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Research Article

Oil Production from *Yarrowia lipolytica* Po1g Using Rice Bran Hydrolysate

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The purpose of this study was to produce microbial oil from *Yarrowia lipolytica* Po1g grown in defatted rice bran hydrolysate. After removing oil from rice bran by Soxhlet extraction, the bran is subjected to acid hydrolysis with various sulfuric acid concentrations (1–4% v/v), reaction times (1–8 h), and reaction temperatures (60–120°C). The optimal conditions for maximum total sugar production from the hydrolysate were found to be 3% sulfuric acid at 90°C for 6 h. Glucose was the predominant sugar (43.20 ± 0.28 g/L) followed by xylose (4.93 ± 0.03 g/L) and arabinose (2.09 ± 0.01 g/L). The hydrolysate was subsequently detoxified by neutralization to reduce the amount of inhibitors such as 5-hydroxymethylfurfural (HMF) and furfural to increase its potential as a medium for culturing *Y. lipolytica* Po1g. Dry cell mass and lipid content of *Y. lipolytica* Po1g grown in detoxified defatted rice bran hydrolysate (DRBH) under optimum conditions were 10.75 g/L and 48.02%, respectively.

1. Introduction

The increasing industrialization and motorization of the world has led to a steep rise for the demand of petroleum-based fuels which are obtained from limited reserves. These finite reserves are highly concentrated in certain regions of the world. Therefore, those countries not having these resources are facing energy/foreign exchange crisis, mainly due to the import of crude petroleum. Hence, it is necessary to look for alternative fuels which can be produced from resources available locally within the country such as alcohol, biodiesel, and vegetable oils [1].

Biodiesel is a clean, biodegradable, renewable, and nontoxic fuel which contributes no net carbon dioxide or sulfur to the atmosphere and emits less pollutants than conventional diesel. However, the high cost of raw material (70–75%) for biodiesel production has become one of the major obstacles for its development and wide applications [2]. On the other hand, consumption of a large amount of vegetable oils as raw material for biodiesel production would result in a shortage in edible oils and leads to the soar of food price. Adoption of animal fat, used frying oil, and waste cooking oil as feedstock is a good strategy to reduce the cost. However, these limited resources cannot meet the increasing needs for clean renewable fuels [3, 4].

Recently, there has been an increasing interest in looking for new oil sources for biodiesel production. Among them, microbial oils, namely single cell oils (SCOs), have attracted great attention worldwide. Oils from oleaginous microorganisms including bacteria, yeasts, moulds, and microalgae [5] are now considered as promising candidates due to their specific characteristics such as being unaffected either by seasons or by climates, having high lipid contents, ability to be produced from a wide variety of sources with short period of time, and their similar fatty acid compositions to that of vegetable oils [6, 7]. However, the high production cost of SCO makes microbial oils less economically competitive. As a result, the production of microbial oils from wastes or renewable materials is significantly important [8].

The oleaginous yeast *Y. lipolytica* is one of the most extensively studied "nonconventional" yeasts found in environments rich in hydrophobic substrates, such as alkanes or lipids, and has developed sophisticated mechanisms for the efficient use of hydrophobic substrates as its sole carbon source [9]. One of the most striking features of this yeast is the presence in its genome of several multigene families involved in these metabolic pathways. The complexity and multiplicity of these genes enable *Y. lipolytica* to use and valorize a wide range of HS. Using these mechanisms, this yeast is able to accumulate large amount of lipids [10]. Studies about the uses of *Y. lipolytica* showed its potential application for the production of reserve lipids with composition resembling that of cocoa butter [11], lipids with particular structures (e.g., oils enriched in essential polyunsaturated fatty acids), and nonspecific oils for use as renewable starting materials for the synthesis of biofuels [12].

Lipid biosynthesis from sugars and related substrates is a secondary anabolic activity, conducted after essential nutrient (usually nitrogen) depletion in the medium. Due to this exhaustion, the carbon flow is directed towards the accumulation of intracellular citric acid that is used as acetyl-CoA donor in the cytoplasm. Acetyl-CoA generates cellular fatty acids and subsequently triacylglycerols. SCO produced by *Y. lipolytica* cultivated under specific growth conditions could be directly converted into biodiesel, as its fatty acid composition is similar to the one from common vegetable oils [13]. Studies related to the production of specialty lipids by the yeast *Yarrowia lipolytica* during growth on various fatty agroindustrial residues utilized as substrates showed that the potential for growth on stearin (a low-cost industrial derivative of tallow composed of saturated free fatty acids) resulted in significant biomass production. This was accompanied by notable intracellular accumulation of lipid which occurred as a primary anabolic activity regardless of the extracellular nitrogen availability in the medium [14].

In order to reduce the cost of microbial oil production from *Y. lipolytica*, low-cost raw materials, such as rice hull [15], industrial fats [16], industrial sugars [17], lignocellulosic residues [18], and raw glycerol [19, 20], have been used as substrates. Rice hull, one of the agricultural residues, is used for microbial oil production. According to Economou et al. [15], acid hydrolysis of rice hull using sulfuric acid resulted in hydrolysate that was used as feedstock for microbial lipids production with the oleaginous fungus *Mortierella isabellina*. Results from kinetic experiments showed the maximum oil accumulation into fungal biomass to be 64.3% and suggested rice hull as cheap source of carbon.

Rice bran is also one of the most abundant agricultural by-products in the world. Typical rice bran is composed of about 15–19.7% lipids, 34.1–52.3% carbohydrates, 7–11.4% fiber, 6.6–9.9% ash, and 10–15% proteins. After oil is being removed, the residual defatted rice bran (DRB) powder contains significant amount of starchy and cellulosic polysaccharides. Enzymatic and chemical hydrolysis is used to break the polysaccharides into smaller molecules which will be used as carbon sources for microorganisms [21].

The purpose of this study was to investigate the possibility of using cheap and easily available defatted rice bran hydrolysate as a nutrient source for *Y. lipolytica* Po1g for microbial oil production. The effects of acid concentration, reaction time, and temperature on the hydrolysis of DRB were investigated. The effects of different types of acid hydrolysates of rice bran on the growth and lipid content were also studied. To the best of our knowledge, this is the first report to use defatted rice bran hydrolysate to culture *Y. lipolytica* Po1g for microbial oil production.

2. Materials and Methods

2.1. Materials. All solvents and reagents were either high-performance liquid chromatography (HPLC) or analytical reagent grade, obtained from commercial sources. For HPLC analysis, all the standards were purchased from Acros Organics (USA) and Sigma Aldrich (USA). Thin layer chromatography (TLC) aluminium plates (20×20 cm) were obtained from Merck KGaA (Darmstadt, Germany). Qualitative filter papers (grade no. 2, 0.26 mm thickness, 80% collection efficiency) were acquired from Advantec, MFS Inc. (Dublin, CA).

2.2. Defatted Rice Bran Hydrolysate (DRBH) Preparation

2.2.1. Raw Material and Pretreatment. Fresh rice bran was purchased from a local rice mill in Taoyuan County, Taiwan. Bran collected from the mill was stored in a freezer at 4°C before use. Prior to defatting, it was dried at 50°C for 24 h until weight was constant. Defatting of rice bran was carried out in a Soxhlet extractor with hexane as the solvent at 60°C for 6 h. The defatted rice bran was kept at 4°C for future use.

2.2.2. Acid Hydrolysis of Defatted Rice Bran. The defatted rice bran was hydrolyzed, following the method of Zhu et al. [4] with minor modification, by using H_2SO_4 (1%, 2%, 3%, or 4%, v/v) with a bran to acid ratio of 1 : 8 (g/mL). The effect of temperature on hydrolysis was investigated at 60, 70, 80, 90, 100, and 120°C. The effect of hydrolysis time was also investigated. After hydrolysis, the mixture was subjected to vacuum filtration to obtain the defatted rice bran hydrolysate (DRBH).

2.3. Detoxification of DRBH. To reduce the concentration of inhibitors in DRBH, neutralization with $Ca(OH)_2$ was employed. $Ca(OH)_2$ was slowly added to the DRBH by stirring the mixture at room temperature until the pH was adjusted to 6.5. Then, the hydrolysate was repeatedly vacuum filtrated until the precipitated particles were removed. After its composition was analyzed by HPLC, the detoxified hydrolysate was stored in a refrigerator at 4°C for further use as nutrient for microbial fermentation.

2.4. Fermentation

2.4.1. Microorganism. *Y. lipolytica* Po1g cells were obtained from YEASTERN Biotech Co. Ltd. (Taipei, Taiwan). The strain is a derivative of the wild-type strain W29 (ATCC 20460) by a series of genetic modifications. The cells were

maintained on a sterilized yeast-, peptone-, and dextrose-(YPD-) agar plate containing 10 g/L yeast extract (Bacto, France), 10 g/L peptone (Bacto, France), 20 g/L glucose (Acros Organics, USA), and 20 g/L agar (Acros Organics, USA) at 4°C for further microbial fermentation.

2.4.2. Media, Inoculum Preparation, and Large-Scale Fermentation. The preculture of *Y. lipolytica* Po1g was incubated on a sterilized YPD-agar plate and rejuvenated by incubation in 25 mL YPD medium containing yeast extract (10 g/L), peptone (10 g/L), and D-glucose (20 g/L) for 24 h at 26°C in an orbital shaker incubator model LM-570 (Chemist Scientific Corp, Taiwan) and then inoculated to cultures in 500 mL Erlenmeyer flasks at an inoculum to medium ratio of 1 : 10 (v/v).

Fermentation was carried out in several 500 mL Erlenmeyer flasks each containing 250 mL detoxified DRBH with initial pH 6.5. To investigate the effect of different nitrogen sources on growth of cells and lipid content, either urea (5 g/L, Acros Organics, USA) or peptone (5 g/L) was added to the DRBH. The effect of the concentration of fermentable sugars in DRBH on microbial growth and lipid content was studied by diluting the sugar concentration obtained at optimum conditions (48.41 g/L) and adjusted to 20 g/L, 30 g/L, or 40 g/L.

The flasks were then incubated in an orbital shaker incubator at 150 rpm and 26°C. Cell biomass was harvested by centrifugation at 3500 g for 30 min. The cell mass was washed twice with deionized water and dried in an oven at 50°C until constant weight. All media and flasks were autoclaved at 121°C for 30 min before the microbial fermentation.

2.5. Analytical Methods

2.5.1. Cell Concentration Determination. For monitoring yeast growth, optical density (OD) of the diluted fermentation broth was measured at 600 nm using a UV/Vis spectrometer V-550 model (Jasco, Japan), and cell concentration was determined from a calibration curve of absorbance versus dry cell weight.

2.5.2. Analysis of Protein, Sugar, and Inhibitor Concentration in DRBH. The solubilized protein content of the hydrolysates was determined by the Bradford method [22]. Absorbance at 595 nm was measured by using a UV-VIS spectrophotometer V-550 model (Jasco, Japan), and then comparison to a standard curve provided a relative measurement of protein concentration.

Concentrations of D-glucose, D-xylose, and L-arabinose in the hydrolysates were determined by HPLC (Jasco, Japan) equipped with a PU-1580 pump, a DG-4400 degasser, an Alltech 2000 ELSD detector, and a Zorbax NH_2 column (5 μm particle size, 250 mm × 4.6 mm, Agilent Technologies, USA). The sample was diluted appropriately with deionized water, filtered through a 0.22 μm PVDF syringe filter (Test high) and then injected into the HPLC under the following conditions. The temperature of the ELSD detector was 80°C, and nitrogen flow rate was 2 mL/min. The column temperature was 25°C. Acetonitrile: water (80 : 20, v/v) was the eluent mobile phase with a flow rate of 1 mL/min, and the injection volume was 25 μL.

The concentrations of 5-hydroxymethylfurfural (5-HMF) and furfural in the hydrolysate were determined by HPLC (Jasco, Japan) equipped with a PU-2089 pump combined with degasser, an UV 2077 UV detector, and a Luna C-18 column (5 μm particle size, 250 mm × 4.6 mm, Phenomenex, USA). The sample was diluted appropriately with deionized water, filtered through a 0.22 μm PVDF syringe filter (Test high), and then injected into the column under the following conditions: 25°C column temperature, acetonitrile : water : acetic acid (11 : 88 : 1, v/v/v) with a flow rate of 0.8 mL/min, injection volume of 25 μL, and the absorption wavelength was 276 nm. The concentrations of these compounds were calculated by using calibration curves obtained from standard D-glucose, D-xylose, L-arabinose, furfural, and 5-HMF solutions.

The amount of total reducing sugars in the hydrolysate was measured by the dinitrosalicylic acid (DNS) method based on a colorimetric reaction between the sugars and dinitrosalicylic acid. DNS reagent (0.5 mL) was added to the appropriately diluted sample (0.5 mL) in a capped brown bottle to prevent DNS from being affected by light. Then, the mixture was heated to 100°C for 5 min, and after cooling to room temperature in a cold water bath, the absorbance was monitored with a spectrophotometer at 540 nm. The results were calculated based on calibration curve of standard D-glucose.

2.5.3. Lipid Analysis. Extraction of total lipid was performed by using Soxhlet extractor with hexane and methanol (1 : 1, v/v) for 4 h. The extracted lipid was subjected to silica gel thin layer chromatography (TLC) analysis to identify its neutral lipid content. After that, the crude microbial lipid was dewaxed and degummed according to the methods described by Rajam et al. [23], and Vandana et al. [24]. Crude microbial oil was dissolved in hot water at 60°C, and the water soluble fraction was separated from the insoluble fraction by vacuum filtration. The insoluble fraction was then dissolved in acetone and kept at 60°C for 1 h to obtain clear solution. After allowing the content to cool to room temperature, the solution was then kept at 5°C for 24 h to crystallize the remaining waxes. The insoluble fraction was separated by vacuum filtration. The dewaxed and degummed lipid was analyzed by gas chromatography (GC-17A, Shimadzu, Japan) for its neutral lipid composition as well as its fatty acid profile. The GC was equipped with a flame ionization detector and a DB-17HT capillary column (0.25 cm × 30 m, Agilent Technologies Inc., USA). The column temperature was programmed to increase from 80°C to 365°C at 10°C/min and kept at 365°C for 29 min. Nitrogen was used as the carrier gas at a flow rate of 0.80 mL/min. The split ratio was 1 : 50 (v/v). The temperatures of injector and the detector were both maintained at 370°C. Twenty milligrams sample was dissolved in 1 mL ethyl acetate, and 0.5 μL sample was taken and injected into the GC. Standards

Table 1: Composition of DRBH with different reaction time and H_2SO_4 concentration at 90°C.

H_2SO_4 (%v/v)	Reaction time (h)	Concentration (g/L)				
		Glucose	Xylose	Arabinose	HMF	Furfural
2%	2	13.36 ± 0.89	1.88 ± 0.42	1.13 ± 0.28	0.05 ± 0.02	0.004 ± 0.003
	4	16.25 ± 0.94	2.34 ± 0.28	1.46 ± 0.19	0.16 ± 0.01	0.012 ± 0.002
	6	21.26 ± 0.90	3.20 ± 0.28	1.97 ± 0.35	0.21 ± 0.02	0.020 ± 0.003
	8	25.57 ± 0.76	3.84 ± 0.11	2.41 ± 0.16	0.28 ± 0.02	0.029 ± 0.020
3%	2	26.87 ± 0.64	4.61 ± 0.11	2.82 ± 0.07	0.13 ± 0.01	0.006 ± 0.002
	4	36.67 ± 0.32	5.45 ± 0.043	2.23 ± 0.01	0.25 ± 0.01	0.015 ± 0.001
	6	43.20 ± 0.28	4.93 ± 0.03	2.09 ± 0.01	0.32 ± 0.05	0.025 ± 0.003
	8	40.12 ± 0.49	3.89 ± 0.05	1.46 ± 0.07	0.41 ± 0.02	0.041 ± 0.003
4%	2	28.80 ± 0.15	4.26 ± 0.02	2.83 ± 0.01	0.23 ± 0.01	0.011 ± 0.002
	4	37.98 ± 0.19	4.60 ± 0.02	2.07 ± 0.81	0.40 ± 0.01	0.030 ± 0.003
	6	43.64 ± 0.49	4.37 ± 0.16	1.87 ± 0.12	0.60 ± 0.08	0.053 ± 0.009
	8	36.75 ± 1.79	3.78 ± 0.28	1.32 ± 0.21	0.88 ± 0.07	0.078 ± 0.005

of saturated and unsaturated fatty acids (Sigma-Aldrich, USA) were used for the identification of fatty acids in the lipid. All data were averages of triplicate determinations.

3. Result and Discussion

3.1. Acid Hydrolysis of DRB. Huang et al. [25] studied the production of microbial oil from *T. fermentans* grew in sulfuric-acid-treated rice straw hydrolysate (SARSH). The hydrolysate (SARSH) contained a maximum of 35.2 g/L fermentable monosaccharides including glucose, xylose, and arabinose, and the concentration of pentoses was about six times higher than hexose.

In this study, the composition of the defatted rice bran hydrolysate (DRBH) obtained by using 1% H_2SO_4 at different temperature (60°C–120°C) for 1 h showed that glucose was the predominant sugar in the hydrolysate. This result suggests the presence of a higher proportion of starch and glucans in DRB along with other minor pentoses such as xylose and arabinose, which are mainly derived from xylans and arabinoxylans, respectively. The maximum fermentable sugars concentration from DRBH under these conditions was 5.96 ± 0.43 g/L out of which glucose, xylose, and arabinose are 3.16 ± 0.22 g/L, 1.36 ± 0.12 g/L, and 0.74 ± 0.01 g/L, respectively. However, the results suggest that hydrolysis of DRB for 1 h with 1% H_2SO_4 was not efficient to hydrolyze the DRB to produce fermentable sugars. The optimum temperature was 90°C, and this was used as a basis for the next hydrolysis reactions with different acid concentrations.

Table 1 shows the effects of H_2SO_4 concentrations (2%, 3%, and 4%) and hydrolysis time (2–8 h) on the composition of DRBH at 90°C. The concentration of total fermentable sugars using 2% H_2SO_4 was much lower than that of 3% and 4%, which shows that higher acid concentration could break down the polymerized structure of hemicellulose, amylase, and amylopectin more efficiently. The highest total released sugar concentration (50.22 ± 0.34 g/L) was obtained when DRB was hydrolyzed with 3% H_2SO_4 at 90°C for 6 h. These optimum conditions resulted in glucose (43.20 ± 0.28 g/L), xylose (4.93 ± 0.03 g/L), arabinose (2.09 ± 0.01 g/L), HMF (0.32 ± 0.05 g/L), and furfural (0.025 ± 0.003 g/L). Compared to the total fermentable sugars from SARSH (35.2 g/L), our results showed that rice bran has higher concentration of fermentable sugars than sulfuric-acid-pretreated rice straw hydrolysate. On the other hand, the highest monosaccharide sugar concentration obtained in this study (43.20 ± 0.28 g/L) is glucose, while that of SARSH (25.5 g/L) is xylose. This indicates rice bran is a better source of glucose than rice straw for microbial fermentation.

The total sugar obtained in this work was also much higher than that of enzymatic hydrolysis of DRB which resulted in 37 g/L total sugars when defatted rice bran was subjected to saccharification with a mixture of amylase and cellulase [26]. Therefore, dilute acid hydrolysis breaks down the lignocelluloses better than enzymes and can be considered as an efficient method to hydrolyze the DRB to obtain sugars for utilization in microbial fermentation.

The concentration of monosaccharides increases as time increases from 2 h to 8 h using 2% acid concentration. However, at an acid concentration of 3% or 4%, glucose concentration increased with time until 6 h and then started to decrease. The same trend was observed for xylose and arabinose. The rapid increase in the concentration of inhibitors with longer hydrolysis time indicates the decomposition of monosaccharides into less desirable compounds under the severe acid hydrolysis condition (4% H_2SO_4). A similar work also reported that sugar concentration increases with longer hydrolysis time and higher acid concentration until the optimum conditions were reached and decreases thereafter [27].

3.2. Effect of Detoxification on the Composition of DRBH. The presence of inhibitory substances in the fermentation medium can restrict both sugar utilization and growth of microorganisms. It has been reported that oleaginous microorganisms could not grow and reproduce well in the rice straw hydrolysate obtained by acid hydrolysis without

Table 2: Chemical composition of DRBH before and after detoxification. The hydrolysate used for detoxification was obtained by using 3% H_2SO_4 at 90°C for 6 h.

Type of DRBH		Concentration (g/L)				
		Glucose	Xylose	Arabinose	HMF	Furfural
2%	Before detoxification	25.57 ± 0.76	3.84 ± 0.11	2.41 ± 0.16	0.28 ± 0.02	0.029 ± 0.020
	After detoxification	25.40 ± 0.87	3.53 ± 0.43	2.03 ± 0.11	0.22 ± 0.01	0.023 ± 0.001
3%	Before detoxification	43.20 ± 0.28	4.93 ± 0.03	2.09 ± 0.01	0.32 ± 0.05	0.025 ± 0.003
	After detoxification	42.15 ± 0.39	4.71 ± 0.14	1.99 ± 0.09	0.24 ± 0.04	0.019 ± 0.005
4%	Before detoxification	43.64 ± 0.49	4.37 ± 0.16	1.87 ± 0.12	0.60 ± 0.08	0.053 ± 0.009
	After detoxification	42.99 ± 0.44	4.16 ± 0.75	1.70 ± 0.33	0.47 ± 0.16	0.044 ± 0.003

detoxification. Low lipid content was obtained after culturing *T. fermentans* for 8 days owing to both sugar utilization and lipid accumulation being inhibited by various inhibitors [25]. Therefore, detoxification of DRBH is necessary to reduce the amount of inhibitors and achieve higher fermentability of sugars. Various methods such as overliming, neutralization, ion exchange, steam stripping, and treatment with activated carbon have been applied to detoxify the lignocellulosic hydrolysate. However, the most economical way to detoxify lignocellulosic hydrolysate was neutralization by calcium hydroxide [28].

Detoxification of DRBH by using $Ca(OH)_2$ was applied since it can easily remove the residual SO_4^{2-} by forming $CaSO_4$ precipitate and efficiently reducing the level of inhibitors simultaneously. The decrease in the concentrations of HMF and furfural is due to the reaction between calcium ions and furans, producing complex ions [29].

During the hydrolysis of DRB, the concentrations of both HMF and furfural increased with increasing acid concentrations. Using 2% sulfuric acid, the concentrations of HMF and furfural were 0.28 g/L and 0.029 g/L, respectively, and when the acid concentration increased from 3% to 4%, the concentration of HMF and furfural also increased from 0.32 g/L to 0.60 g/L and from 0.025 g/L to 0.053 g/L, respectively.

The maximum concentration of total released sugars from nondetoxified DRBH at optimum conditions was 50.22 ± 0.34 g/L, out of which the concentration of glucose was 8.76 times higher than that of xylose. HMF and furfural were the main inhibitors in the hydrolysate before neutralization. As shown in Table 2, the concentration of monosaccharides before and after detoxification by $Ca(OH)_2$ did not show a significant change. However, the concentrations of HMF and furfural decreased by 25.0% and 24.0% (in the 3% DRBH), respectively, which indicates that detoxification by lime can effectively reduce the amount of inhibitors and can provide a potential alternative medium for better microbial fermentation.

3.3. Growth of Y. lipolytica Po1g in Different Types of DRBH Media. DRB is rich in carbohydrates, fiber, and proteins and can be regarded as a potential nutrient source for microbial oil production [21]. Therefore, detoxified DRBH can be a potential nutrient source for growing *Y. lipolytica* Po1g.

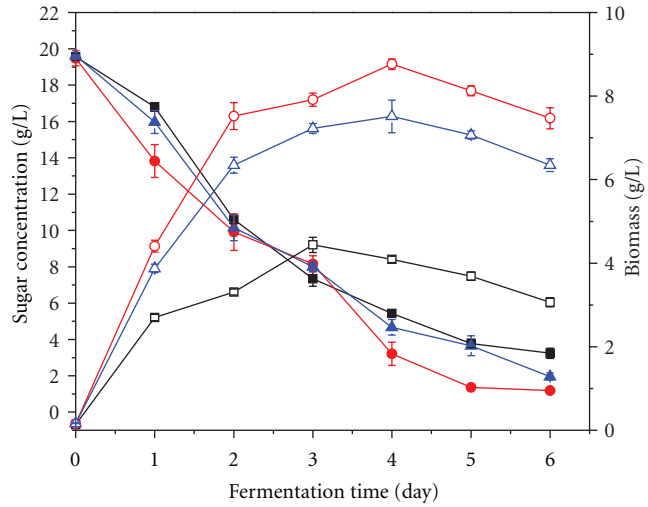


Figure 1: Effect of different types of DRBH on the growth of *Y. lipolytica* Po1g. Culture conditions: growth in Erlenmeyer flasks; initial total sugar concentration: 20 g/L; pH: 6.5; incubation temperature: 26°C; agitation speed = 150 rpm; biomass concentration: (□) 2%, (○) 3%, (△) 4%; sugar consumption: (■) 2%, (•) 3%, (▲) 4%.

Three different types of detoxified DRBHs were obtained from rice bran hydrolysis with three different acid concentrations (2%, 3%, and 4%) and named 2%, 3%, and 4%, respectively. The DRBHs were then used as the basic media for the growth of *Y. lipolytica* Po1g. The concentrations of total fermentable sugars in all three DRBH media were made constant (20 g/L) by appropriately diluting the corresponding initial detoxified DRBHs with deionized water. The protein contents of DRBHs obtained by using 2%, 3%, and 4% sulfuric acid were analyzed to be 1.09, 2.85, and 3.05 g/L, respectively. To study the possibility of using the hydrolysates as direct nutrient sources, comparison of the growth profile of *Y. lipolytica* Po1g in these media was made, and the lipid contents of the maximum biomass concentrations were determined.

As can be seen in Figure 1, yeast growth was poor (maximum biomass concentration = 4.43 g/L, 3rd day) in the 2% DRBH medium. The corresponding cellular lipid content obtained was 21.66%.

Increases in biomass concentration were observed when *Y. lipolytica* Po1g grew in the DRBH obtained by hydrolysis using 3% H_2SO_4. The highest biomass concentration (8.76 g/L) was observed on the 4th day. The cellular lipid content obtainable is 30.13% and is relatively higher than the one from the medium that contained the DRBH from 2% H_2SO_4. The sugar concentration in the medium prepared by hydrolysis of rice bran with 3% H_2SO_4 also decreased faster due to an increase in biomass. As the biomass increases, sugar consumption also increases. On the other hand, both biomass and lipid contents decreased slightly to 7.51 g/L and 27.73%, respectively, when *Y. lipolytica* Po1g was cultivated in the DRBH obtained by hydrolysis with 4% H_2SO_4.

According to a study conducted by Economou et al. [15], rice hulls hydrolysate was used as the sole carbon and energy source for oleaginous fungus *M. isabellina*. In their study, an increase in acid concentration from 0.03 M to 0.09 M increased the extractable oil amount from 36% to 64.3%, and it was observed that in the rice hulls hydrolysate obtained from sulfuric acid concentration higher than 1 M, the fungus failed to grow, probably due to the high concentrations of toxic compounds in the culture medium. The same trend was observed in our study. The highest acid concentration (4%) might have led to higher concentration of inhibitors during hydrolysis. This shows that neutralization of this hydrolysate with $Ca(OH)_2$ alone did not reduce the inhibitors concentration, and these inhibitors affected the growth of the yeast cells.

A review about lipid accumulation properties of *Y. lipolytica* shows that the study of *de novo* lipid accumulation as a result of the conversion of carbohydrate substrates, such as glucose, provides insight into the regulation of the entire lipid synthesis pathway. The accumulation of lipids produced from these substrates is triggered by nutrient limitation. Nitrogen limitation is the principal type of limitation governing lipid accumulation [30]. Our results also suggested that limited nitrogen content in the media (3% DRBH) led to better growth and lipid accumulation while the protein content (1.09 g/L) in the medium from 2% H_2SO_4 was not enough for the growth of the cells, which limited biomass concentration to 4.43 g/L.

3.4. Effect of DRBH Sugar Concentration on Biomass and Microbial Oil Content. To investigate the effect of sugar concentrations on microbial growth and lipid accumulation, three different concentrations of total fermentable sugars (20 g/L, 30 g/L, and 40 g/L) were prepared by appropriate dilution of the detoxified DRBH (type 3%, Table 2) with an initial total sugar concentration of 48.41 g/L. The total protein content in each of these media was adjusted to 5 g/L by addition of appropriate amount of yeast extract.

As shown in Table 3, the lowest cellular lipid content (30.13%) was obtained when the *Y. lipolytica* Po1g was incubated in the medium with the lowest sugar concentration (20 g/L). However, the highest lipid content (48.02%) and lipid yield (5.16 g/L) were observed when the sugar concentration in the medium was 30 g/L. Both biomass concentration and cellular lipid contents decreased when the total sugar concentration was 40 g/L.

Table 3: Effects of sugar concentration on microbial oil production by *Y. lipolytica* Po1g.

Sugar concentration (g/L)	Maximum biomass (g/L)	Lipid content (%)	Lipid yield (g/L)
20	8.76 ± 0.13	30.13 ± 0.56	2.64 ± 0.08
30	10.75 ± 0.19	48.02 ± 0.61	5.16 ± 0.15
40	9.33 ± 0.16	40.97 ± 0.74	3.82 ± 0.13

Zhu et al. [4] investigated cell growth and lipid accumulation of *T. fermentans* in molasses with different total sugar concentrations ranging from 5% to 30%. Both biomass and lipid content increased with the total sugar concentration, and the maximum biomass (29.9 g/L) and lipid content (31.8%) were attained at 15% total sugar concentration. Further rise in the total sugar concentration beyond 15% led to the decrease in biomass and lipid content. The same trend was observed when *Y. lipolytica* Po1g was cultivated in DRBH with different sugar concentrations which increased both biomass and lipid content when sugar concentration was raised from 20 g/L to 30 g/L. But further increase to 40 g/L resulted in lowering of biomass concentration and lower lipid content. This may be due to substrate inhibition in which excess sugar concentration in the DRBH media inhibits the growth of the microorganism during the early stages of growth.

Another study on the biochemical behavior of wild-type or genetically modified *Y. lipolytica* strains cultivated on commercial glucose and nitrogen-limited cultures showed that carbon-excess conditions favored the secretion of organic acids into the growth medium [17]. Cultivation of *Y. lipolytica* Po1g using 40 g/L in this study also resulted in reduction of biomass concentration, which may most probably be due to the secretion of organic acids.

3.5. Effect of Addition of Different Nitrogen Sources on Growth and Microbial Oil Production. Factors such as carbon and nitrogen sources have significant influences on cell growth and lipid accumulation of oleaginous microorganisms [31]. To study the effect of addition of different nitrogen sources on biomass concentration and microbial oil production, three different DRBH media with the same total fermentable sugar concentration (30 g/L) and the same initial protein content (2.82 g/L) were prepared. No external nitrogen source was added to the first one, but either peptone (5 g/L) or urea (5 g/L) was added to the other two media.

As it can be seen from Figure 2, the medium without additional nitrogen source was the best for yeast cells to accumulate the highest lipid content (48.02%) with a lipid yield of 5.16 g/L. The addition of urea did not significantly affect (P-value = 0.27) biomass production (10.85 g/L) but caused reduction in cellular lipid accumulation to 14.19%. Compared to urea, *Y. lipolytica* Po1g grew better, and the maximum biomass concentration was 13.53 g/L when the nitrogen source was peptone. The cellular lipid content (26.43%) obtained when cells grew in peptone was much higher than the one with urea, indicating that urea is not a

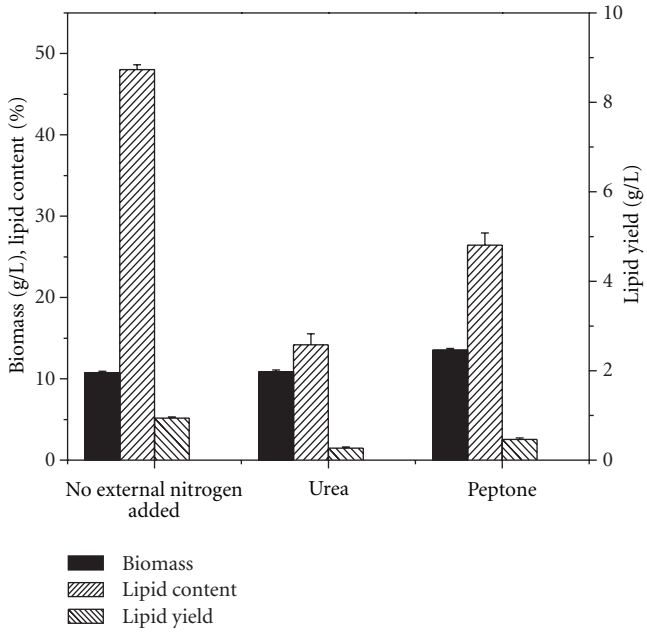


Figure 2: Effects of nitrogen addition on microbial oil production by *Y. lipolytica* Po1g grown in DRBH medium. Culture conditions: growth in Erlenmeyer flasks; initial total sugar concentration: 30 g/L; pH: 6.5; incubation temperature: 26°C; agitation speed = 150 rpm.

good nitrogen source for microbial oil production from *Y. lipolytica* Po1g grown in detoxified DRBH medium.

Microbial oil production with both nitrogen supplements showed similar tendencies in which the microorganisms did not accumulate more lipids but produced more biomass than the one without external nitrogen source. Nitrogen is essential for the syntheses of proteins and nucleic acids which are required for cellular proliferation. However, this synthesis will be repressed when nitrogen source is limited, and carbon source will be channeled to lipid synthesis causing accumulation of lipid in the cells [32]. Therefore, nitrogen limitation is effective in enhancing the accumulation of lipid in *Y. lipolytica* Po1g grown in DRBH media.

Similar results by Zhu et al. [4] also showed that the cellular lipid content of *T. fermentans* grown in a medium with urea as the nitrogen source is 2.4 times lower than that when peptone was the nitrogen source. The result of this study shows that the yeast tended to channel sugar into cellular lipid production when it was cultivated on the nitrogen-limited detoxified DRBH resulting in the highest cellular lipid content. On the other hand, according to Papanikolaou et al. [17], growth of either wild-type or genetically modified strains of *Y. lipolytica* under nitrogen-limited culture conditions, with glucose or similarly metabolized compounds used as substrates, was not accompanied by significant lipid accumulation. Carbon-excess conditions favored the secretion of organic acids into the growth medium.

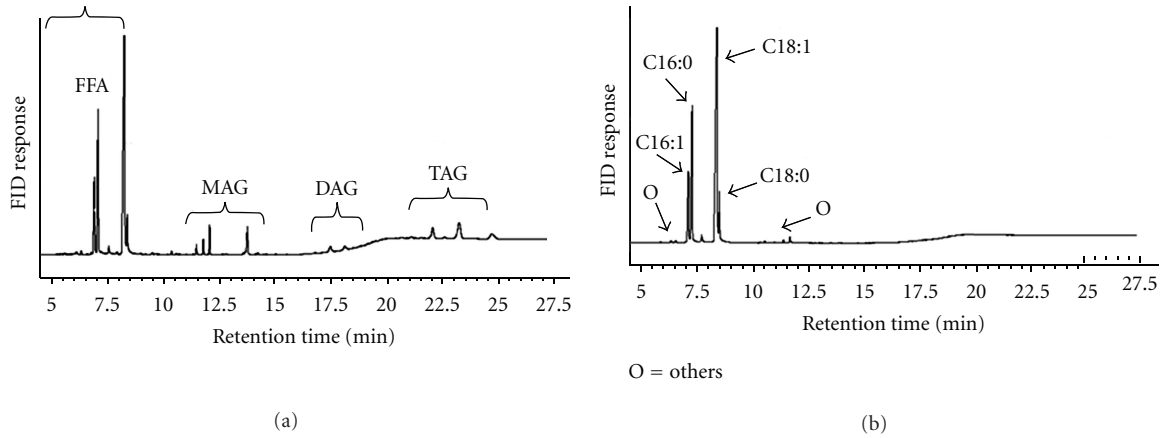
In general, various *Y. lipolytica* strains, despite being incapable of producing lipid via the *de novo* lipid accumulation mechanism, are able to accumulate huge amounts of lipid when growth is carried out on various hydrophobic carbon sources as substrates, since accumulation of lipid from fat materials is a completely different process from that from sugars [13].

3.6. Cellular Lipid Analysis. Crude microbial oil from *Y. lipolytica* Po1g cells grown in a medium with detoxified DRBH (3%) was investigated by TLC, and the contents were determined gravimetrically. After dewaxing and degumming, the neutral lipid content and the fatty acid profile were determined by GC 17A.

The crude microbial oil consisted of gum (52.77%), neutral lipids (41.92%), and wax (5.31%). The results of analysis of lipids isolated from the biomass of the yeast *C. lipolytica* grown in medium with methanol or glucose as the only carbon source also showed that polar lipids make more than half of the total cell lipids for both carbon sources (52.3 and 64.2%, resp.) [32]. This is in agreement with the results of our work. On the other hand, Makri et al. [19] found that neutral lipids are the major components in *Y. lipolytica* when grown in a glycerol medium. According to their study, lipid composition in *Y. lipolytica* showed specific trends with time that largely reflect the physiological role of individual lipids. The increase of neutral lipids during lipogenic phase and their subsequent decrease in citric acid production phase may explain the physiological role of neutral lipids, which is energy storage in times of plenty and energy provision in times of shortage. In fact, during lipid turnover phase, neutral lipids are preferentially degraded, while some quantities of polar lipids are synthesized.

The GC chromatograms (Figures 3(a) and 3(b)) show that the major components of neutral lipid are free fatty acids (FFA, 79.56%), monoacyl glycerides (MAG, 8.91%), diacyl glycerides (DAG, 2.15%), and triacyl glycerides (TAG, 8.59%). Media, type of strain, and cultivation time have significant effect on the amount of FFA in lipids from *Y. lipolytica*. As reported by Papanikolaou et al. [16], growth of *Y. lipolytica* on medium containing hydrophobic compounds as (co)substrates resulted in the accumulation of storage lipid containing considerable quantities of FFA (30 to 40 wt% of total lipids). They also found that large quantities of FFA did not result in lethality or growth deficit, which is similar to our result. According to Juliano [21], rice bran is composed of about 15–19.7% lipids, which depends on factors such as type and origin of rice. The solvent used or extraction time used in this study might have an effect in which some amount of oil has not been removed from rice bran. The excess oil may remain in the medium and contribute to the increase in the FFA amount that can be extracted from *Y. lipolytica* Po1g. The reason for the high FFA content needs further investigation.

The fatty acid profile of the neutral lipid consisted of oleic acid (C18 : 1, 59.91%), palmitic acid (C16 : 0, 20.46%), palmitoleic acid (C16 : 1, 11.41%), and stearic acid (C18 : 0, 5.39%), with 2.83% as unidentified fatty acids. The main cellular fatty acid of *Y. lipolytica* lipid produced during growth on glucose or glycerol media also showed similar results, and oleic acid, palmitic acid, palmitoleic acid, and



(a) (b)

Figure 3: GC chromatograms of neutral lipid before saponification (a) and fatty acid profile after saponification with KOH and ethanol (b).

Table 4: Lipid contents of different strains of *Y. lipolytica* grown in different agroindustrial sources.

Strain	Carbon source	Biomass (g/L)	Lipid content (%)	Lipid yield (g/L)	Reference
Y. lipolytica	Industrial derivative of animal fat	12.5	54	3.8	[16]
C. lipolytica	8% molasses	NA	59.9	NA	[33]
Y. lipolytica LGAM (7)1	*Teucrium polium L. aqueous* extract	9.3	33	3.12	[34]
Y. lipolytica LGAM (7)1	Glucose	9.3	25	2.3	[34]
Y. lipolytica	Industrial fats	8.7	44.0	3.8	[11]
Y. lipolytica ACA-YC 5033	Commercial glucose	5.5	14	NA	[17]
C. lipolytica	Mixtures of stearin, glucose, and glycerol	11.4	30	3.4	[35]
C. lipolytica	1% methanol	NA	4.9	NA	[32]
Y. lipolytica Po1g	Sugarcane bagasse hydrolysate	11.42	58.5	6.68	[18]
Y. lipolytica Po1g	Defatted rice bran hydrolysate	10.75	48.02	5.16	This study

stearic acid were the major fatty acids [17, 19]. The combined content of saturated and monounsaturated fatty acid is more than 97.17%, making neutral lipids obtained from the culturing of *Y. lipolytica* Po1g in rice bran hydrolysate ideal feedstock for biodiesel production.

Table 4 gives comparison of maximum biomass concentration, lipid content, and lipid yield of *Y. lipolytica* Po1g grown in DRBH media with those from different strains of *Y. lipolytica* grown in different nutrient sources. In terms of biomass concentration, DRBH is a better nutrient source for the *Y. lipolytica* Po1g compared to commercial glucose used to cultivate *Y. lipolytica* ACA-YC 5033. This indicates the potential of defatted rice bran as a nutrient source for oleaginous microorganisms.

According to Karatay and Dönmez [33], *C. lipolytica* can accumulate lipids (59%, w/w) when grown in 8% molasses medium. However, the maximum lipid concentration of *Y. lipolytica* Po1g in detoxified DRBH is 48.02%. On the other hand, our work shows better results compared to that of *Y. lipolytica* cultivated in methanol and industrial fats which resulted in a lipid content of 4.9% and 44%, respectively. Lipid yield of *Y. lipolytica* Po1g (5.6 g/L) is more promising when compared to the results of different strains of *Y. lipolytica*.

In our previous study, *Y. lipolytica* Po1g cells were cultivated in sugarcane bagasse hydrolysate, and the maximum biomass, lipid content, and lipid yield attainable were 11.42 g/L, 58.5%, and 6.68 g/L, respectively [18]. Therefore, under different media, different strains of *Y. lipolytica* have different lipid contents and lipid yields.

4. Conclusion

Defatted rice bran is an undervalued agricultural by-product which can be hydrolyzed by dilute acids not only to release carbon sources from starch and lignocellulose but also enough nitrogen sources for growing microorganisms. Detoxification of the defatted rice bran hydrolysate is required to reduce the level of inhibitors in the fermentation medium. Detoxified DRBH was found to be an effective medium for cultivating *Y. lipolytica* Po1g, and high cellular lipid content was obtained. The composition of the neutral lipid obtained is similar to those of most staple vegetable oils indicating its potentials for biodiesel production.

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