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Statistical Optimization for Cost-Effective Production of Yeast-Bacterium Cell-Bound Lipases Using Blended Oily Wastes and Their Potential Applications in Biodiesel Synthesis and Wastewater Bioremediation

Fidia Fibriana [1,2], Apichat Upaichit [1,3,*] and Benjamas Cheirsilp [3]

[1] Molecular Biotechnology Laboratory, Faculty of Agro-Industry, Prince of Songkla University, Hat Yai 90110, Thailand
[2] Department of Integrated Science, Faculty of Mathematics and Natural Sciences, Universitas Negeri Semarang, Semarang 50229, Indonesia
[3] Center of Excellence in Innovative Biotechnology for Sustainable Utilization of Bioresources, Faculty of Agro-Industry, Prince of Songkla University, Hat Yai 90110, Thailand
* Correspondence: apichat.u@psu.ac.th or upaichit@gmail.com

Abstract: Oily wastes have been widely used to produce lipases, but there is insufficient knowledge on their use to efficiently produce cell-bound lipases (CBLs). This research aimed to optimize yeast–bacterium CBLs production using blended oily wastes by statistical optimization and their potential applications in biodiesel production and wastewater bioremediation. The co-culture of *Magnusiomyces spicifer* AW2 and *Staphylococcus hominis* AUP19 produced CBLs as high as 4709 U/L with cell biomass of 23.4 g/L in a two-fold diluted palm oil mill effluent (POME) added by 2.08% (*v*/*v*) waste frying oil, 1.72.0% (*w*/*v*) ammonium sulfate, 0.1% (*w*/*v*) Gum Arabic as an emulsifier (initial pH at 7.0) within 24 h. The CBLs were successfully applied as whole-cell biocatalysts to produce biodiesel through esterification and transesterification with 76% and 87% yields, respectively. Direct application of CBLs for bioremediation of heat-treated various POME concentrations achieved 73.3% oil and grease removal and 73.6% COD removal within 3 days. This study has shown that the blended oily wastes medium was suitable for low-cost production of yeast–bacterium CBLs and their potential applications in solvent-free biodiesel production and wastewater bioremediation. These strategies may greatly contribute to economical green biofuel production and waste biotreatment.

Keywords: waste valorization; whole-cell biocatalysts; mixed lipases; fatty acid methyl esters; bioremediation; statistical approach

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1. Introduction

Lipases catalyze the breakdown of fatty acids, diglycerides, monoglycerides, and glycerol from fats and oils [1,2]. These enzymes catalyze ester synthesis, esterification, and transesterification reactions. They are widely employed in the food industry, detergent formulation, wastewater treatment, and biodiesel production [3,4]. Recently, microbial cell-bound lipases (CBLs) have received more attention than secreted lipases due to a milder operating condition and easy re-utilization [5,6]. However, the high-cost operation in enzyme production always becomes a bottleneck; therefore, cheaper substrates, such as agro-industrial wastes, can be an alternative to achieve a low-cost production [7]. It has been recently reported that palm oil mill effluent (POME), waste frying oil (WFO), molasses (MO), and crude glycerol (CG) were utilized in biotechnological processes to form certain products, including microbial lipids and lipases [5–10]. Even though many attempts have been made to use POME as a growth medium for microorganisms to produce lipases, an optimization of POME combined with another agro-industrial waste as a means of waste valorization for lipase production has not been discovered (Table 1).

Table 1. Palm oil mill effluent (POME) as a growth medium for lipase production.

Strain	POME Medium	Nutrient Supplementation	Product and Applications	Ref.
Magnusiomyces spicifer AW2 *Staphylococcus hominis* AUP19 *Magnusiomyces spicifer* AW2 + *Staphylococcus hominis* AUP19 (Co-culture)	25%, 50%, 100% (*v*/*v*) POME	-	Cell-bound lipases through the bioremediation approach for biodiesel synthesis	[5]
Yarrowia lipolytica TISTR5151	Raw POME 50% (*v*/*v*) POME	1 g-N/L $(NH_4)_2SO_4$	Cell-bound lipase and lipid for biodiesel synthesis	[6]
Candida cylindracea ATCC14830	POME	0.45% (*w*/*v*) Peptone 0.65% (*v*/*v*) Tween-80	Lipase	[11]
Pseudomonas aeruginosa	POME	0.4% (*v*/*v*) Olive oil 0.9% (*w*/*v*) Peptone 0.9% (*v*/*v*) Tween-80	Dry extract lipase	[12]
Candida antartica lipase B *Fusarium heterosporum* *Bacillus thermocatenulatus*	2.0% (*w*/*v*) POME	2.0% (*w*/*v*) Polypeptone 0.5% (*w*/*v*) KH_2PO_4 0.1% (*w*/*v*) $NaNO_3$ 0.05% (*w*/*v*) $MgSO_4.7H_2O$	Immobilized whole-cell lipases for biodiesel production	[13]
Magnusiomyces spicifer AW2 + *Staphylococcus hominis* AUP19 (Co-culture)	50% (*v*/*v*) POME	2.08% (*v*/*v*) Waste frying oil 1.72.0% (*w*/*v*) $(NH_4)_2SO_4$ 0.1% (*w*/*v*) Gum Arabic	Cell-bound lipases for biodiesel synthesis and bioremediation	This study

Some previous findings state that using untreated POME or POME in high concentrations inhibited microbial growth due to the high concentration of toxic compounds. In contrast, the dilution of POME positively improved microbial survivability in this kind of waste [5,14,15]. However, the dilution reduced the oil and grease concentrations, the primary carbon source that induces microbial lipase production. Therefore, nutrient supplementation to the POME medium is needed to boost lipase synthesis. POME-based medium added with other nutrient sources could induce high lipase production. For example, *Candida cylindracea* ATCC 14,830 produced lipase in POME containing peptone and Tween-80, and it revealed lipase activity 5.19-fold higher than the one obtained from the non-optimized medium [11]. Another example, the lipase from *Pseudomonas aeruginosa* was successfully produced in a POME-based medium with olive oil, peptone, and Tween-80 [12]. The immobilization of *Candida antartica*, *Fusarium heterosporum*, and *Bacillus thermocatenulatus* whole-cell lipase was performed in a POME medium with supplementation of polypeptone, KH_2PO_4, $NaNO_3$, and $MgSO_4.7H_2O$ [13]. Even though the supplementation successfully enhanced lipase production, the olive oil, peptone, and other components are costly for the large production scale. Therefore, the idea of waste combination as the low-cost medium for optimum lipase production is interesting to study.

In microbial cultivation, the pure culture for lipase production is easy to control and monitor. However, the co-culture allows each microbial specialty to obtain optimal products [16]. Limited studies have found that the yeast *Magnusiomyces spicifer* could produce high CBL activity [5,17], whereas lipases from the bacterium *Staphylococcus* spp. have broad substrate specificity with many potential applications [5,18]. The co-culture of microbial strains isolated from the same habitat is relatively easier to explore than the engineered microbial flora. Previously, the co-cultivation of *Magnusiomyces spicifer* AW2 and *Staphylococcus hominis* AUP19 was investigated in a combined approach for bioremediation and valorization of POME into lipases. *M. spicifer* AW2 and *S. hominis* AUP19 were isolated from palm oil-contaminated wastes, producing synergistic effects and increasing the yield

of target products. *M. spicifer* AW2 produces short hyphae and serves as the dispersion factor for *S. hominis* AUP19, helping each other for substrate utilization [5,17]. The 50% POME was suitable for yeast and bacterium co-culture growth with high survivability, bioremediation efficiency, and CBLs production [5]. The use of 50% POME was continued in this research, where the co-culture lipases production was optimized using the combination of one variable at a time (OVAT), Plackett–Burman design (PBD), and response surface methodology (RSM) to achieve an efficient bioprocess optimization at a minimum cost [19].

Biodiesel is an environmentally friendly transportation fuel. Biodiesel has a higher cetane number, produces less smoke, contains smaller particles, is biodegradable, and is harmless. Chemical techniques employ acidic and basic catalysts, whereas biological approaches employ lipases as biocatalysts. Biodiesel production using biological methods utilizing lipases has gained popularity since it is safer and can be made with low-cost feedstock containing a high concentration of fatty acids [6,20]. Additionally, regarding the application of lipase in biodiesel production, CBLs reduce the cost of production since the enzymes will be easily separated from the product, allowing the enzymes to be reused without cell reproduction. The enzyme activity can be maintained and has a tolerance for high temperatures and organic solvents [21]. The application of the yeast–bacterium CBLs system for enzymatic biodiesel generation uses various lipases, each with particular characteristics that can function on several substrates [22]. Natural oils comprise triglycerides with different fatty acids, and the reaction mixture consists of triglycerides and free fatty acids. Hence, a mixture of cell-bound lipases with different specificities (hydrolysis and conversion) that act upon several substrates could be an optimal biocatalyst system [23,24]. The one-step reaction by the combined use of lipases took a shorter time to reach the maximum biodiesel yield than the two-step reaction due to the synergic effect of the mixed lipases (Lipase AY performed hydrolysis, whereas Lipase AK converted fatty acids to biodiesel) [25].

It has been reported that many microorganisms can biodegrade POME. The microbial system produces lipid-degrading enzymes, such as *Candida* and *Bacillus* species isolated from solid and liquid wastes from grease traps showed lipolytic activity in decreasing the oil and grease (O&G) of POME samples; O&G removal varied from 56 to 79% after 72 h of treatment. The consortium of five degrading yeasts from this research produced O&G removal at 84% in POME samples after 48 h [26]. However, the biodegradation rate depends on the physiological state of the microorganisms, which are sensitive to variable environmental factors. Therefore, the idea of using membrane-bound lipases in this research could potentially overcome the problems related to the physiological state of microorganisms.

The present study investigated the optimization of CBLs production via valorization of oily wastes and their potential applications as whole-cell biocatalysts in biodiesel synthesis via esterification and transesterification reactions as well as in palm oil mill effluent bioremediation. This research is the first report on the statistical optimization of blended oily wastes valorization into CBLs by yeast–bacterium co-culture with the investigation of their potential applications in solvent-free biodiesel synthesis and direct application of wet cells in wastewater bioremediation. The utilization of cheap agro-industrial oily wastes as microbial substrate alternatives for value-added whole-cell lipases production with potential applications is highlighted in this work.

2. Materials and Methods

2.1. Samples and Chemicals

Palm oil mill effluent (POME) was obtained from the palm oil mill industry, Larp Tavee Palm Oil Co., Ltd., (Satun, Thailand). Crude glycerol (CG) was kindly provided from the biodiesel pilot plant, Prince of Songkla University, Thailand. Waste frying oil (WFO) was acquired from local fried chicken stalls (Songkhla, Thailand) and molasses (MO) was purchased from a local retailer located in Songkhla, Thailand. The refined palm oil product (Sime Darby Oils Morakot PCL, Bangkok, Thailand) was purchased from the local

supermarket. Other chemicals and reagents used in this study were of analytical grade supplied by Kemaus Chemicals Elago Enterprises Pty Ltd. (New South Wales, Australia), Loba Chemie Pvt. Ltd. (Mumbai, India), Ajax Finechem Pty Ltd. (New South Wales, Australia), Nacalai Tesque, Inc. (Kyoto, Japan), RCI Labscan Co., Ltd. (Bangkok, Thailand), and various components were purchased from HiMedia Laboratories Pvt. Ltd. (Maharashtra, India) which procured through local suppliers in Hatyai, Thailand.

2.2. Palm Oil Mill Effluent (POME)-Based Media Preparation and Their Characteristics

The CBLs production medium was prepared by centrifuging raw POME at 4000× *g*, 4 °C, for 15 min to eliminate solids. Then, the POME supernatant was diluted at a ratio of 1:1 by adding distilled water to obtain 50% POME (POME50). The physicochemical characteristics of POME50 and waste frying oil used in this study were determined in the previous research [5,27], whereas the properties of crude glycerol and molasses were characterized by our research group, Baloch et al. [10]. The characteristics of POME50, crude glycerol, waste frying oil, and molasses used in this study are presented in Supplementary Materials Table S1. In this research, the oil and grease (O&G) and chemical oxygen demand (COD) of POME (before and after cultivation) were determined to obtain the O&G removal and COD removal values. O&G (mg/L), COD (mg/L), and nitrogen (mg/L) were analyzed using the EPA Method 1664, closed reflux method, and Total Kjeldahl Nitrogen (TKN) method, respectively. Whereas pH was analyzed following the standard method [5].

2.3. Microbial Strains and Inoculum Preparation

In this study, the CBL-producing microorganisms, *Magnusiomyces spicifer* AW2 (MF135611) and *Staphylococcus hominis* AUP19 (MW595703) were attained from the Laboratory of Molecular Biotechnology, Faculty of Agro-Industry, Prince of Songkla University, Thailand. The seed culture was prepared by following the previous experimental report where the mixed yeast–bacterium seed culture (ratio 1:1) at 10^7 cells/mL was inoculated into the growth media at a total concentration of 10% (*v*/*v*) [5].

2.4. Microbial Growth and Cell Biomass (CBM) Determination

The viable yeast and bacterium cells in the co-culture system were calculated by cell number determination using serial dilution and cell counting on a basal standard medium (BSM) agar plate. Samples were collected from the culture broth every 24 h for 5 days. The colonies grown on the agar plate were observed and counted as colony forming unit (CFU) per mL (CFU/mL) [15]. Two milliliters of culture broth were centrifuged at 8000× *g* for 15 min at 4 °C to obtain cell pellets. Then, the pellets were washed twice with sterilized 0.1 M phosphate buffer pH 7.0 to remove the remaining medium. Finally, it was dried at 60 °C until the constant weight was obtained and CBM was expressed in g/L [5].

2.5. The Lipases Hydrolytic Activity Assays

To determine the lipases hydrolytic activity assay, 1 mL culture broth was centrifuged at 4000× *g* for 10 min at 4 °C. The supernatant (200 µL) was taken for extracellular lipases (ECLs) activity assay, whereas the cell pellets were for CBLs activity measurement. The cell pellets were washed twice using phosphate buffer pH 7.0 to remove impurities. The hydrolytic activities of ECLs and CBLs were determined using the modified cupric acetate method with 10% (*v*/*v*) palm oil in isooctane as the substrate. The reaction of all samples and control was performed by shaking at 300 rpm for 30 min at room temperature (30 ± 2 °C). The reaction was stopped by adding 0.3 mL 6 N HCl. Then, 1 mL of the upper layer was withdrawn and mixed with 0.4 mL cupric acetate solution. The free fatty acid content dissolved was determined at 715 nm against the control, which contained no free fatty acid. The hydrolytic activity was calculated by measuring the amount of fatty acid from a standard curve of palmitic acid. The standard curve was prepared by dissolving various weights of palmitic acid in isooctane to obtain various concentrations (µmol/mL).

The solution was mixed with a cupric-acetate reagent, and the absorbance of the upper isooctane layer of palmitic acid solution was measured at 715 nm against the control. The results were plotted to form the standard curve and equation. The enzyme activity at one unit is defined by the enzyme required to release 1 µmol palmitic acid per min under the specified conditions [5,17,28]. ECLs activity was measured based on the volume of the enzyme (U/L), whereas CBLs activity was measured based on hydrolytic activity and dry cell weight (U/g). The U/g was then converted to U/L by multiplying with cell biomass in g/L. The calculation was performed following Equations (1)–(3).

$$\text{ECLs Activity (U/L)} = \frac{\text{OD(715 nm)} \times \text{V.Total of 1st step(mL)} \times \text{V.Total of 2nd step(mL)} \times \text{Dilution Factor}}{\text{Slope} \times \text{V.Upper layer(mL)} \times \text{V.Enzyme and buffer(mL)} \times \text{Reaction Time(min)}} \times 1000 \quad (1)$$

$$\text{CBLs Activity (U/g)} = \frac{\text{OD(715 nm)} \times \text{V. Total of 1st step(mL)} \times \text{V. Total of 2nd step(mL)} \times \text{Dilution Factor}}{\text{Slope} \times \text{V. Upper layer(mL)} \times \text{W. Cell biomass(g/mL)} \times \text{Reaction Time(min)}} \quad (2)$$

$$\text{CBLs Activity (U/L)} = \text{CBLs activity (U/g)} \times \text{Cell biomass (g/L)} \quad (3)$$

2.6. Experimental Design and Statistical Analysis

2.6.1. Selection of Medium Composition for Valorization Process of Agro-Industrial Wastes into CBLs by One Variable at a Time (OVAT)

The pure cultures of *M. spicifer* AW2 and *S. hominis* AUP19, as well as their co-culture, were cultivated in a 250 mL Erlenmeyer flask containing 90 mL of POME50-based media for the selection of medium composition for CBLs production. Firstly, the POME-based media including 50% POME (POME50), POME50 + 2.0% (*v*/*v*) crude glycerol (CG), POME50 + 2.0% (*v*/*v*) waste frying oil (WFO), and POME50 + 2.0% (*v*/*v*) molasses (MO) were prepared and sterilized using autoclave at 121 °C and 15 psi for 15 min. All the prepared media were adjusted to pH 7.0 and added with 10% (*v*/*v*) microbial seed culture to obtain a 100 mL total volume of culture broth. Then, the mixtures were incubated at room temperature (30 ± 2 °C), shaking at 150 rpm for 24 h. After incubation, the CBLs were harvested from each media and measured for their hydrolytic activities and cell biomass. Then, the substrate showing high CBLs production was selected and then supplemented with 0.5% (*w*/*v*) nitrogen sources, including urea (UR), ammonium sulfate (AS), ammonium nitrate (AN), yeast extract (YE), beef extract (BE), and soytone (SY). The prepared medium was treated under the same conditions, and the CBLs were harvested for measurement. Next, the substrate with the best nitrogen source producing high CBLs activity was selected and studied for the effect of surfactant, i.e., Gum Arabic (GA), Tween-80 (TW), and Triton X-100 (TX). Each surfactant was added to the medium at 0.1% (*v*/*v*) and mixed well using a blender to obtain a homogenous culture broth. The same conditions were applied, and the POME-based medium containing oily waste, nitrogen source, and surfactant with the highest CBLs production was selected. Further, the components in this medium were screened for the key factors that significantly affect enzyme production using the Plackett–Burman design (PBD).

2.6.2. Experimental Design by Plackett–Burman Design (PBD) and Optimization of Key Parameters by Response Surface Methodology-Central Composite Design (RSM-CCD)

Plackett–Burman design (PBD) aimed to screen the key factors affecting oily waste substrate valorization to CBLs. Five independent variables, including WFO, AS, GA, inoculum size, and initial pH, were examined with each factor at low level (−1) and high level (+1). The low and high levels of each factor were selected based on other studies which used oily media, such as refined vegetable oil, waste frying oil, and POME as the substrate for microbial growth or bioremediation process [6,9,29–33]. The CBLs (U/L), CBM (g/L), and O&G removal (%) were the dependent variables. In total, 12 experimental runs were generated by Design-Expert® Version 13 Software Trial (Stat Ease Inc., Minneapolis, USA) to determine the responses (Table 2).

Table 2. The design matrix of Plackett-Burman design (PBD) for evaluating significant factors influencing CBLs, CBM, and O&G removal.

Run	Factor: A Waste Frying Oil % (*v*/*v*)	Factor: B Ammonium Sulfate % (*w*/*v*)	Factor: C Gum Arabic % (*w*/*v*)	Factor: D Inoculum Size % (*v*/*v*)	Factor: E Initial pH	CBLs Activity (U/L)		CBM (g/L)		O&G Removal (%)	
						Act. *	Pred. **	Act. *	Pred. **	Act. *	Pred. **
1	4.0	0.5	0.5	20.0	4.00	3413	2879	16.6	13.7	49.5	43.3
2	4.0	2.0	0.1	20.0	7.00	3132	3027	17.9	16.1	45.4	45.4
3	4.0	2.0	0.1	10.0	4.00	1558	1732	6.9	8.7	27.2	27.1
4	2.0	0.5	0.5	10.0	7.00	4342	4098	17.8	18.2	53.9	56.2
5	4.0	0.5	0.1	10.0	7.00	3451	3349	17.1	15.9	48.2	51.5
6	4.0	0.5	0.5	20.0	7.00	3299	3822	16.9	19.9	54.4	56.5
7	2.0	0.5	0.1	20.0	4.00	2908	3389	7.9	10.4	49.7	48.4
8	2.0	2.0	0.5	10.0	7.00	3172	3424	17.9	17.2	51.1	45.0
9	4.0	2.0	0.5	10.0	4.00	1807	1852	10.3	11.5	26.0	26.9
10	2.0	2.0	0.1	20.0	7.00	3981	3657	15.3	15.6	51.9	50.3
11	2.0	0.5	0.1	10.0	4.00	3160	3036	10.8	9.2	43.5	43.2
12	2.0	2.0	0.5	20.0	4.00	2876	2835	13.2	12.2	30.0	37.0

* Actual value from the experiment. ** Predicted value from the software output.

Next, the effect of all factors on the responses was calculated by analysis of variance (ANOVA). The most significant effect on the responses was selected and optimized using response surface methodology-central composite design (RSM-CCD) (Table 3). Twenty experiment runs (Table 4) were conducted based on the experimental runs generated by the same software. The significant difference between the predicted value and the actual value was determined using ANOVA. Finally, the validation experiment was performed following the conditions predicted by the CCD. Then, the responses were applied to predict the polynomial model using standard regression.

Table 3. Maximum and minimum levels of variables used for optimizing the production of CBLs and CBM with O&G removal in RSM-CCD.

Variable	Unit	Level Code				
		−1.68	**−1**	**0**	**1**	**+1.68**
Waste Frying Oil	% (*v*/*v*)	1.32	2.00	3.00	4.00	4.68
$(NH_4)_2SO_4$	% (*w*/*v*)	0	0.5	1.25	2.0	2.51
Initial pH	-	2.98	4.00	5.50	7.00	8.02

Table 4. RSM-CCD matrix with actual and predicted values of the CBLs, CBM, and O&G removal by the co-culture of *M. spicifer* AW2 and *S. hominis* AUP19 in blended oily wastes media.

Run	Factor: A Waste Frying Oil % (*v*/*v*)	Factor: B Ammonium Sulfate % (*w*/*v*)	Factor: C Initial pH	CBLs Activity (U/L)		CBM (g/L)		O&G removal (%)	
				Act. *	Pred. **	Act. *	Pred. **	Act. *	Pred. **
1	4.00	2.00	7.00	4293	4476	21.6	22.2	57.7	57.8
2	3.00	1.25	5.50	4375	4399	18.3	20.4	52.1	54.7
3	4.00	2.00	4.00	2885	2951	9.9	11.8	47.6	49.2
4	3.00	1.25	2.98	1705	1716	5.2	3.7	43.9	42.4
5	2.00	0.50	4.00	2618	2554	7.2	7.9	42.6	44.3
6	3.00	1.25	5.50	4245	4399	22.5	20.4	55.3	54.7
7	4.00	0.50	4.00	3504	3269	15.0	13.6	50.8	50.0
8	3.00	1.25	5.50	4088	4399	19.9	20.4	55.6	54.7
9	3.00	1.25	5.50	4474	4399	22.1	20.4	56.1	54.7
10	3.00	1.25	5.50	4314	4399	18.7	20.4	54.7	54.7
11	4.00	0.50	7.00	3682	3492	16.8	15.7	51.6	51.8
12	1.32	1.25	5.50	4404	4074	22.3	19.9	56.0	53.2
13	3.00	1.25	5.50	4766	4399	20.4	20.4	53.9	54.1
14	3.00	2.51	5.50	4426	3941	21.3	17.3	56.0	53.2
15	2.00	2.00	7.00	4663	5016	23.6	26.4	58.2	60.9
16	3.00	1.25	8.02	4296	4117	21.0	20.7	58.9	57.9
17	4.68	1.25	5.50	4059	4221	20.6	21.2	55.1	55.3
18	3.00	0.00	5.50	2941	3257	9.4	11.5	48.2	48.3
19	2.00	2.00	4.00	2074	2383	5.9	8.3	42.7	44.3
20	2.00	0.50	7.00	3832	3885	18.3	17.6	53.9	54.7

* Actual value from the experiment. ** Predicted value from the software output.

All culture media for PBD and RSM-CCD experiments were prepared in 250 mL Erlenmeyer flasks with a total volume of 100 mL each, which consisted of 10% (*v*/*v*) seed culture and 90 mL POME-based media. Each media composition was prepared following the guideline of each run set by the software for PBD and CCD experiments. The pH of the media was adjusted by adding 50% (*w*/*v*) NaOH or 98% (*v*/*v*) H_2SO_4 solutions. Then, each medium was mixed well and sterilized before adding the seed culture. Finally, the mixtures were incubated at room temperature (30 ± 2 °C) with shaking at 150 rpm for 24 h [5]. The CBLs were harvested and measured for their activity, whereas the culture broth was collected for cell biomass determination and O&G removal. All experiments in each run were performed in triplicate.

2.7. Time Course Study of CBLs Production under Optimal Conditions

The time-course experiment for further investigation of the growth profile of the yeast–bacterium co-culture was conducted. At a 1:1 (10% *v*/*v*) ratio, the yeast–bacterium seed culture was inoculated to the optimum medium of 90 mL in a 250 mL Erlenmeyer flask with the total volume of the culture broth of 100 mL. The CBLs and ECLs activities were assayed using the cupric acetate method (U/L). The cell growth was determined from CBM calculation (g/L) and microbial colony observation on basal standard medium (BSM) agar and BSM^+ containing 10% (*v*/*v*) tartaric acid agar plates [5]. The colonies grown on the agar plate were observed and counted (CFU/mL) [15]. The ECLs and CBLs activities, CBM, cell number, pH changes, and the O&G removal were measured every 24 h for 5 days.

2.8. Application of CBLs in Biodiesel Production

The CBLs were harvested from the optimized culture broth by centrifugation at 4000× *g*, 4 °C for 10 min. The palm oil substrate (0.2 g) was mixed with methanol (oil to methanol molar ratio at 1:3) for the transesterification reaction, whereas oleic acid (0.55 g) was mixed with methanol (oil to methanol molar ratio at 1:1) for the esterification reaction. The wet cells of 45 U (approximately 45 mg dried cell weight or 10^8 cells/mL) suspended in 10% (*v*/*v*) 0.1 M phosphate buffer pH 7.0 (based on the total volume of the substrate) were added to each substrate mixture. The reaction was conducted in a thermomixer at room temperature (30 ± 2 °C), shaking at 300 rpm for 72 h [5]. After incubation, 40 µL of the upper phase was withdrawn every 24 h, and the reactions were prolonged to 96 h. The upper phase samples were transferred to a 1500 µL GC vial and mixed with 50 µL methyl heptadecanoate (C17:0) as an internal standard. Then, the final volume was adjusted to 500 µL by adding n-heptane. Finally, the biodiesel formed was analyzed using gas chromatography, GC (Hewlett Packard Plus 6850 series, Agilent, Santa Clara, CA, USA) equipped with the capillary column, 30 m length, 320 µm I.D., 0.25 film thickness (Agilent J&W Select Biodiesel GC Column, Agilent, Santa Clara, CA, USA), and a flame ionization detector (FID). The GC-FID conditions were set according to Baloch et al. [17]. The weight percentage of the fatty acid methyl esters (FAME) was determined by Equation (4).

$$C = \frac{(\sum A) - AEI}{AEI} \times \frac{CEI \times VEI}{m} \times 100\% \quad (4)$$

where *A* is the total peak area from methyl ester in C14 to that in C24, *A*EI is the peak area corresponding to methyl heptadecanoate, *C*EI is the concentration in milligrams per milliliter of methyl heptadecanoate solution, *V*EI is the volume, in milliliters of methyl heptadecanoate solution, and m is the mass, in milligrams of the sample.

2.9. Palm Oil Mill Effluent (POME) Bioremediation by CBLs

The CBLs obtained were investigated for their performance in removing O&G and COD in palm oil mill effluent (POME). The effect of various concentrations of sterilized POME (raw POME, POME100, POME50, and POME25) was studied. Raw POME was collected directly from the palm oil mill site, brought to the laboratory, and centrifuged at 4000× *g*, 4 °C, for 15 min to eliminate solids. The centrifuged POME (POME100) sample

was stored at −20 °C until further use. The POME was diluted with water at 1:1 to obtain POME50 and at 1:3 to obtain POME25. Before the bioremediation process, the effluents were sterilized by autoclave at 121 °C and 15 psi for 15 min. The physicochemical characteristics of raw POME, POME100, POME50, and POME25 after dilution were measured [5]. The CBLs were harvested from the optimized medium (24 h) by taking 10 mL of culture broth. The CBLs were collected by centrifugation at 4000× *g*, 4 °C, for 10 min and washed twice with sterilized 0.1 M phosphate buffer pH 7.0 to remove the remaining medium. Then, the pellets were added with prepared effluents until the volume reached 10 mL. The 10% (*v*/*v*) cell suspension was then inoculated to the 250 mL Erlenmeyer flasks containing effluents at 90 mL. The oil and grease (O&G) and chemical oxygen demand (COD) removals of POME were determined after 3 days of cultivation using the EPA Method 1664 and the closed reflux APHA method, respectively. Whereas pHs of the effluents before and after the bioremediation process were measured using the standard method [5].

2.10. Data Analysis

All experiments in this research were performed in triplicate. The statistical analyses of PBD and RSM-CCD results were performed by one-way analysis of variance (ANOVA), and the responses were applied to predict the polynomial model using standard regression in Design-Expert® Version 13 Software Trial (Stat Ease Inc., Minneapolis, MN, USA). The significant difference between the predicted and actual values was determined using Duncan's multiple range tests ($p < 0.05$) with IBM SPSS Statistical Subscription.

3. Results and Discussion

3.1. Selection of Medium Composition for Valorization Process of Agro-Industrial Wastes into CBLs by One Variable at A Time (OVAT)

This research used the low-cost agro-industrial wastes, i.e., palm oil mill effluent (POME) and POME added with 2.0% (*v*/*v*) agro-industrial wastes as the growth media for yeast *Magnusiomyces spicifer* AW2, bacterium *Staphylococcus hominis* AUP19, and yeast–bacterium CBLs production. POME at 50% (*v*/*v*) or POME50 was utilized as the base medium to reduce high chemical oxygen demand (COD) concentration and inhibitors that can inhibit the optimal growth of yeast and bacterium strains [5,14]. The results are shown in Figure 1 where the production of lipases and cell biomass by the pure culture of *M. spicifer* AW2 (Figure 1a) and pure culture of *S. hominis* AUP19 (Figure 1b) are compared to the yeast–bacterium co-culture (Figure 1c). The CBLs produced by yeast strain, bacterium strain, and co-culture at 24 h were highest in POME50+waste frying oil (WFO), followed by POME50, POME50+crude glycerol (CG), and POME50+molasses (MO). The co-culture produced the highest CBLs activity at 3474 U/L with CBM at 19 g/L and ECLs at 691 U/L, followed by yeast CBL at 2979 U/L and bacterium CBL at 1452 U/L. The 24 h incubation was the best for CBLs production since the CBLs were already synthesized and attached to the cell membrane, which could be detected at the early logarithmic phase (12 h). Then, the enzyme accumulation began during the logarithmic phase (18 h), peaked at the late log phase (24–36 h), and released to the medium as ECLs at the stationary phase (after 36 h) [5].

It could be suggested from this experiment that the addition of WFO induced the synthesis of lipases. The effect of WFO on the lipase production investigated in this research was similar to the lipase production by *Rhizopus arrhizus*, where its intracellular lipase activity was improved by 30% to 520 U/g when compared to the product from the medium without waste cooking oil supplementation [34]. The lipase production by *Y. lipolytica* cells using waste soybean frying oil collected from a restaurant proved that the oil effectively induced cell-wall-associated lipase production with activity at 211 $U/g_{\text{-dried cell weight}}$ [35]. Interestingly, it was found that adding molasses to the medium might alter microbial adaptation and drastically decrease all CBLs production in yeast, bacterium, and their co-culture form. By adding the molasses, the lipid-rich habitat was shifted to a sugar-rich environment and changed the response of the strains toward lipase production. As observed in this research, the microorganism preferred to utilize sugars more than lipids or

fatty acids in the medium and produced low lipase activities. Generally, microorganisms utilize the simplest carbon source, such as monosaccharides, for their metabolism and later break down more complex molecules [10]. In this research, the presence of molasses in the growth medium lowered the lipase production rate because of an increase in the medium viscosity. High viscosity in the medium lowers the oxygen transfer rate, thus affecting the osmolarity and reducing microbial growth [36].

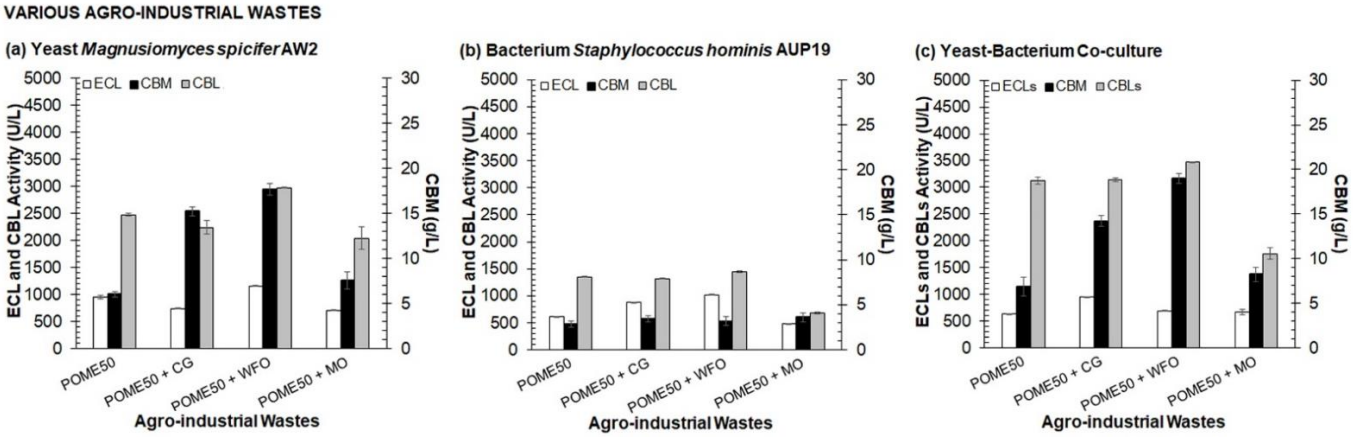

Figure 1. Selection of medium composition by one variable at a time (OVAT) for CBLs production in various agro-industrial wastes, including 50% POME (POME50), POME50 + 2.0% (*v*/*v*) crude glycerol (CG), POME50 + 2.0% (*v*/*v*) waste frying oil (WFO), POME50 + 2.0% (*v*/*v*) molasses (MO) by pure *M. spicifer* AW2 (**a**), pure *S. hominis* AUP19 (**b**), and co-culture *M. spicifer* AW2 + *S. hominis* AUP19 (**c**) at room temperature (30 ± 2 °C), with shaking at 150 rpm for 24 h.

Further, it was found that adding nitrogen sources, including urea (UR), ammonium sulfate (AS), ammonium nitrate (AN), yeast extract (YE), beef extract (BE), and soytone (SY), enhanced the CBL activity in yeast and bacterium pure cultures, and the presence of AS in the medium gave the highest CBLs production in the pure cultures and co-culture system compared to other nitrogen sources as shown in Figure 2.

Figure 2a shows that AS, AN, BE, and SY enhanced the CBLs production, whereas SY significantly improved CBM production by yeast *M. spicifer* AW2. Meanwhile, the bacterium *S. hominis* AUP19 produced high CBLs activity in the presence of AS, YE, and BE, with the highest CBM production in oily waste medium containing YE (Figure 2b). The nitrogen sources improved the yeast–bacterium CBLs production with high activity, except UR, which did not enhance the production compared to the control experiment. Meanwhile, AS improved the production of CBLs with activity up to 4198 U/L in the co-culture form, whereas YE significantly affected CBM production at 27 g/L (Figure 2c). Based on these findings, the cells rapidly utilize the inorganic nitrogen, whereas organic nitrogen is essential for cell growth and amino acid supply for cell metabolism and protein synthesis. Similarly, AS enhanced lipase production by *Y. lipolytica* strain TISTR5151 to 3353 U/L lipase yield [6].

The effect of various surfactants, including Gum Arabic (GA), Tween 80 (TW), and Triton X-100 (TX) added in the medium at 0.1% (*v*/*v*), was investigated on CBLs production. It was found that GA enhanced the CBLs production in pure and co-cultures (Figure 3). By adding GA, the yeast CBL activity was improved from 4029 to 4122 U/L, but not significant (Figure 3a), whereas the bacterium CBL activity was significantly enhanced from 2301 to 2696 U/L (Figure 3b). The combination of yeast–bacterium in a co-culture system could improve CBL production with the highest activity at 4460 U/L (Figure 3c). In this research, GA acted as an emulsifier for the oil in the medium. The emulsifier agent addition in oily substrates facilitates the enzyme-substrate interaction both in solution and at the interface by incorporating it into the oil layer and performing an ideal oil-water emulsion stabilizer which enhances the accessibility and availability of the substrates to the enzyme active

sites [37]. Therefore, waste frying oil (WFO), ammonium sulfate (AS), and Gum Arabic (GA) were selected as the variables for further optimization steps.

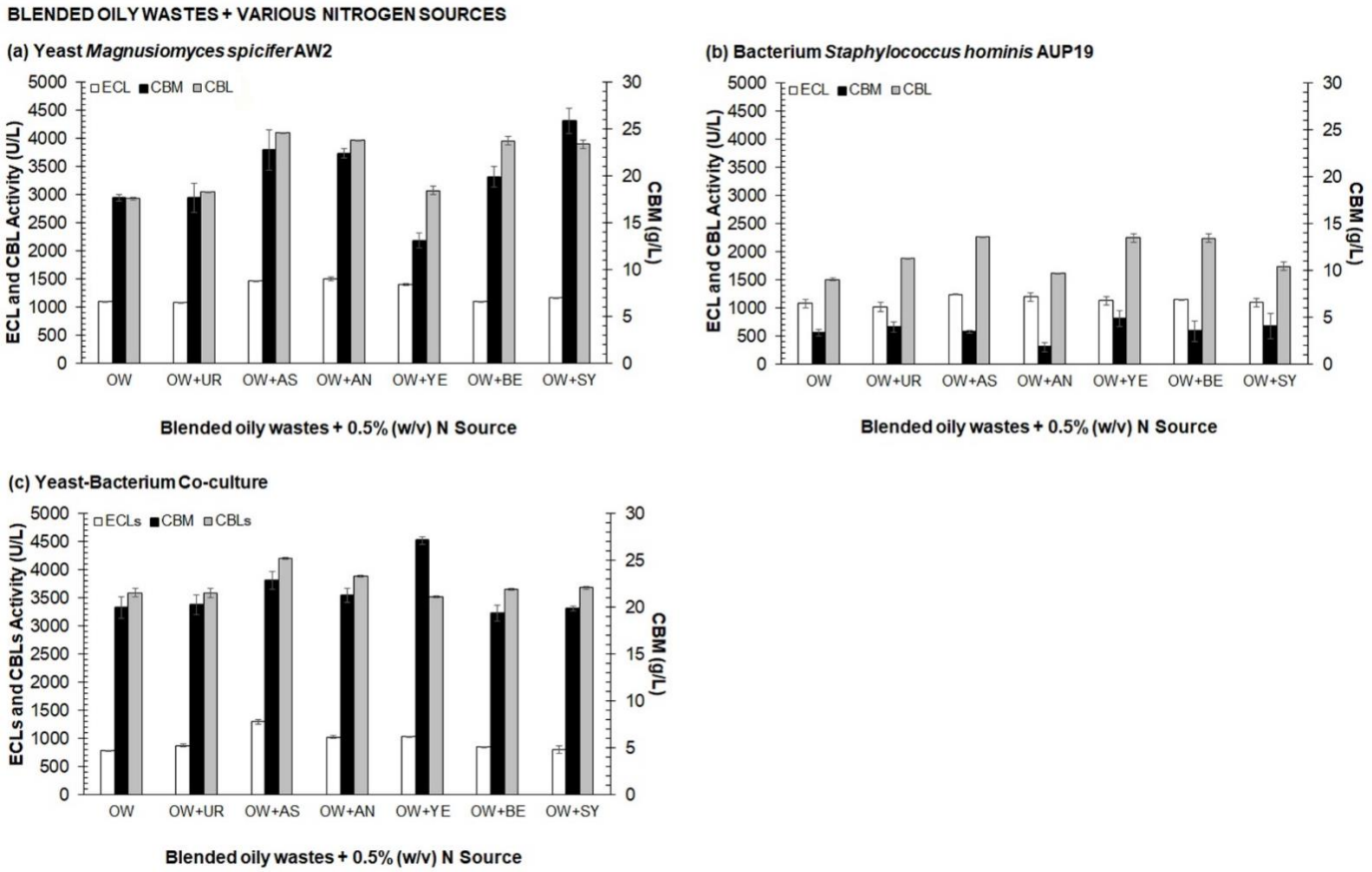

Figure 2. Selection of medium composition by one variable at a time (OVAT) for CBLs production in blended oily wastes (POME50 + 2.0% (*v*/*v*) WFO) + 0.5% (*w*/*v*) nitrogen sources, including urea (UR), ammonium sulfate (AS), ammonium nitrate (AN), yeast extract (YE), beef extract (BE), and soytone (SY) by pure *M. spicifer* AW2 (**a**), pure *S. hominis* AUP19 (**b**), and co-culture *M. spicifer* AW2 + *S. hominis* AUP19 (**c**) at room temperature (30 ± 2 °C), shaking 150 rpm for 24 h.

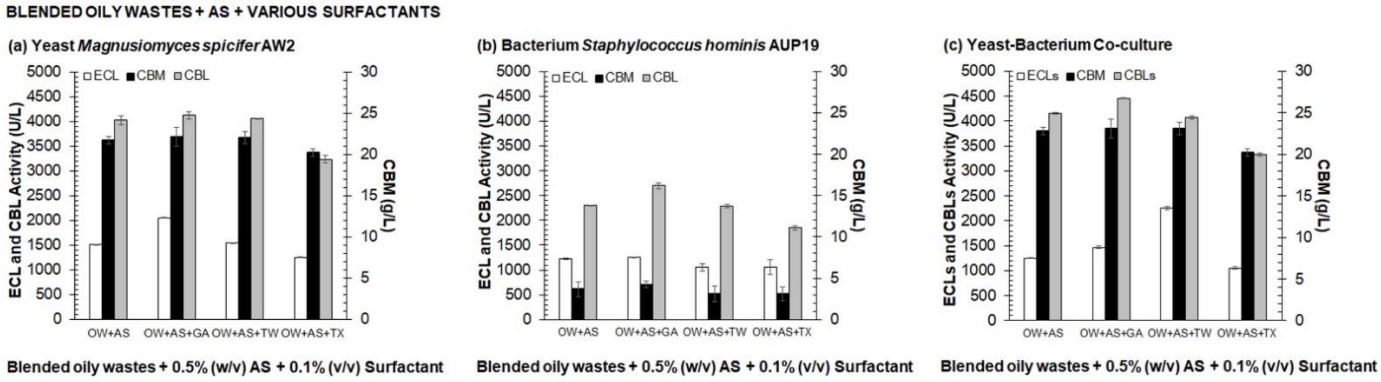

Figure 3. Selection of medium composition by one variable at a time (OVAT) for CBLs production in POME50 + 2.0% (*v*/*v*) WFO + 0.5% (*w*/*v*) AS + 0.1% (*v*/*v*) surfactants, including Gum Arabic (GA), Tween 80 (TW), and Triton X-100 (TX) by pure *M. spicifer* AW2 (**a**), pure *S. hominis* AUP19 (**b**), and co-culture *M. spicifer* AW2 + *S. hominis* AUP19 (**c**) at room temperature (30 ± 2 °C) with shaking at 150 rpm for 24 h.

3.2. Screening of Significant Parameters Using Plackett-Burman Design (PBD)

Based on the results obtained from one variable at a time (OVAT), the medium containing POME50 added with 2.0% (*v*/*v*) waste frying oil (WFO), 0.5% (*w*/*v*) ammonium sulfate (AS), and 0.1% (*v*/*v*) Gum Arabic (GA) was used for the Plackett–Burman design (PBD) experiments. The POME50 was added with 2.0% (*v*/*v*) and 4.0% (*v*/*v*) waste frying oil as the low and high levels as the substrate for lipase production. These concentrations were selected because POME50 has already contained 5562 mg/L or 0.56% (*w*/*v*) O&G. Some research used refined oil- or waste frying oil-based medium and applied 0.5–8.0% (*v*/*v*) of the oily compound as the sole carbon source. The wide lipase production using these WFO concentrations depends on culture conditions, co-substrates added, and metabolites target. In cultures with Gum Arabic as an emulsifier, 1.0% (*w*/*v*) WFO was sufficient to obtain maximum lipase activity by *Y. lipolytica* [29]. The lipase production by *Bacillus cereus* ASSCRC-P1 in waste frying oil-based medium used 4.0% (*v*/*v*) and 8% (*v*/*v*) as the low and high levels in its statistical optimization strategy. Notably, 8% (*v*/*v*) oil gave optimum lipase activity [38]. Palm oil at 0.5% (*w*/*v*) was added to an undiluted POME medium to provide high oil concentrations for yeast–bacterium co-culture growth [39]. The ammonium sulfate concentrations ranging from 0.2 to 5.0% (*w*/*v*) were added to the oil- and POME-based media and gave high microbial survivability [6,29,40,41]. For example, Rodriguez-Mateus et al. [26] described 0.3% (*w*/*v*) ammonium sulfate as the optimum concentration for O&G degradation by *Candida* and *Bacillus* species. The CBL hydrolytic activity of *R. mucilagenosa* increased with the concentration of palm oil and Gum Arabic between 1.90 and 2.20% (*w*/*v*) and 0.37 and 0.45% (*w*/*v*) [42]. The microbial inoculum size is also an important factor, or a small inoculum lengthens the lag phase, and lowered the lipase production, whereas a large inoculum results in excessive strain niche overlap with too much biomass production [31,43,44]. Generally, 5.0–20.0% (*v*/*v*) inoculum size was added to the growth medium and investigated for its effect on metabolite target production, and the results depend on the strain characteristics. Therefore, 10% (*v*/*v*) and 20% (*v*/*v*) of inoculum size were applied as the low and high levels in PBD (Table 2). Using waste cooking oil and olive oil as carbon sources and adding 5.0% (*v*/*v*) inoculum gave the highest lipase production by *B. subtilis* at 4.96 U/mL [45]. The investigation of 5.0–20.0% (*v*/*v*) inoculum level of *B.* marisflavi NA8 to produce bioflocculant in POME showed that 5.0% (*v*/*v*) inoculum size yielded 6.4 g/L of product [31]. The pH 4.0 and 7.0 were chosen for PBD based on the previous research conducted, where the ECL, CBL, and cell growth of *M. spicifer* AW2 and *S. hominis* AUP19 were at the highest level in the POME media pH 7.0, and a substantial reduction was found at pH 3.8 [5].

The value of CBLs activity, CBM production, and O&G removal obtained in PBD are given in Table 2. The CBLs activity produced by the co-culture of *M. spicifer* AW2 and *S. hominis* AUP19 was found to vary from 1558 to 4342 U/L in the 12 experimental runs conducted, which shows the influence of medium components mainly on CBLs production. Based on the ANOVA results (Supplementary Materials Tables S2–S4), all models in the PBD experiment were significant (p-value < 0.05). Table S2 shows that the p-value of the model for CBLs production at 0.0229 was significant, with R-Squared 0.8383 indicating that the model could explain 83.33% of the variability in the responses. The significant factors for CBLs production were A-WFO, B-AS, and E-initial pH, whereas C-GA and D-inoculum size were insignificant for CBLs production. Table S3 shows the PBD model for CBM production where the p-value of 0.0419 was significant and the R-squared 0.7987 showing that the model could explain 79.87% of the variability in the responses. However, the E-initial pH was the only factor significantly affecting yeast–bacterium co-culture CBM production. The O&G removal PBD model showed a p-value of 0.0110, indicating the significance of the model at this level (Table S4). The R-squared for the O&G removal model was 0.8753 representing that the model could explain 87.53% of the variability in the responses. The significant factors affecting O&G removal were B-AS and E-initial pH. The regression equation predicted the factors that affected the response. Therefore, when

including the insignificant factors (p-value greater than 0.05), the model equation for all responses can be generated as:

$$\text{CBLs (U/L)} = 2161.11475 - 314.88758 * A - 449.73198 * B + 120.13389 * C + 35.31252 * D + 314.07504 * E \quad (5)$$

$$\text{CBM (g/L)} = -3.24259 + 0.24167 * A - 0.63333 * B + 2.81667 * C + 0.11833 * D + 2.06481 * E \quad (6)$$

$$\text{O\&G removal (\%)} = 29.33333 - 2.45000 * A - 7.51111 * B - 0.16667 * C + 0.51667 * D + 4.38889 * E \quad (7)$$

where A is waste frying oil, B is ammonium sulfate, C is Gum Arabic, D is inoculum size, and E is initial pH.

The PBD approach also allowed reliable factors for fermentation in the medium or nutrient components in this research. In this case, WFO, AS, and initial pH were the most significant effects on CBLs production, whereas initial pH affected the CBM production significantly, and AS, together with initial pH, had a significant effect on the O&G removal. Therefore, three factors (WFO, AS, and initial pH) were selected and further employed to optimize the production of CBLs and CBM with O&G removal using response surface methodology-central composite design (RSM-CCD). All remaining factors (POME50, GA 0.1% w/v, inoculum size 10% v/v, shaking at 150 rpm, incubation at RT for 24 h) were kept constant. Similarly, the PBD also allowed the evaluation of fermentation medium components for lipase production by *Pseudomonas fluorescens*. The results of PBD analysis showed the most significant parameters affecting bacterium lipase production [46].

3.3. Statistical Optimization Procedure Using Response Surface Methodology-Central Composite Design (RSM-CCD)

The optimized level of each variable (WFO, AS, and initial pH) was investigated using response surface methodology-central composite design (RSM-CCD), and the responses (the production of CBLs and CBM with O&G removal) for each run were specified by the software; the predicted responses are shown in Table 3.

The CBLs activity was produced in the range of 1705 to 4766 U/L. While the CBM was produced between 5.2 and 23.6 g/L and O&G removal was achieved, ranging from 42.6 to 58.9% (Table 4). The annotation of the function of each parameter are WFO (A), AS (B), and initial pH (C).

Figure 4 shows the plots described by the model for yeast–bacterium CBLs production. The plots formed as the results of the interaction between WFO with AS (Figure 4a), WFO with initial pH (Figure 4b), and AS with initial pH (Figure 4c). The interaction between WFO with initial pH (AC) and AS with initial pH (BC) significantly improved the CBLs production with high enzyme activity. Adding WFO at any concentration from 1.32 to 4.68% (v/v) helped the co-culture to improve the production of CBLs, indicating that the yeast and bacterium have consumed enough oil and are in optimum condition (Figure 4a). Further increases in WFO concentration led to a decrease in CBLs response. The oily substrate seems essential for obtaining optimum lipase production, which poses high activity. However, the oil concentration must be controlled because the high viscosity of oil can affect the oxygen transfer rate, and the accumulation of free fatty acids can decrease the pH, which leads to inhibition of lipase production [29]. At the same time, the addition of AS at 0.68 to 2.51% (w/v) increased the CBLs activity (Figure 4a). AS enhanced microbial growth, where a higher concentration increased the production of CBLs and CBM. In addition, the O&G removal was also improved by adding AS. This result demonstrates that oil biodegradation can be strongly affected by the nitrogen provided to support yeast and bacterium growth [47]. The bioremediation of oil-contaminated wastewater usually involves nitrogen supplementation as ammonia [48].

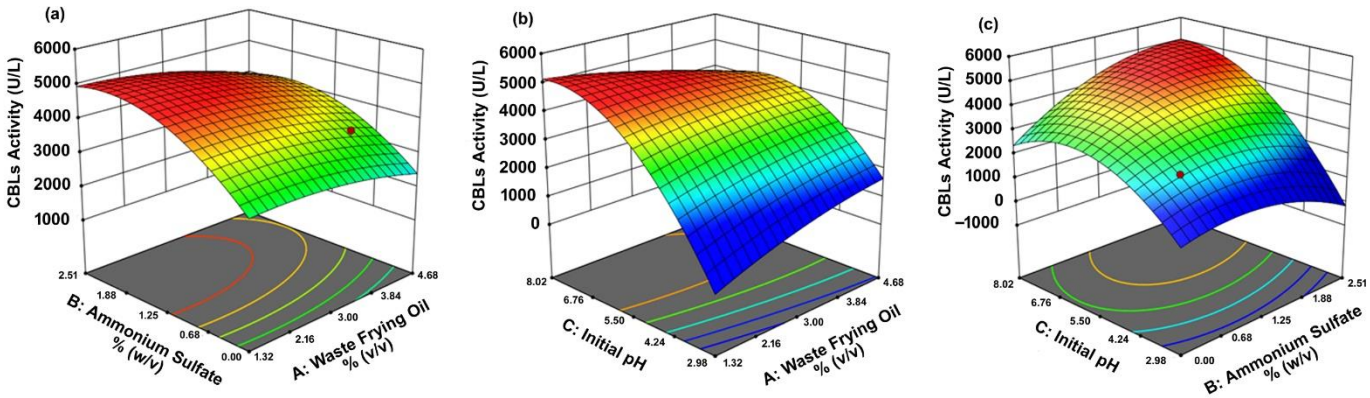

Figure 4. Response surface plots described by the model for the CBLs production by the co-culture of *M. spicifer* AW2 and *S. hominis* AUP19 in oily wastes medium, representing the interactive effect between variables, i.e., AB: waste frying oil (WFO) with ammonium sulfate (AS) (**a**), AC: WFO with initial pH (**b**), and BC: AS with initial pH (**c**). The interaction of WFO with initial pH or AC and AS with initial pH or BC were significant, with a *p*-value of 0.0416 and 0.0206, respectively.

The three-variable models expressing the interactions among the variables representing the responses were obtained, and ANOVA was employed to evaluate the statistical significance of equations for the quadratic response model (Supplementary Materials, Tables S5–S7). The final three-variable model with the interactions between the variables representing the production of CBLs and CBM with O&G removal are as follows:

$$\text{CBLs (U/L)} = -8430.42 + 1618.87 * A + 56.8879 * B + 3201.78 * C - 49.0215 * AB - 184.676 * AC + 289.442 * BC - 83.0406 * A^2 - 492.227 * B^2 - 230.336 * C^2 \quad (8)$$

$$\text{CBM (g/L)} = -57.5457 + 7.96846 * A + 3.67754 * B + 19.0455 * C - 0.73024 * AB - 1.27738 * AC + 1.8455 * BC + 0.0609913 * A^2 - 3.73308 * B^2 - 1.28688 * C^2 \quad (9)$$

$$\text{O\&G removal (\%)} = -6.14215 + 9.20921 * A + 0.65642 * B + 13.07802 * C - 0.26409 * AB - 1.33380 * AC + 1.49348 * BC - 0.15020 * A^2 - 2.43320 * B^2 - 0.71604 * C^2 \quad (10)$$

where A is waste frying oil, B is $(NH_4)_2SO_4$, and C is initial pH.

The analysis for CBLs activity showed the $p > F$-value of the model at 0.0002, and for CBM at 0.0004 and O&G removal at 0.0005, indicating that the obtained models were significant for all responses. The optimized condition was tested and resulted in CBLs production, CBM production, and O&G removal at the level of 4709 U/L, 23.4 g/L, and 58.3%, respectively, with %error below 5% in all experiments (Table 5). Therefore, this model was suitable for achieving high CBLs production and O&G removal as a combined approach of valorization and bioremediation processes at the same time.

Table 5. The best solution for operational conditions. The conditions were obtained from the software to confirm optimization capability by the co-culture of *M. spicifer* AW2 and *S. hominis* AUP19.

Factor: A Waste Frying Oil % (*v*/*v*)	Factor: B Ammonium Sulfate % (*w*/*v*)	Factor: C Initial pH	Responses [a]								
			CBLs Activity (U/L)			CBM (g/L)			O&G Removal (%)		
			Act. *	Pred. **	Error (%) [b]	Act. *	Pred. **	Error (%) [b]	Act. *	Pred. **	Error (%) [b]
2.08	1.72	7.00	4708.9	4954.9	4.9	23.4	25.6	4.7	58.3	59.8	2.5

[a] Actual value: mean ± S.D. (n = 3), [b] (Difference between the predicted value and actual value/Predicted value) × 100, * Actual value from the experiment, ** Predicted value from the software output.

3.4. Time Course Study of Yeast–Bacterium CBLs Production under Optimal Conditions

Figure 5 shows the time course of the oily wastes medium valorization to produce CBLs (Figure 5a), ECLs (Figure 5b), CBM (Figure 5c), with O&G removal (Figure 5f) under optimal conditions. The CBLs production by the co-culture was maximal at 24 h of

incubation (Figure 5a), and compared to the non-optimized medium (POME50) [5], the activity was improved from 3860 U/L to 4709 U/L (1.2-fold). The co-culture reached the stationary phase after 72 h with no flocculation found since nitrogen source, and residual nutrients in POME were available. The ECLs were produced at the stationary phase, starting at 72 h (Figure 5b). The co-culture CBM was stable until day 5 (Figure 5c). The yeast–bacterium synergistic effect was observed from the pattern of cell growth (Figure 5d) and the pH changes during incubation (Figure 5e). The synergistic interaction improved the production of CBLs and CBM, with O&G removals at 1.5, 1.8, and 2 times higher than in the pure cultures. The yeast to bacterium ratio at 1:1 produced the synergistic interaction when utilizing the O&G in the medium. The synergy of microbial strains affected the growth and metabolic patterns [49].

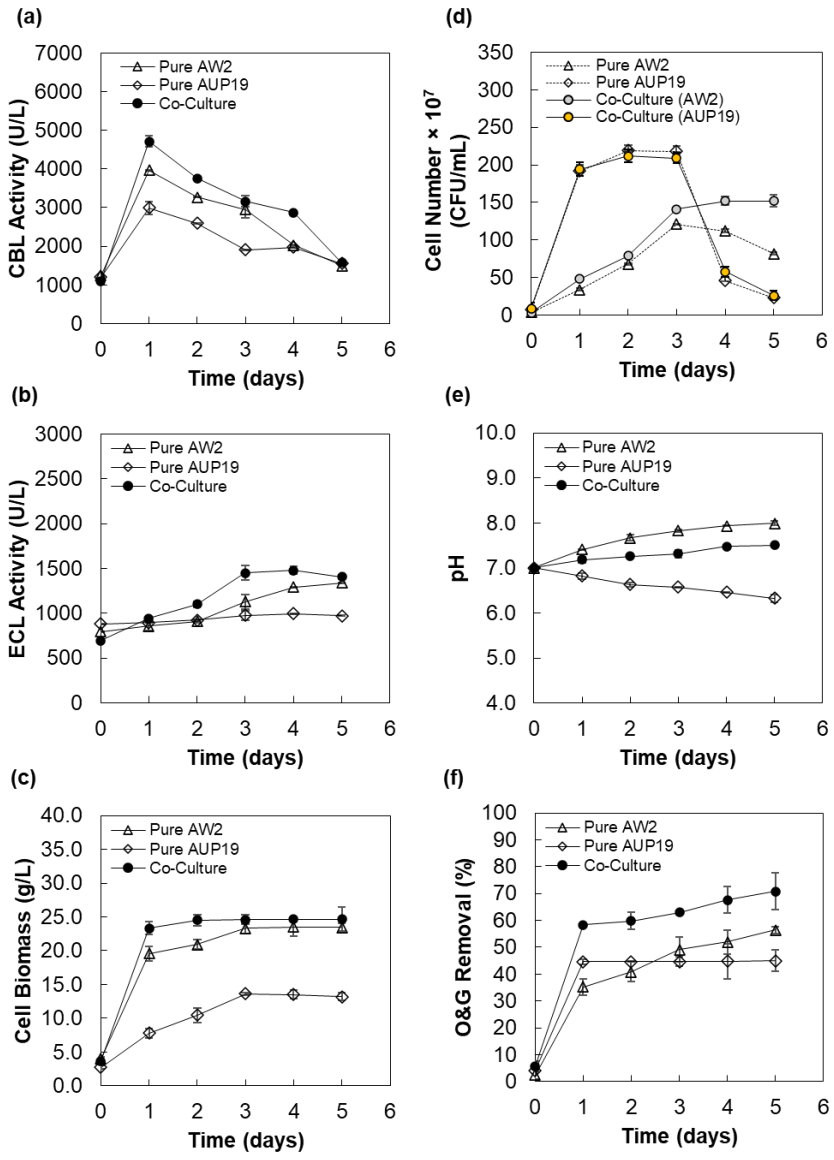

Figure 5. Time course of the blended oily wastes valorization into CBLs (**a**), ECLs (**b**), CBM (**c**), and the profile of cell number (**d**), pH changes (**e**), and O&G removal (**f**) by a pure culture of *M. spicifer* AW2, pure culture of *S. hominis* AUP19, and co-culture of *M. spicifer* AW2 and *S. hominis* AUP19 under the optimized conditions of POME50 with the addition of 2.08% (*v*/*v*) waste frying oil, 1.72.0% (*w*/*v*) ammonium sulfate, 0.1% (*w*/*v*) Gum Arabic, initial pH at 7.0, the incubation time for 5 days at room temperature (30 ± 2 °C), shaking at 150 rpm, inoculum size at 10% (*v*/*v*) with the yeast–bacterium ratio at 1:1.

Based on the cell number in the pure culture (Figure 5d), the bacterium grew faster than the yeast and reached the stationary phase on days 2–3, whereas the yeast grew slower than the bacterium and reached the stationary phase on days 3–4. The bacterium cells declined after 84 h of cultivation, whereas the yeast cell numbers declined after 72 h. The O&G removal efficiency was highest on day 5 (Figure 5f), following the trend of yeast growth in the pure culture. Therefore, it could be assumed that the yeast took advantage of bacterium cells after reaching its death phase starting at 84 h. The bacterium cell lysis mechanisms released the cell components, which could help the yeast growth until day 5. There is evidence that CBLs and CBM increased during their co-habitation in utilizing the O&G in the growth medium. The same pattern was found in the co-culture of *M. spicifer* AW2 and *S. hominis* AUP19 in POME50 medium pH 7.0, where the CBLs activity achieved 3860 U/L, whereas the O&G removal was 1.1-fold higher than the monocultures [5]. In Figure 5e, *S. hominis* AUP19 might release the acids during cultivation which caused the pH to drop from 7.0 to 6.3. In contrast, the pH of *M. spicifer* AW2 culture increased from 7.0 to 7.9. Whereas in the co-culture, the pH increased to 7.4. The co-culture performance in O&G removal (Figure 5f) was better than the single culture form with the rising trend of activities until 5-day cultivation.

The drop in pH of the medium is probably due to acid production, which can be associated with ammonia and oil metabolism. Oil degradation by the hydrolytic activity of microbial lipase forms the organic acids and fatty acids, reducing the culture pH [48,50]. The fatty acids (FAs) profile of refined palm oil, waste frying oil, and POME oil is shown in Supplementary Materials, Table S8. The alkaline pH in the pure culture medium might be because the yeast could assimilate the organic acids, fatty acids, and phenolic compounds present in POME, which led to the formation of hydroxide ions and increased the pH. During the cultivation of *Lipomyces starkeyi*, the POME medium pH reached 7.5, which was caused by the degradation of the phenolic compounds and turned the medium into a basic condition [15].

The co-culture system was also observed using morphological observation on agar plates (Figure 6) to confirm their growth in the medium. It can be observed that *M. spicifer* AW2 and *S. hominis* AUP19 were evenly distributed and could grow normally and stably. It was confirmed that the microbial growth on the agar plate indicated no inhibition found between yeast–bacterium interaction. It suggests that the artificially developed yeast–bacterium co-culture had a synergistic effect on CBLs production where the bacterium colonies appeared smaller than the yeast, and the yeast had short hyphae structure [5].

3.5. Application of CBLs in Biodiesel Synthesis

Based on the time course of CBLs production, the CBLs obtained from 24 h cultivation in the optimized medium gave the highest activity. Then, the CBLs were harvested and applied to synthesize fatty acid methyl esters (FAME) using oleic acid for the esterification reaction and palm oil for the transesterification reaction with methanol as the acyl acceptor for 0–96 h. A direct increase in FAME production from oleic acid was observed with the increased reaction time until 48 h; further increases in reaction time did not significantly improve biodiesel yield. In comparison, the FAME production from palm oil showed optimum results at 96 h. The mixed cells of *M. spicifer* AW2 and *S. hominis* AUP19 effectively converted the substrate to biodiesel of 76% from oleic acid and 87% from palm oil at 48 h and 96 h reaction times, respectively (Table 6). The yeast–bacterium CBLs were produced in a 50% POME medium, and 3860 U/L hydrolytic activity was obtained in the previous study [5]. In the current study, CBLs activity of 4709 U/L was achieved after medium optimization by supplementing 50% POME with 2.08% (*v*/*v*) waste frying oil, 1.72.0% (*w*/*v*) ammonium sulfate, and 0.1% (*w*/*v*) Gum Arabic. The initial pH was adjusted to 7.0. It should be noted that the CBLs production improved the enzyme activity by 1.2-fold compared to the non-optimized CBLs obtained in the previous study [5]. This improvement positively affected biodiesel yield production, increasing up to 3%. Interestingly, the esterification reaction took a shorter time to achieve a 76% yield, whereas 96 h was the

best reaction time for the transesterification reaction. The transesterification takes a more extended reaction period since the reaction step must pass the hydrolysis stage first and then go to the esterification process.

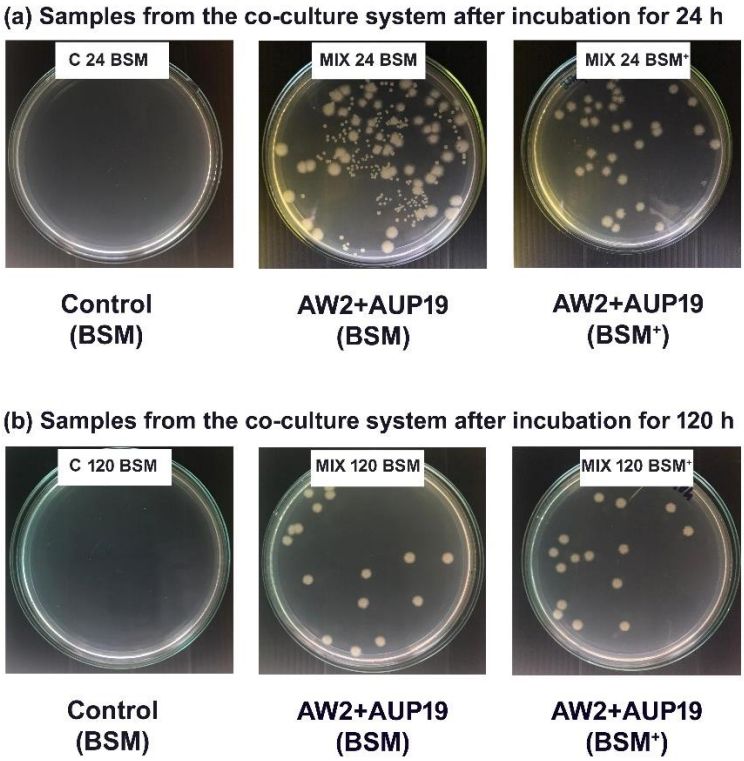

Figure 6. Cell-bound lipase (CBL)-producing strains observation on the agar plates (BSM agar and BSM^+ containing 10% (*v*/*v*) tartaric acid agar plates). Tartaric acid was used to inhibit bacterium growth to observe the pattern of yeast growth. The samples were taken from the co-culture system at 24 h (**a**) and 120 h (**b**) and serially diluted to 10^{-7} before inoculation on agar medium.

Table 6. Fatty acid methyl esters (FAME) from esterification and transesterification reactions by the CBLs obtained from co-culture of *M. spicifer* AW2 and *S. hominis* AUP19 in the optimized and non-optimized media [5] at 24, 48, 72, and 96 h of reaction time, vigorous shaking at 300 rpm, and room temperature (30 ± 2 °C).

Reaction Time (h)	FAME (%)			
	Esterification		Transesterification	
	CBLs from Optimized Medium	CBLs from Non-Optimized Medium *	CBLs from Optimized Medium	CBLs from Non-Optimized Medium *
24	$35.5 \pm 0.5^{A,a}$	$35.6 \pm 0.3^{A,a}$	$58.4 \pm 0.5^{A,a}$	$57.0 \pm 2.4^{A,a}$
48	$76.1 \pm 0.2^{B,b}$	$58.2 \pm 1.5^{A,b}$	$65.5 \pm 1.5^{A,b}$	$64.9 \pm 2.3^{A,b}$
72	$76.0 \pm 0.1^{B,b}$	$73.5 \pm 3.3^{A,c}$	$85.1 \pm 0.9^{B,c}$	$82.5 \pm 0.3^{A,c}$
96	$76.2 \pm 0.1^{B,b}$	$73.4 \pm 1.2^{A,c}$	$87.7 \pm 0.5^{B,d}$	$84.1 \pm 0.1^{A,d}$

Values are presented as mean ± SD (n = 3), Different superscript uppercase letters in the same row indicate significant differences ($p < 0.05$), Different superscript lowercase letters in the same column indicate significant differences ($p < 0.05$), * Data obtained from the previous study [5].

Most product generations use single lipase instead of mixed lipases [51]. However, some authors have reported that the enzyme mixtures have the application potential to improve the product yield, i.e., the one-step reaction by the combination of lipases from *C. rugosa* and *P. fluorescens* gave a shorter time to reach the maximum biodiesel yield due to the synergic effect of the mixed lipase [25]. Binhayeeding et al. [52] used the mixed immobilized lipase from *C. rugosa* and *Rhizomucor miehei* to convert waste cooking oil to

biodiesel. The mixed catalysts gave the highest biodiesel yield at 96.5% and could be reused for more than six cycles. The use of mixed cell-bound lipases (CBLs) obtained from POME without medium optimization in biodiesel synthesis was previously investigated in our work [5], where the mixture of POME-derived CBLs from yeast and bacterium gave 73.5% and 82.5% biodiesel yield from oleic acid and palm oil within 72 h, respectively, higher than single lipase. The mixtures of different lipases with different characteristics are used mainly to reduce the lag time. Therefore, the synergetic strategy of using two lipases has a vital role and advantages for high efficiency and being environmentally friendly.

3.6. Application of CBLs in Palm Oil Mill Effluent (POME) Bioremediation

Yeast–bacterium CBLs obtained from the oily waste medium were directly employed in the bioremediation of sterilized POME without pH adjustment, mainly for O&G and COD removals. The sterilization was aimed to show the mixed CBLs potential in the bioremediation process. Waste bioremediation by microbial enzymes is an environmentally friendly solution over chemical and physical waste treatment. The use of yeast–bacterium CBLs was compared to single yeast CBL and bacterium CBL. Figure 7 shows the results of POME treatment in various POME concentrations.

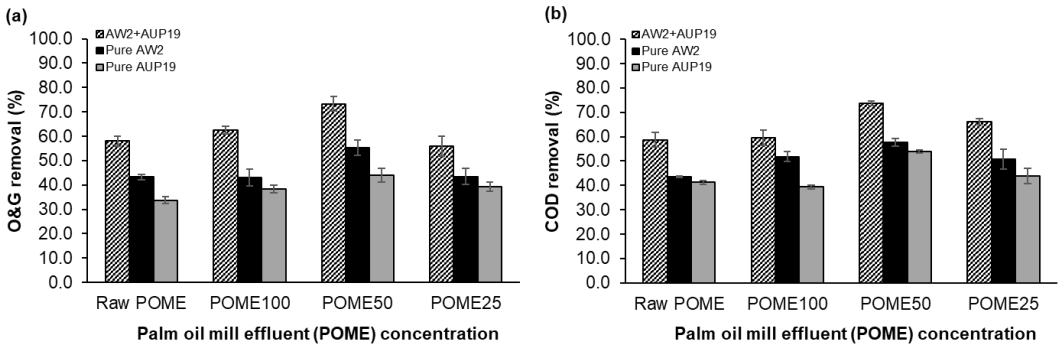

Figure 7. Bioremediation of POME under sterilized conditions in various POME concentrations, i.e., raw POME, POME100, POME50, and POME25. The parameters of O&G removal (**a**) and COD removal (**b**) were measured after inoculation with CBLs obtained from the oily waste medium at room temperature (30 ± 2 °C), 150 rpm, for 72 h.

POME contains some inhibitors, including a high concentration of O&G and COD. Raw POME was obtained directly from the sampling site, and then it was brought to the laboratory. POME100 was obtained after the centrifugation process of raw POME to remove the solid and debris. In this experiment, POME100 was diluted to obtain various concentrations, i.e., POME50 and POME25. The pH of effluents was not adjusted, and it was 3.8. The physicochemical characteristics of raw POME were 67,145 mg/L COD, 1462 mg/L TKN, 11,382 mg/L O&G; POME100 were 50,581 mg/L O&G, 526 mg/L TKN, and COD 7733 mg/L; POME50 were 29,747 mg/L COD, 277 mg/L TKN, 5562 mg/L O&G; whereas POME25 were 18,889 mg/L COD, 277 mg/L TKN, and 2783 mg/L O&G. The O&G and COD of various POME concentrations (before and after cultivation) were determined to obtain the O&G removal and COD removal values [5]. It was found that the dilution was effectively helping to reduce the inhibitors. The mixed CBLs obtained from the co-culture cultivation at 24 h were harvested and directly added at 10% (*v*/*v*) in a two-fold dilution of POME, giving the best O&G removal at 73.3% (Figure 7a) and COD removal at 73.6% (Figure 7b). The CBLs could retain their activity in the acidic environment for 72 h and gave a relatively high O&G removal. The pH of the effluents changed drastically from 3.8 to 6.7 in raw POME, 6.8 in POME100, 7.6 in POME50, and 7.9 in POME25. According to the standard quality of discharge POME characteristics, the palm oil mill industries must treat their POME before disposal to have a pH from 5.0 to 9.0 after biotreatment [53].

The previous investigation showed that *M. spicifer* AW2 and *S. hominis* AUP19 had high survivability in 100% and 50% POME, and both strains had a much lower growth rate

of 25% POME [5]. The COD level in the 50% POME was lower at 29,747 mg/L, and it may have a more suitable composition for yeast and bacterium strains than the 100% POME or raw POME. On the other hand, the lowest POME concentration of 25% significantly slowed the growth since the nutrient concentration was reduced with dilution [54]. The combined strategy of POME bioremediation and lipid synthesis by *Lipomyces starkeyi* showed the same research findings, where POME at 50% supported a higher growth rate of microorganism and 21% lipid production with a higher bioremediation rate [15]. Additionally, POME treatment was successfully performed by *Humicola insolens* D2, *Thermomyces lanuginosus* E4, and *Rhizopus oryzae* ST29, which attained an optimum COD concentration at 22.6 g/L by 50% dilution of POME [14]. The co-culture of *Candida rugosa* and *Yarrowia lipolytica* showed high triglyceride and COD removal efficiency of 98.5% and 60.3%, respectively, in undiluted POME for 120 h [39].

Using biological remediation employing microorganisms could be the solution to chemical and physical waste treatment. Recently, the biological degradation of fats by lipase enzyme-producing microorganisms has been applied (Table 7). *Candida and Bacillus* species isolated from solid and liquid wastes from grease traps showed lipolytic activity in decreasing the oil and grease of POME samples with oil and grease (O&G) removal varying from 56 to 79% after 72 h of treatment. The consortium of lipid-degrading yeasts from this research produced O&G removal at 84% in POME samples after 48 h [26].

Table 7. Co-culture and microbial cells application for wastewater bioremediation.

Microorganisms	Wastewater	Modes of Interactions	% Removal	Ref.
Magnusiomyces spicifer AW2 + *Staphylococcus hominis* AUP19 (Starter cultures added into POME)	POME and diluted POME with pH adjustment to 7.0	Yeast–bacterium A synergic effect of the microbial interactions	80.1% O&G removal, 75.9% COD removal after 72 h	[5]
Candida palmioleophila *Bacillus* sp.	POME	Yeast–bacterium A synergic effect of the microbial interactions	84% O&G removal after 48 h	[26]
Micrococcus species *Bacillus* species *Pseudomonas* species *Staphylococcus aureus* *Aspergillus niger* *Aspergillus fumigatus* *Candida* species *Fusarium* species *Mucor* species *Penicillium* species	Raw POME	Bacteria-Fungi Mutual interactions for POME degradation	40% COD removal after 180 h	[55]
Micrococcus luteus 101PB *Stenotrophomonas maltophilia* 102PB *Bacillus cereus* 103PB *Providencia vermicola* 104PB *Klebsiella pneumonia* 105PB *Bacillus subtilis* 106PB *Aspergillus fumigatus* 107PF *Aspergillus nomius* 108PF *Aspergillus niger* 109PF *Meyerozyma guilliermondii* 110PF	POME	Bacteria-Fungi Mutual interactions in biodegradation	90.2.0% BOD removal, 91.1% COD removal, and 92.2.0% TSS removal	[56]

Table 7. *Cont.*

Microorganisms	Wastewater	Modes of Interactions	% Removal	Ref.
Pseudomonas fluorescens *Flavobacterium* sp. *Micrococcus* sp. *Bacillus subtilis* *Aspergillus niger* *Aspergillus tamari* *Aspergillus* sp. *Penicillium* sp. *Trichoderma* sp. *Mucor* sp.	Diluted and enriched POME	Bacteria-Fungi Mutual interactions for POME degradation	90.3% COD removal after 7 days	[57]
Magnusiomyces spicifer AW2 + *Staphylococcus hominis* AUP19 (Cell-bound lipases added into POME)	POME and diluted POME without pH adjustment	Yeast–bacterium A synergic effect of the microbial interactions	73.3% O&G removal, 73.6% COD removal after 72 h	This study

The waste from palm oil mill industries, i.e., palm oil mill effluent or POME, was treated by locally isolated microorganisms, including *M. spicifer* AW2 and *S. hominis* AUP19. The yeast–bacterium co-culture could remove 80.1% O&G and 75.9% COD after 3 days of incubation in POME50. It should be noted that in the previous experiment (Table 7), the fresh starter cultures were inoculated into the POME media with pH adjustment to 7.0 [5]. In the present study, the treatment using these yeast–bacterium mixed wet cells or mixed CBLs gave >70% O&G and COD removals in the effluents at the original pH (3.8), slightly lower than the previous study. The growth of yeast–bacterium in the blended oily wastes medium and the pH changes may alter the metabolic profiles of the microbial strains. pH was also found to affect the balance between fungal and bacterial growth. In this case, bacterial growth is highly influenced by pH because of their prokaryotic cell structure [58]. The *Micrococcus* species, *Bacillus* species, *Pseudomonas* species, *Staphylococcus aureus*, *Aspergillus niger*, *Aspergillus fumigatus*, *Candida species*, *Fusarium species*, *Mucor species*, and *Penicillium* species were used to remove chemical oxygen demand (COD), biochemical oxygen demand (BOD), and color (ADMI). The results indicated that the microorganisms are prospective for POME biodegradation [55]. The microbial consortium isolated from POME was also influential in degrading POME. The combination of bacteria and fungi gave the BOD, COD, and TSS reduction efficiency, which varied from 84 to 92.0% in 1000 mL POME [56]. At optimum conditions, the mutual interactions between microorganisms were found to be more beneficial for POME remediation process than individual species, where the 90.3% COD removal was achieved by the consortium [57].

4. Conclusions

Valorization of blended oily wastes containing palm oil mill effluent (POME), waste frying oil (WFO), ammonium sulfate (AS), and Gum Arabic (GA) was successfully conducted to produce optimum cell-bound lipases (CBLs) from the yeast–bacterium co-culture by combining one variable at a time and a statistical approach through a green bioprocess. Through oil and grease (O&G) content utilization in the medium, yeast–bacterium CBLs were co-produced with synergistic effect and statistically optimized. The mixed CBLs were suitable for esterification and transesterification reactions with high fatty acid methyl ester (FAME) yield. The CBLs successfully performed the bioremediation of POME to remove O&G and chemical oxygen demand (COD) in the palm oil mill effluents (POME). In conclusion, it is promising to develop sustainable and green processes to produce membrane/cell-associated lipolytic enzymes via valorization of oily waste with potential application in biofuel production and bioremediation.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/fermentation8080411/s1; Table S1. Characteristics of POME50, crude glycerol, waste frying oil, and molasses used in the present study; Table S2. ANOVA for the factorial model of CBLs obtained from experimental design using PBD; Table S3. ANOVA for a factorial model of CBM obtained from experimental design using PBD; Table S4. ANOVA for a factorial model of O&G removal obtained from experimental design using PBD; Table S5. ANOVA for a factorial model of CBLs obtained from experimental design using RSM-CCD; Table S6. ANOVA for a factorial model of CBM obtained from experimental design using RSM-CCD; Table S7. ANOVA for a factorial model of O&G removal obtained from experimental design using RSM-CCD; Table S8. Fatty acid composition of palm oil, waste frying oil, and oil extracted from POME.

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Abbreviations

AN: Ammonium nitrate; AS: Ammonium sulfate; BE: Beef extract; BSM: Basal standard medium; CBL/s: Cell-bound lipase/s; CBM: Cell biomass; CCD: Central composite design; CFU: Colony forming unit; CG: Crude glycerol; COD: Chemical oxygen demand; ECL/s: Extracellular lipase/s; FAME: Fatty acid methyl ester; GA: Gum Arabic; MO: Molasses; O&G: Oil and grease; OVAT: One variable at a time; OW: Oily wastes; PBD: Plackett-Burman design; POME: Palm oil mill effluent; RSM: Response surface methodology; SY: Soytone; TKN: Total Kjeldahl nitrogen; TW: Tween 80; TX: Triton X-100; UR: Urea; WFO: Waste frying oil; YE: Yeast extract.

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