cheap raw materials for producing them we can overcome this problem (6).

2. MATERIAL AND METHODS

1- Yeast strain

Yeast strain which we used in this investigation was the isolated strain *Rhodotorulla aurantiaca*

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2- Preparation of inoculums

The oleaginous yeast colonies were first streaked on to YEPD plates and grown for 2 days. After that they were transferred in to 250 ml Erlenmeyer flask containing 50 ml of inoculation medium which contains (g per liter) Glucose 15, NH_4Cl 5, KH_2PO_4 1, $MgSO_4.7H_2O$ 0.5, and yeast extract 0.5 and grown at 28ºC on a shaker at 150 rpm for 48h(13).

3- Preparation of lipid production

5 ml of inoculums was transferred to 45 ml of nitrogen-limited medium containing (g per liter): Glucose 35, NH_4Cl 2, KH_2PO_4 7, NaH_2PO_4 2, $MgSO_4.7H_2O$ 2, yeast extract 1, $CaCl_2$ 0.15, $MnSO_4.H_2O$ 0.06, $ZnSO_4.7H_2O$ 0.02 g/L and $FeCl_3.6H_2O$ 0.15 g/L in 250 ml Erlenmeyer flask and incubated in a rotary shaker at 150 rpm and 28ºC for 72h (8, 13, 14).

4- Qualitative analysis by sudan black staining

Evaluating of lipid production by these yeasts was done by Sudan black B staining. Lipid bodies were seen as black droplets in the cells under light microscope.

5- Lipid extraction

Extraction of lipid was done according to Bligh & Dyer with modification (13). 40 ml of sample was centrifuged at 6000 rpm for 10 min. After that the yeasts were washed with 40ml of distilled water. We repeated this stage and then 8ml of 4M HCl was added and incubated at 70ºC for 2h. Then acid hydrolyzed mass was shaked with 16ml chloroform/ methanol mixture (1:1) at room temperature for 3h. At the end we centrifuged at 5000 rpm for 5 min at room temperature to separate the aqueous upper phase and organic lower phases. Then we recovered the lower phase that contain lipid with Pasteur pipette and evaporated chloroform/ methanol mixture in the vacuum. After that the dry lipid weighed.

6- Determination of yeast dry biomass

Portions of 5ml cultures were harvested by centrifugation at 6000 rpm for 20 min. harvested biomass was washed twice with 5ml of distilled water and then dried at 80ºC to constant mass. The biomass was determined gravimetrically (8).

7- Analysis by Thin Layer Chromatography

We use silica gel plates with lipid standards such as triolein as reference substance for Triacylglycerol. The solvent was n-hexane-diethyl ether-acetic acid (90:10:2). The bands were observed after staining the TLC plate by Iodine vapor. So the qualitative and semi quantitative analysis of intracellular lipid was carried out by thin-layer chromatography (1).

8- Lipid production using Xylose as carbon source

Glucose was substituted by xylose as carbon source. Other substrates did not change.

9- Medium optimization by one factorial method

Effects of nutrient composition such as xylose and ammonium Sulfate concentrations, KH_2PO_4, $MgSO_4$ and pH were investigated.

Xylose concentration was varied at 35, 55, 75, 95, 115g/L. Effect of combined organic Nitrogen source (yeast extract and peptone at 1 g/L) and inorganic compounds (sulfate ammonium and chloride ammonium at 1g/L) on lipid production were investigated. KH_2PO_4 varied from 1 to 3, 5 and 7 g/L, MgSO4 from 0.5 to 1, 1.5, 2 , 2.5 g/L and pH from 4 to 5, 6 and 7(15).

10- Lipid production using rice bran and corn stalk as Carbon source

Before using rice bran and corn stalk we must prepare them by acid hydrolysis. For this purpose the materials were ground, then hydrolysis by using sulfuric acid 5% at solid: Liquid ratio of 1:8 and then autoclaved at 110°C for 20 minutes. Then we centrifuged the suspension to remove unhydrolyzed residues (4).

11- Transesterification of the extracted oil in to biodiesel

Transesterification was done by using sulfuric acid as catalyst in flask containing 30:1 molar ratio of methanol to extracted oil, 170 rpm, 5-5.5 h as reacting time, after that two layer were formed, the upper layer which contain biodiesel separate with petroleum ether(4).

3. RESULTS AND DISCUSSION

1- Qualification analysis with sudan black staining and TLC

Figure 1 shows *Rhodotorulla aurantiaca* cells under light microscope after sudan black B staining, lipid bodies are seen as black particles in the cells. This figure also shows TLC plate in which we can see the bands toward the standard.

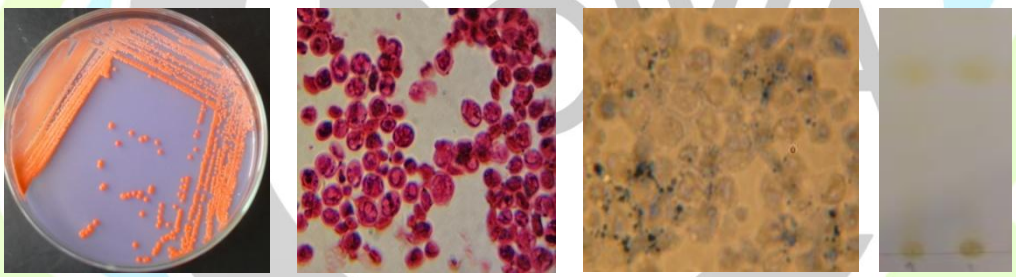

Figure1. The first picture shows photo of *Rhodotorulla aurantiaca* colonies, the next two pictures show sudan black B staining under light microscope, the last one is TLC picture.

2- Optimization of lipid production

Lipid accumulation accrues in the medium with excess amount of carbon and limited nitrogen condition. Table 1 shows the results of lipid yield on glucose and xylose as carbon sources. Absolutely lipid yield on glucose was better but because lipid production on xylose is important for us and has relation with lipid production on agricultural residues, xylose was selected for the next steps.

Table 2 shows the result of lipid production different cultivation conditions. Results from the current investigation showed that using yeast extract with ammonium salt caused higher lipid production. Li et al (2007) and Yong- Hong (2006) reported that ammonium salt is a good nitrogen source for lipid production (9, 18).

Lipid accumulation on glucose as carbon source was better but xylose assimilating by this strain is important because it has a straight relation with ability of lipid production in lignocellulosic materials. Maximum lipid yield was at 75g/L of Xylose and 1 g/L ammonium sulfate. Phosphate limitation beside nitrogen limitation can help lipid accumulation in yeast cells so according to the results 1g/l of KH_2PO_4 was chosen for the next step, this is in agreement with results obtained by kraisintu (2010). $MgSO_4$ has no specific effect on lipid production. According to the results the optimum pH for maximum lipid yield was 6. The influence of pH on lipid production depends to the yeast strain and also carbon source in the medium. Angerbauer (2008) and many investigators have shown that pH can effect on lipid production. Syed (2006) reported that lipid production decrease greatly in pH=4 and pH=8, and pH between 5 to 6 has good effect on lipid yield (16). Table 3 shows lipid production on agricultural rasidues. Dai et al(2007) cultivated *Rhodotorulla glutinis* on corn stalk and rice straw and reported 2.01g/L and 0.21g/L lipid production on these carbon sources respectively.

Table1. Results of lipid yield by *Rhodotorulla aurantiaca* with glucose and xylose as carbon sources

condition	Lipid production (g/l)	Biomass (g/L)	%lipid productivity
Carbone source			
Glucose	6.3	17.45	36.10
Xylose	5.15	17.16	30.01

Table2. results of lipid production by *Rhodotorulla aurantiaca* cultivated in Nitrogen limited medium at different conditions.

condition	Lipid production (g/l)	Biomass (g/L)	%lipid productivity
Nitrogen source			
Yeast extract and $(NH_4)_2SO_4$	5.41	15.55	34.79
Yeast extract and NH_4Cl	5.25	15.32	34.26
Peptone and $(NH_4)_2SO_4$	5.18	15.15	34.19
Peptone and NH_4Cl	5.08	14.87	34.16
xylose concentration(g/l)			
35	5.35	15.50	34.51
55	6.52	16.21	40.22
75	7.13	16.2	44.01
95	5.84	13.9	42.01

115	5.71	14.23	40.12
$(NH_4)_2SO_4$ (g/l)			
0.5	6.9	15.97	43.20
1	7.45	16.55	45.01
1.5	6.7	16.14	41.51
KH_2PO_4(g/L)			
1	7.76	17.16	45.22
3	7.7	17.7	43.50
5	7.45	17.86	41.71
7	7.01	17.05	41.11
$MgSO_4.7H_2O$(g/L)			
0.5	7.81	17.16	45.51
1	7.9	17.32	45.61
1.5	7.75	17.07	45.40
2	7.67	17.19	44.61
2.5	7.56	16.8	45.00
pH			
5	6.83	15.14	45.11
5.5	7.2	15.92	45.22
6	7.98	17.34	46.02
6.5	7.32	16.19	45.21

Table3. Lipid production with rice bran and corn stalk as carbon sources

condition	Lipid production (g/l)	Biomass (g/L)	%lipid productivity
Rice bran	7.12	16.18	44.00
Corn stalk	6.54	16.76	39.02

4. CONCLUSION

The results of this study showed that we can reduce agricultural wastes by using oleaginous yeasts. In this way we can find renewable source of energy and also decrease the cost of biodiesel production. This process has many environmental and economical benefits. *Rhodotorulla aurantiaca* produced 7.98g/L lipid with cellular lipid content of 46% of dry biomass in medium with xylose as carbon source. It also had good results of 7.12g/L and 6.54g/L

lipid production on rice bran and corn stalk respectively. These results make us hopeful for biodiesel production using oleaginous yeasts and agricultural residues in future.

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