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Combined effect of phosphorus, magnesium, yeast extract on lipid productivity of *Yarrowia lipolytica* grown with molasses

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Abstract−Inadequate global fossil fuel reserves have forced researchers to investigate alternative fuel sources, and oleaginous microorganisms have attracted attention with their potential. Since high lipid production yield is an important criterion for suitable fuel production, in this study an oleaginous yeast, *Yarrowia lipolytica*, was selected and the lipid and biomass productivity under molasses (M20) substrate and nutrient supplementation was investigated. The effect of phosphorus as dipotassium hydrogen phosphate (K_2HPO_4), magnesium as magnesium sulphate ($MgSO_4$), and yeast extract supplementation to molasses (M20) were evaluated individually and in combination. In addition, two quadratic models, using Box-Wilson central composite design, were used to correlate the phosphorus, magnesium and yeast extract concentrations that would achieve the highest biomass and lipid productivity. The study has shown that molasses (M20) supplemented with 336 mg/L K_2HPO_4, 0.17 g/L $MgSO_4$ and 4.54 g/L yeast extract had the highest biomass productivity (80.7 mg/L/hour) and the highest lipid productivity (28.3 mg/L/hour). These productivity results were 1.44-fold and 2.42-fold higher than those of yeast extract-peptone-dextrose (YPD) broth, respectively. With enhanced biomass and lipid productivity, *Yarrowia lipolytica* can thus be used effectively in the fermentation industry.

Keywords: *Yarrowia lipolytica*, Molasses, Lipid Productivity, Surface Response Methodology

INTRODUCTION

The use of fossil fuels is endangered due to limited global reserves and greenhouse gas emissions [1]. Many parts of the world are now concerned about the rapid depletion of fossil fuels [2]. For these reasons, biofuels, which have become a trend, can be a viable solution to rising oil prices as well as the problem of climate change [3,4]. Microorganisms that can accumulate at least 20% lipid (g lipid/g cell dry weight (CDW)) are referred to as oleaginous microorganisms [4]. Microbial oils or single cell oils (SCOs) are produced by oleaginous microorganisms, such as filamentous fungi, microalgae, bacteria, and yeasts [5]. Phototrophic microalgae are affected by the climate and heterotrophic microalgae use less sugar as a carbon source than yeast. This can be problematic. Fungi, on the other hand, suffer from a high oxygen demand and a low tolerance to metal ions. Bacteria are extremely difficult to collect due to their small size. As a result, yeasts are the best microorganisms for biofuel production [6]. The most common oleaginous yeast species are *Cryptococcus*, *Cutaneotrichosporon*, *Cyberlindnera*, *Lipomyces*, *Rhodotorula* (*Rhodosporidium*), *Schwanniomyces*, *Trichosporon*, and *Yarrowia* spp. [7,8]. *Yarrowia lipolytica* can produce lipids up to 68 percent of cell dry weight (CDW) when using low-cost carbon substrates [9]. Hydrophobic and hydrophilic substrates can be used to grow *Yarrowia lipolytica*. The yeast *Yarrowia lipolytica* digests these substrates by releasing their enzymatic secretions. As a result of digestion, *Yarrowia lipolytica* produces valuable cellular metabolites such as single cell oil [10].

The yeast *Yarrowia lipolytica* has a wide range of applications and it is an important strain in biotechnology [11,12]. The common ways of energy generation from yeast are fermentation, microbial electrolysis cell (MEC) and microbial fuel cells (MFC). In addition to generating energy, these technologies also treat wastewater and reduce CO_2 emissions [13]. MFC can generate bioelectricity and biohydrogen by oxidizing organic materials via bacteria or enzymes [14-16]. However, MEC differs from MFC in that it operates anoxically and generates hydrogen in the cathode with a high energy input [13,17-20]. Moreover, high biomass productivity with increased lipid productivity is essential for large-scale fungal fermentation. The lipid content of microbial species is critical for converting waste streams to energy [21]. Higher biomass productivity in less time could provide sustainable lipid. The enhanced oil productivity and oil accumulation could be used to produce industrially derived lipids, such as aliphatic compounds and oleochemicals [21]. Medium and long-chain oleochemicals are used in soaps, biodiesel, herbicides, lubricant production and as polymer additives. Therefore, the lipid with enhanced yield is the industrially desired by-product [21].

Studies have shown that different ions and minerals have different effects on cell growth and lipid deposition. Ca^{2+}, Cu^{2+}, Fe^{3+}, Mg^{2+}, Mn^{2+}, and Zn^{2+} are ions that frequently appear in studies [7]. This can be explained by the fact that nutrient deficiency suppresses cell proliferation and thereby converts the remaining carbon source into storage lipid. Because when the limiting nutrient source is depleted, cell growth is suppressed, resulting in the stor-

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age of the remaining carbon source as lipids [22]. Furthermore, the addition of nutrient supplements to waste streams can promote the growth of microorganisms. Olive mill effluent [23], palm oil mill effluent [24] and dairy wastewater [25] have been used for the growth of *Yarrowia lipolytica*. Industrial wastewater generally lacks the necessary nutrients and nutrient levels for yeast growth. On the other hand, molasses contains significantly more organic carbon than other wastewaters.

Molasses, as a byproduct of sugar refining, is a widespread microbial fermentation substrate, with a high content of biodegradable sugars [23]. It can be produced as a nutrient-rich substrate with a yield of 5 tons per 100 tons of sugarcane processing [24]. The use of molasses reduces the environmental impact, considering energy consumption and pollution levels, and can result in lower costs compared to synthetic media [23]. It can be used as a suitable substrate for a variety of end products, including ethanol, succinic acid, polymers, and mannitol [24]. Sugar-rich molasses does not require any significant physical or chemical pre-treatment (such as sterilization, filtration and hydrolysis) prior to fermentation. This fact renders sugar-rich molasses ideal for the production of ethanol, fulvic acid, butanol and biohydrogen [25-28].

Phosphorus plays a critical role in the cell, including the formation of nucleic acid, phospholipid and coenzyme structures [29]. The effect of phosphorus on yeast cells in combination with a nitrogen source is not fully understood. Studies have shown both positive and negative effects of lipid storage in phosphorus-limited or phosphorus-enriched oleaginous microorganisms. When phosphorus is limited in yeast nutritional supplementation and nitrogen is not limited, the lipid storage of oleaginous yeast improves [30]. But in the study by Wierzchowska et al. [31] when the nitrogen to phosphorus (N/P) ratio decreased from N/P=4.3 : 1 to N/P=1 : 1 by supplementing a phosphorus source, KH_2PO_4, the biomass increased. Therefore, phosphorus is a necessary element for the cellular growth of microorganisms [31]. In addition, yeast extract contains about 1-2.5% phosphorus, 10-12% nitrogen (rich source of organic nitrogen) and 0.2% magnesium as well as other micro and macronutrients [22,30]. Consequently, phosphorus supplementation in the form of phosphorus compounds or as part of yeast extract can improve lipid storage capacity in yeast culture when low phosphorus-containing molasses substrate is considered. Aside from phosphorus, magnesium supplementation to the growth medium may improve biomass and lipid productivity. Cell dry weight increased as yeast extract and magnesium sulphate ($MgSO_4$) concentrations increased in a study with *Rhodosporidium toruloides* A29; and the optimal $MgSO_4$ concentration was found to be 0.25 g/L, as there was no further increase in CDW, although the yeast extract concentration increased [32]. Consequently, phosphorus addition to the growth medium in the form of K_2HPO_4 or yeast extract and the magnesium addition can lead to the accumulation of higher storage lipid yields without biomass loss. The combined effects of more than one nutrient supplement can result in high lipid yields. Cordova et al. [33] used a combined copper and iron supplement and there was a three-fold increase in total lipids. The supplementation of three different nutrient sources such as $MgSO_4$, K_2HPO_4 and yeast extract could provide enhanced lipid yield with synergistic effect on lipid accumulation.

The biomass and lipid productivities of microbial populations are important to predict the best growth conditions for strains. For this purpose, researchers prefer response surface methodology (RSM) for this purpose. RSM can optimize many variables with fewer experiments than the traditional method, which starts with one factor at a time. So RSM saves a significant amount of time in this regard. This methodology is still used to ensure optimal composition in many processes ranging from biotechnology to food product development [32]. Numerous studies have been conducted on surface response methodology and yeasts. For instance, Beighbeder et al. [28] studied the initial sugar concentration of molasses using surface response methodology, and sugar concentrations less than 225 g/L resulted in high ethanol yields.

This paper investigates *Yarrowia lipolytica*, an oleaginous fungus, by subjecting it to molasses as a nutrient-enriched fermentation substrate. According to initial characterization, as molasses is low in magnesium and phosphorus, and because cell growth can be hindered in high-carbon substrates, and because phosphorus and magnesium are critical factors in cell growth, the study focused on supplementing both nutrients, considering different magnesium and phosphorus sources. In particular, the addition of phosphorus could lower the nitrogen to phosphorus ratio and stimulate biomass. Since yeast extract is composed of both magnesium and phosphorus, combining with phosphorus and/or magnesium supplementation could enhance the biomass and lipid production. For this purpose, besides individual and combined effects, surface response methodology was evaluated to determine the optimal conditions for biomass and lipid productivity.

MATERIALS AND METHODS

1. Inoculation of *Yarrowia lipolytica*

Molasses from the Burdur Sugar Factory was used in the studies. Characterization of molasses was performed using Standard Methods 4600-P Phosphorus D and ionic concentrations were analyzed using ion chromatography (IC) (Dionex ICS-3000), and other elements were analyzed using ICP-OES (Perkin Elmer). The results are summarized in Table 1. Sugar analyses in molasses samples were performed according to the simple sugar analysis application, which was prepared by reference to the Waters brand Xbridge Amide HPLC columns application notes. During the sample preparation phase, 0.5 g sample was weighed and 2 ml (50 : 50 v/v) acetonitrile:water mixture was added. After vortexing for 15 seconds, it was kept on a shaker mixer for 30 minutes. It was then centrifuged at 13,200 rpm for 5 minutes. After filtering through a 0.45 μm filter, it was fed to the HPLC system. The HPLC system consists of Waters 2690 Controller, a Waters 2410 Differential Refractive Index Detector and a Waters Carbohydrate Amino (NH2) 125 Å. 10 μm. 3.9 mm×300 mm column. Acetonitrile:water (75 : 25) was used as the solvent system. The flow rate was set at 1 ml per minute and this level was maintained for 35 minutes of analysis.

Since light and heavy metal ions such as potassium, calcium and iron are present in relatively high concentrations in molasses, the osmotic inhibition of cell growth occurs. Several strategies, such as sulfuric acid treatment and activated carbon treatment, could be used to reduce the concentration of inhibitory compounds [34].

Table 1. The characterization of molasses

Parameters	Results	RSD (%)
Total sugar (%)	52.8	5.4
Fermentable sugar content (%)	23.9	2.4
Fructose (g/L)	5.96	0.4
Glucose (g/L)	6.33	1.3
Sucrose (g/L)	102.15	21.5
Maltose (g/L)	4.62	1.3
Carbon content (%)	23.46	0.2
Nitrogen to phosphorus ratio (N/P)	286 : 1	
Dry solids (%)	82.4	0.8
Ash content (%)	8.44	0.24
Protein content (%)	3.21	0.36
Lipid content (%)	0.34	0.0479
pH	5.9	0.15
Ortho-phosphate (PO_4^{3-}, mg/L)	16.8	0.5
Cu^{2+} (mg/L)	n.d.	n.d.
Fe^{3+} (mg/L)	110.3	0.51
Ca^{2+} (mg/L)	2,493	0.9
Al^{3+} (mg/L)	3.087	2.98
Mn^{2+} (mg/L)	2.824	1.27
Zn^{2+} (mg/L)	2.162	0.73
Ba^{2+} (mg/L)	0.644	1.91
Li^{+} (mg/L)	0.4	4.42
Ni^{2+} (mg/L)	1.711	0.38
Ti^{4+} (mg/L)	0.212	1.29
Pb^{4+} (mg/L)	n.d.	n.d.
Mg^{2+} (mg/L)	19.93	1.61
K^{+} (mg/L)	7,269	0.62

n.d. : not detected

But in this study, molasses was diluted to reduce inhibitory effects during cultivation. *Yarrowia lipolytica* (MUCL 28849, NRRL Y-1094, ATCC 8662) was inoculated in YPD broth (20 g/L D-glucose, 10 g/L yeast extract, and 20 g/L peptone) and cultured at 28±2 °C. The culture medium was sterilized at 121 °C at 1.05 kg/cm^2 for 20 minutes. All experiments were performed in duplicate in 1,000 mL Erlenmeyer flasks with a working volume of 500 mL and continuous aeration (100 L/min). A dilution factor of 5 was used throughout the experiment.

2. Growth, Biomass and Lipid Measurements

The growth of *Yarrowia lipolytica* was monitored by measuring the optical density at 600 nm, and gravimetrically known cell dry weights (CDW)s, that were measured after 1 hour of incubation at 105 °C, and the combined data analyzed for correlation. The equation for the relationship between CDW and OD600 was determined as

$$\text{CDW (mg/L)} = 580.47 \times \text{OD600} - 3{,}240.3;\ R^2 = 0.9941. \quad (1)$$

Lyophilized fungal biomass was used for lipid extraction. Homogenization was carried out using classic Blight-Dyer method, with 1 : 2 : 0.8 parts chloroform:methanol:water (v/v/v) [35]. The lipid extracted from *Yarrowia lipolytica* was correlated using the sulfo-phospho-vanillin (SPV) method.

Table 2. Experimental design

Level	−1	−0.5	0	0.5	1
K_2HPO_4 (C_P) (mg/L)		0	380	790	
$MgSO_4$ (C_M) (g/L)	0	0.1	0.25	0.75	1
Yeast extract (C_Y) (g/L)	0	0.01	0.1	1	10

A total of 2 mL of concentrated sulfuric acid was added to the different amounts of *Yarrowia lipolytica* oil, ranging from 0 to 720 μg. Samples were incubated at 100 °C for 10 minutes before being cooled in an ice bath for 5 minutes. 4 mL of freshly prepared phospho-vanillin reagent was added and incubated at 37 °C for 15 minutes. The absorbances were measured with a spectrophotometer at 530 nm [36,37]. The relationship between fungal lipid and absorbances was calculated as *Yarrowia* lipid (μg)=($OD_{530\,nm}$−0.049)/0.0022, R^2=0.9941. The SPV method was used to determine the lipid content of lyophilized fungal biomass using 5 mg of biomass.

Biomass productivity (BP, mg/L/hour) was calculated using the following equation, where x (mg/L) is the concentration of biomass at the end of the cultivation time (t) and X_0 (mg/L) is the concentration of the biomass at the beginning of the cultivation.

$$\text{BP}\left(\frac{\text{mg}}{\text{L}\cdot\text{hour}}\right) = \frac{X - X_0}{t} \quad (2)$$

The lipid productivity (LP, mg/L/hour) was calculated using the following equation, where L is the lipid content of the biomass in % CDW (Cell Dry Weight).

$$\text{LP}\left(\frac{\text{mg}}{\text{L}\cdot\text{hour}}\right) = \text{BP}\cdot\text{L} \quad (3)$$

3. Surface Response Methodology

The effect of the three independent variables, K_2HPO_4 (C_P), $MgSO_4$ (C_M) and yeast extract (C_Y), on the response variables, biomass productivity (mg/L/hour), and lipid productivity (mg/L/hour), were assessed using multivariable regression analyses. A central composite design was used for the three independent variables and the five-levels. The ranges of the variables tested are shown in Table 2. The experimental data were fitted to a second-order polynomial equation using Design-Expert software version 11.

RESULTS AND DISCUSSION

1. Effect of Molasses and YPD on Biomass and Lipid Productivity of *Yarrowia lipolytica*

The study showed that *Yarrowia lipolytica* had lower biomass and lipid productivity when 20% molasses (M20) was used for cultivation. Molasses (M20) resulted in 1.55-fold lower biomass productivity and 1.54-fold lower lipid productivity compared to YPD broth as shown in Fig. 1(b). This can be attributed to the low levels of phosphorus and other nutrients in molasses. In the study by Yu et al. [38], *Yarrowia lipolytica* strain PGC01003 produced 18.1 g/L biomass under YPD broth, which was 1.24-fold higher than in this study. Wang et al. [39] cultured *Yarrowia lipolytica* with a biomass ranging from 9.9 to 12.6 g/L under pretreated cane molasses. Since 9.4 g/L biomass was produced under M20, the result is

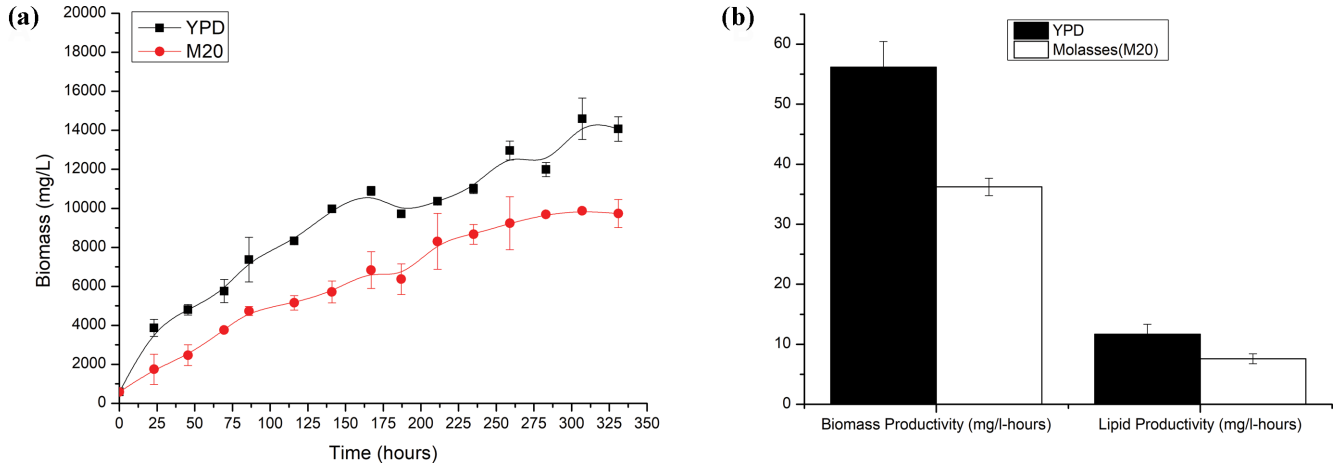

Fig. 1. Growth characteristics of *Yarrowia lipolytica* under molasses and YPD (a) growth curves; (b) biomass productivity and lipid productivity.

in good agreement with previous studies. For instance, Jo et al. [24] examined sugarcane molasses containing 390 g/L sucrose, 138 g/L glucose, and 227 g/L fructose and recombinant *Ralstonia eutropha* reached a biomass of 8.68 g/L in 96 hours of cultivation. Furthermore, Yan et al. [40] combined YPD broth and molasses and found a 1.38-fold increase in lipase productivity as a result of nutrient enrichment. Chacón et al. [25] studied the nutrient-supplemented dilute molasses with a sugar concentration of 45 g/L for butanol production using *Clostridium saccharoperbutylacetonicum*, which has the ability to co-ferment xylose and sucrose. This can be explained by the fact that nutrient enrichment with phosphorus, yeast extract, and other sources can support *Yarrowia lipolytica* growth and byproduct efficiency.

2. Effect of Phosphorus, Magnesium and Yeast Extract on Biomass and Lipid Productivity of *Yarrowia lipolytica*

The addition of nutrients to molasses can alter the low biomass productivity and lipid productivity of *Yarrowia lipolytica*. As shown in Fig. 2, K_2HPO_4 concentrations ranging from 260 to 790 mg/L, $MgSO_4$ concentrations ranging from 0.1 to 1 g/L and yeast extract concentrations ranging from 0.01 to 10 g/L were investigated. In addition, the combined effect of these nutrients on growth at 600 nm was assessed (Fig. 3).

K_2HPO_4 supplementation to molasses (M20) resulted in higher biomass productivity between 1.33-fold and 1.53-fold compared to M20. This situation can be explained by the N/P ratio, since the addition of phosphorus lowered the N/P=645 : 1 of M20 up to the N/P=24 : 1. Thus, in the study by Wierzchowska et al. [31], lowering N/P ratio, in line with these findings, resulted in enhanced biomass. Furthermore, in addition to higher biomass productivity, up to 3.0-fold higher lipid productivity compared to M20 was observed in this study. In a similar study with *Rhodosporidium toruloides*, the addition of 0.4 g/L potassium dihydrogen phosphate (KH_2PO_4) resulted in the highest lipid productivity (8.8 g/L) [41]. However, there are also some contrasting studies in which the best lipid productivity was observed under phosphorus deprivation. For instance, *Cryptococcus curvatus* MUCL 29819 with phosphorus deficiency had the highest lipid content of 30.4% with a lipid concentration of 0.92 g/L, which was 1.44-fold higher than a KH_2PO_4 supplementation and for *Rhodosporidium toruloides* Y4, the best lipid (12.1 g/L) belonged to phosphorus starvation [17]. In another study, molasses supplemented with 5 g/L glucose, 1.5 g/L citric acid, and 2.7 g/L Na_2HPO_4 had the lowest production cost for bacterial cellulose production from *Gluconacetobacter hansenii* [44].

Yeast extract supplementation, with the exception of 1 g/L concentration, resulted in lower phosphorus levels (between 0.25 and 25 mg/L) compared to phosphorus supplementation in the form of K_2HPO_4 and lower magnesium levels (between 0.02 mg/L and 2 mg/L) compared to magnesium supplementation in the form of $MgSO_4$. The effect of the magnesium content of yeast extract can be negligible, and the study only shows the combined effect of low amounts of phosphorus and magnesium supplementation. There are some studies on yeast extract supplementation. In Bellou et al. [42], where yeast extract was used as nitrogen source, 2 g/L yeast extract resulted in 2.1 g/L biomass. When increasing the amount of yeast extract to 4 g/L, the biomass concentration also increased to 8 g/L and the 5 g/L yeast extract resulted in the highest biomass, with 15.5 g/L [42]. But in this study, 1 g/L yeast extract resulted in 1.29-fold higher biomass productivity, which can be attributed to significant changes in N/P ratio under yeast extract supplementation.

Moreover, the combined effect of both nutrients (phosphorus, magnesium and yeast extract) revealed that phosphorus and magnesium supplementation with 790 mg/L K_2HPO_4 and 0.1-1 g/L $MgSO_4$ resulted in a 1.38-fold and 2.02-fold higher biomass, respectively. This can be attributed to growth stimulation by magnesium, which acts as a cofactor for several enzymes that regulate respiration, glycolysis, oxidative phosphorylation and other cellular processes [43].

To evaluate the effect of increasing the amount of phosphorus and/or magnesium by adding them to the yeast extract, yeast extract was combined with magnesium and with a phosphorus supplement. As yeast extract contains about 10% nitrogen, the addition of yeast extract changes the nitrogen to phosphorus ratio (N/P). Phosphorus (790 mg/L K_2HPO_4) in combination with yeast extract (0.01-10 g/L) resulted in high biomass productivity ranging from 1.13-fold to 1.80-fold. This can be attributed to the lowering of the

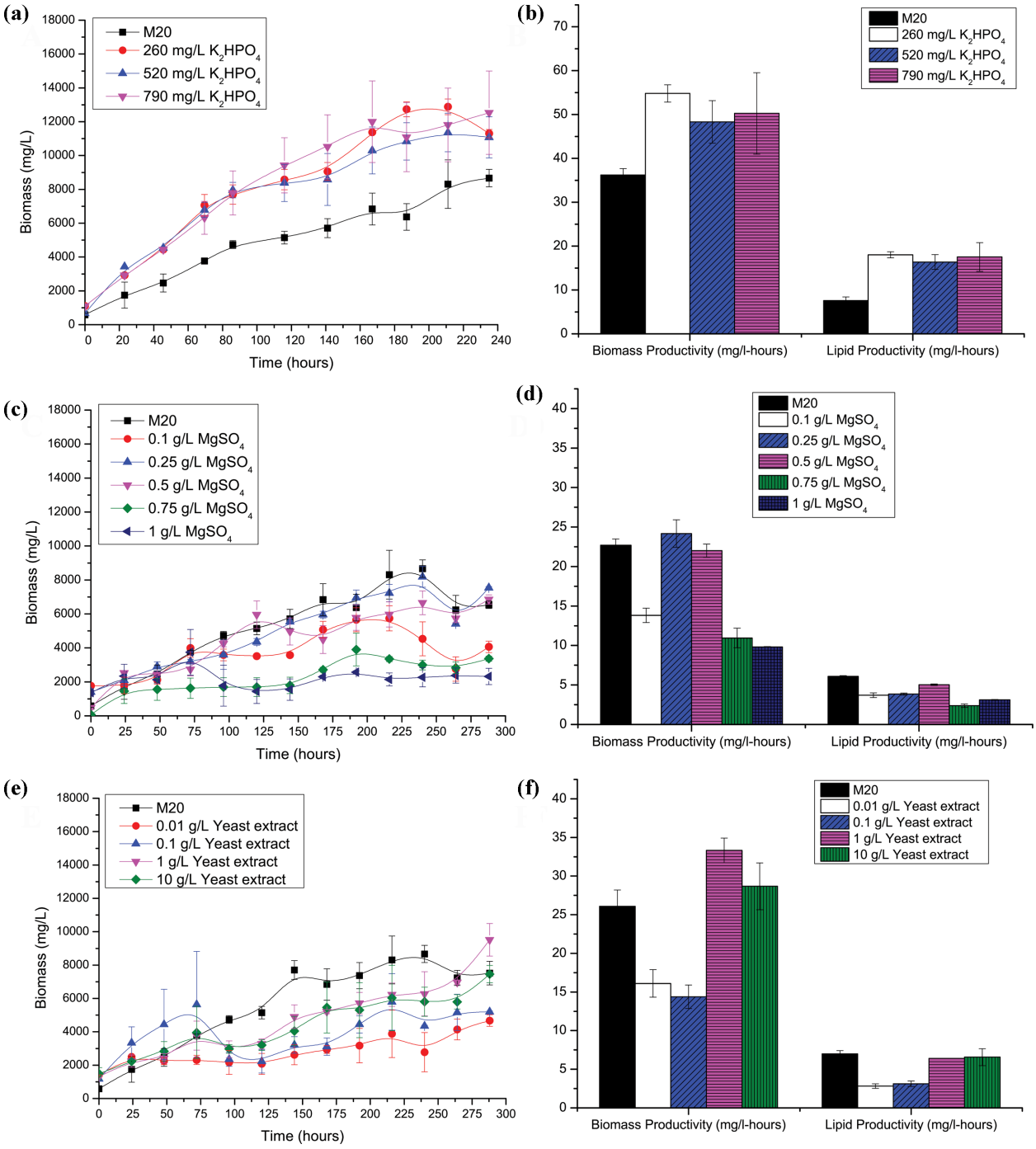

Fig. 2. Growth characteristics of *Yarrowia lipolytica* under molasses with different nutrient supplementation. The data was expressed as growth curves, biomass productivity and lipid productivity of (a), (b) K_2HPO_4 (260-790 mg/L), (c), (d) $MgSO_4$ (0.1-1 g/L) and (e), (f) yeast extract (0.01-10 g/L).

N/P ratio of up to 16 : 1 by phosphorus addition. In particular, by the time the addition of only phosphorus lowered the N/P ratio to 24 : 1, the phosphorus combined with yeast extract lowered the N/P ratio to 16 : 1. The lower N/P ratio is considered a high biomass producing condition in many studies. But the enhanced biomass under combined nutrient supplementation can also be attributed to the increased Mg^{2+} concentration. According to Li et al. [45], the increased lipid productivity with 0.5 g/L $MgSO_4$ and yeast extract (0.1-10 g/L) was attributed to Mg^{2+} ions. *Yarrowia lipolytica* ACADC 50109, on the other hand, demonstrated lipid degradation in the absence of magnesium [46].

3. Surface Response Methodology

Optimization of growth parameters can be an effective strategy to produce high-quality biodiesel with high lipid production efficiency. In this study, three independent variables, C_P, C_M and C_Y, were evaluated, leading to the development of an experimental

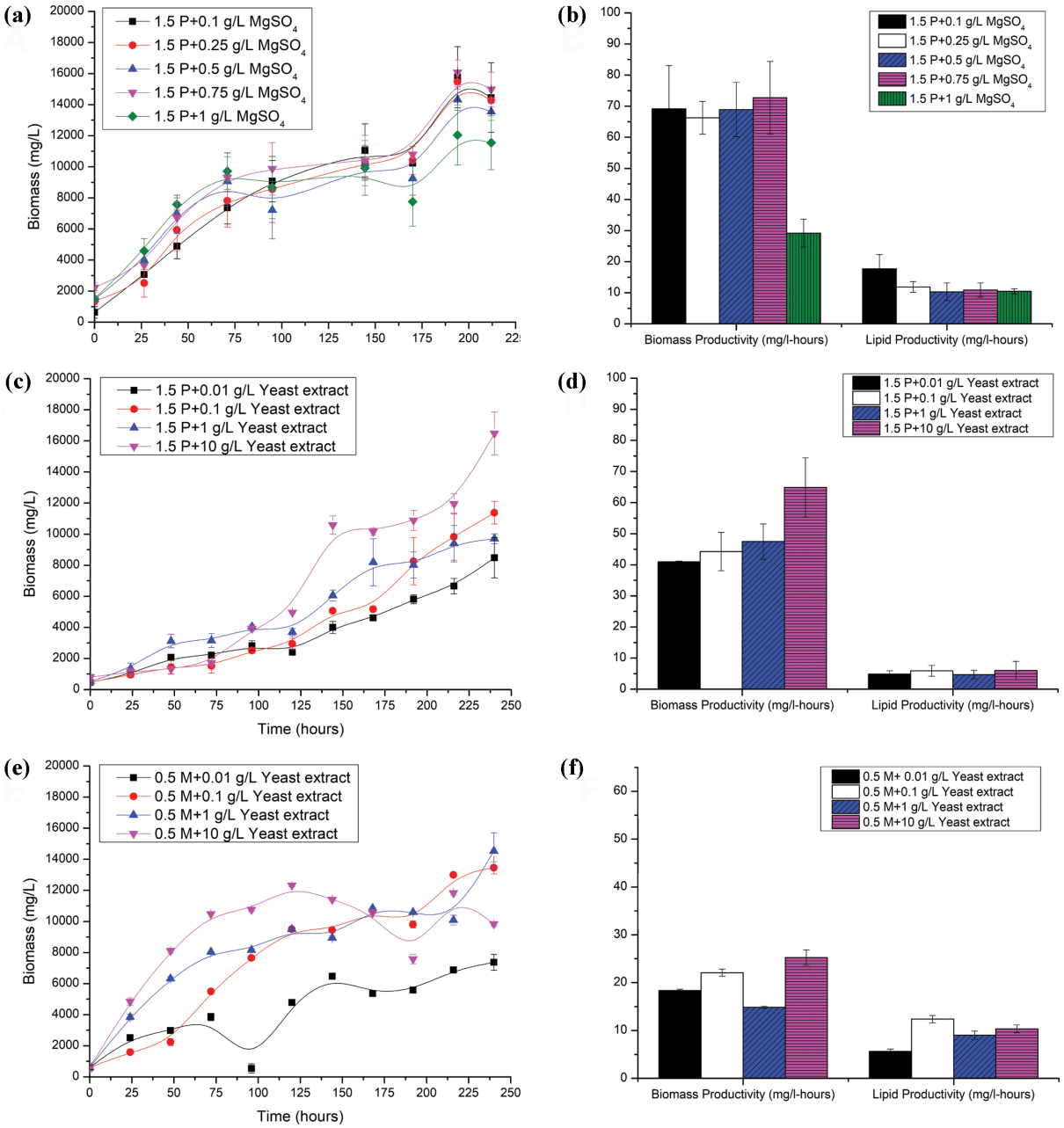

Fig. 3. Growth characteristics of *Yarrowia lipolytica* under molasses with combined nutrient supplementation. The data was expressed as growth curves, biomass productivity and lipid productivity of (a), (b) 790 mg/L K_2HPO_4 with $MgSO_4$ (0.1-1 g/L), (c), (d) 790 mg/L K_2HPO_4 with yeast extract (0.01-10 g/L) and (e), (f) 0.5 g/L $MgSO_4$ with yeast extract (0.01-10 g/L).

design model for high biomass productivity with high lipid productivity (Fig. 4, Table 3). The results of surface response methodology were used to determine the best growth conditions for *Yarrowia lipolytica*.

Eq. (4) defines the regression equation in terms of coded factors for biomass productivity:

$$BP\ (mg/(L{\cdot}hour)) = +89.08 + 25.84{\cdot}A + 2.37{\cdot}B + 5.65{\cdot}C + 10.87{\cdot}A{\cdot}B + 2.57{\cdot}A{\cdot}C - 2.88{\cdot}B{\cdot}C - 30.09{\cdot}A^2 - 6.14{\cdot}B^2 - 11.82{\cdot}C^2 \quad (4)$$

The equation in terms of coded A, B and C which are coded levels of the independent variables, Eq. (4), was then reconstructed in terms of the actual values of the independent variables, as shown in Eq. (5):

$$BP\ (mg/(L{\cdot}hour)) = +17.73650 + 0.183759{\cdot}C_P + 13.29794{\cdot}C_M + 5.92083{\cdot}C_Y + 0.055031{\cdot}C_P{\cdot}C_M + 0.0013{\cdot}C_P{\cdot}C_Y - 1.15149{\cdot}C_M{\cdot}C_Y - 0.000193{\cdot}C_P^2 - 24.54712{\cdot}C_M^2 - 0.472890{\cdot}C_Y^2 \quad (5)$$

According to regression analysis results, the quadratic equation with R^2 of 0.9607 was obtained. The result was confirmed with the ANOVA analysis (Table 4).

The significance of lipid productivity (LP) was evaluated using the quadratic model (Fig. 4). The difference between the R^2 of

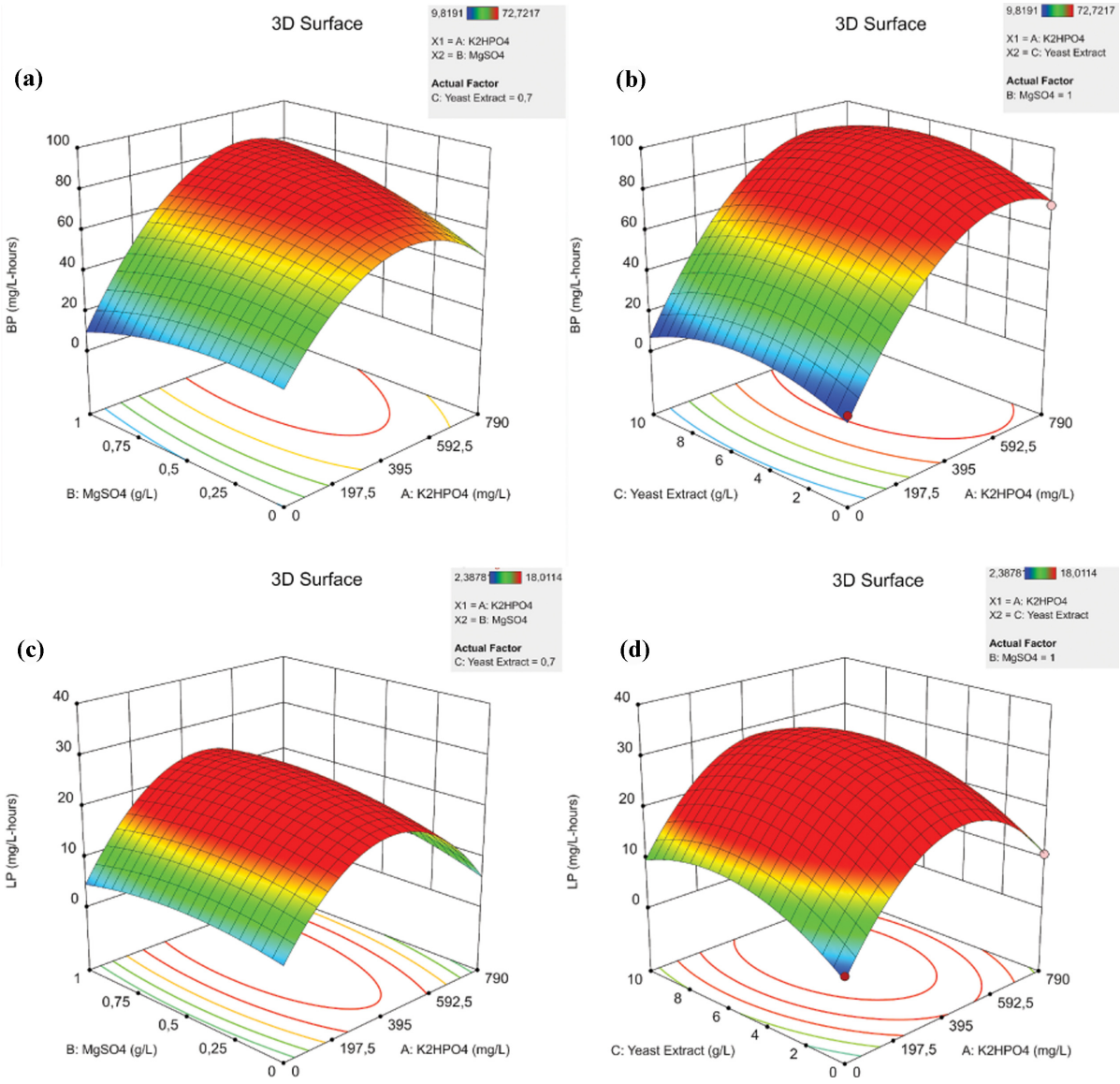

Fig. 4. Surface response methodology results of *Yarrowia lipolytica*: Response surface plots for biomass productivity (a)-(b) and lipid productivity (c)-(d).

0.9487 and the adjusted R^2 of 0.8973 was within a reasonable range (<0.2) (Table 5, Fig. 4(d)). Eq. (6) defines the regression equation in terms of coded factors:

$$\text{LP (mg/(L·hour))} = +31.57 + 1.96 \cdot A + 2.42 \cdot B + 2.18 \cdot C + 1.83 \cdot A \cdot B - 0.5431 \cdot A \cdot C + 0.9189 \cdot B \cdot C - 15.60 \cdot A^2 - 2.32 \cdot B^2 - 6.27 \cdot C^2 \quad (6)$$

where A, B and C are coded levels of the independent variables, phosphate, magnesium and yeast extract, respectively. Eq. (6) was then reconstructed in terms of the actual values of the independent variables, as in Eq. (7):

$$\text{LP (mg/(L·hour))} = +3.02367 + 0.080670 \cdot C_P + 8.61095 \cdot C_M + 2.87089 \cdot C_Y + 0.009260 \cdot C_P \cdot C_M - 0.000275 \cdot C_P \cdot C_Y + 0.367577 \cdot C_M \cdot C_Y - 0.000010 \cdot C_P^2 - 9.26570 \cdot C_M^2 - 0.250934 \cdot C_Y^2 \quad (7)$$

The optimal condition for the highest biomass productivity (80.7 mg/L/hour), and lipid productivity (28.3 mg/L/hour), was derived as C_P=336 mg/L, C_M=0.17 g/L and C_Y=4.54 g/L (Fig. 5-6).

The model was validated by considering this optimal condition for the highest biomass productivity and lipid productivity. The root-mean-square deviation (RMSD) was calculated by finding the difference between experimental and predicted responses, squaring the differences, and dividing by the number of values. Also, the data for biomass and lipid productivity with percentage of increment for each response after optimization were compared with molasses (M20) and YPD and shown in Table 6. The actual biomass and lipid productivity was in agreement with the predicted values. The RMSD percentage was less than 2.75% and 0.71% for biomass productivity and lipid productivity, respectively.

In some studies molasses has been used to produce other forms of energy rather than lipid. For instance, the MFC system could provide both bioelectricity generation and carbon removal from

Table 3. CCD Experimental matrix

	Factor 1	Factor 2	Factor 3	Response 1	Response 2
Run	A: K_2HPO_4	B: $MgSO_4$	C: Yeast extract	Biomass productivity	Lipid productivity
1	−1.000	−1.000	−1.000	36.2174	5.95563
2	−0.266	−1.000	−1.000	54.8072	18.0114
3	0.367	−1.000	−1.000	48.3357	16.3704
4	0.924	−1.000	−1.000	50.2534	17.5258
5	−1.000	−1.000	−0.998	16.1082	2.81955
6	−1.000	−1.000	−0.980	14.3794	3.12152
7	−1.000	−1.000	−0.800	33.3294	6.39619
8	−1.000	−1.000	1.000	28.6824	6.56602
9	−1.000	−0.800	−1.000	13.8194	3.70189
10	−1.000	−0.500	−1.000	24.1667	3.84251
11	−1.000	0.000	−1.000	22.0139	5.02928
12	−1.000	0.500	−1.000	10.9557	2.38781
13	−1.000	1.000	−1.000	9.8191	3.12027
14	1.000	−1.000	−0.998	40.9393	4.86927
15	1.000	−1.000	−0.980	44.2691	5.90967
16	1.000	−1.000	−0.800	47.4623	4.67465
17	1.000	−1.000	1.000	64.876	6.00773
18	−1.000	0.000	−0.998	18.383	5.65035
19	−1.000	0.000	−0.980	22.0846	12.3887
20	−1.000	0.000	−0.800	14.8881	9.0308
21	−1.000	0.000	1.000	25.2337	10.3441
22	1.000	−0.800	−1.000	69.1179	17.6668
23	1.000	−0.500	−1.000	66.2453	11.8709
24	1.000	0.000	−1.000	68.8868	10.3094
25	1.000	1.000	−1.000	72.7217	10.882

Table 4. ANOVA of the quadratic model for biomass productivity

Source	Sum of squares	Degree of freedom	Mean square	F-value	p-Value
Model	7,300.65	9	811.18	18.48	<0.0001
A-K_2HPO_4	3,147.98	1	3,147.98	11.96	0.0072
B-$MgSO_4$	7.64	1	7.64	5.29	0.0470
C-Yeast Extract	67.54	1	67.54	6.68	0.0295
AB	834.31	1	834.31	15.63	0.0033
AC	39.26	1	39.26	1.16	0.3089
BC	12.83	1	12.83	0.8651	0.3766
A^2	646.99	1	646.99	114.98	<0.0001
B^2	87.05	1	87.05	8.20	0.0186
C^2	40.05	1	40.05	7.46	0.0232
Residual	298.50	9	33.17		
Cor total	7,599.15	18			
CV	4.79				
Mean	32.93				
Adeq. precision	16.3427				

carbon-rich wastewaters [47-50]. In one study, *Pseudomonas* sp. cultured in molasses had high energy production of up to 660.82 mW/m^2 power density [51]. Another study found that 5 g/L molasses produced the highest power density of 1,570.68 mW/L in a *Clostridium* sensu stricto dominant microbial consortium [52]. Biocatalysts such as *Actinobacteria*, *Geobacter sulfurreducens*, *Pseudomonas putida*, *Bacillus subtilis* or anaerobic sludge can also ensure enhanced power density in the MFC [20]. As with fermentation,

Table 5. ANOVA of the quadratic model for lipid productivity

Source	Sum of squares	Degree of freedom	Mean square	F-value	p-Value
Model	251.43	9	27.94	18.48	<0.0001
A-K_2HPO_4	18.08	1	18.08	11.96	0.0072
B-$MgSO_4$	8.00	1	8.00	5.29	0.0470
C-Yeast Extract	10.10	1	10.10	6.68	0.0295
AB	23.62	1	23.62	15.63	0.0033
AC	1.76	1	1.76	1.16	0.3089
BC	1.31	1	1.31	0.8651	0.3766
A^2	173.81	1	173.81	114.98	<0.0001
B^2	12.40	1	12.40	8.20	0.0186
C^2	11.28	1	11.28	7.46	0.0232
Residual	13.61	9	1.51		
Cor total	265.04	18			
CV	19.04				
Mean	6.46				
Adeq. precision	17.5369				

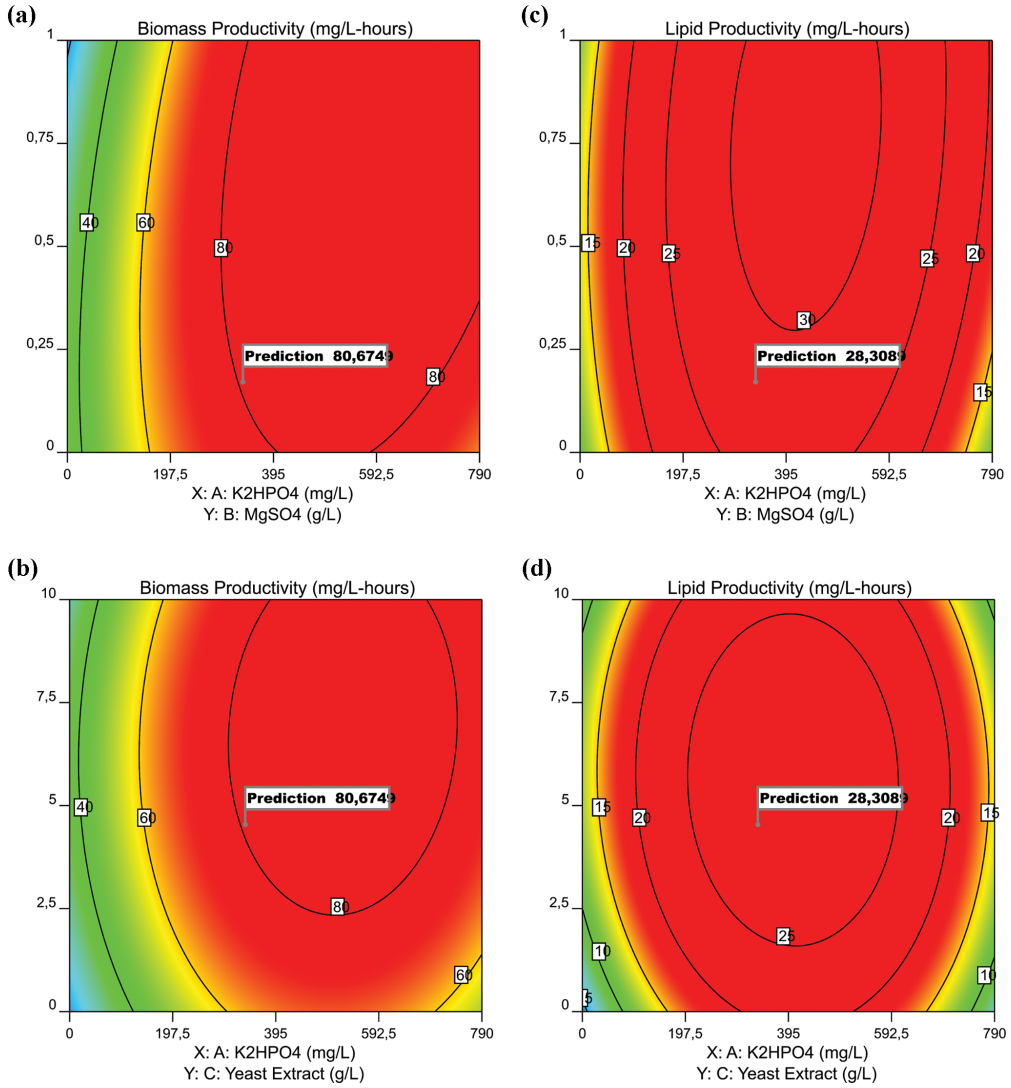

Fig. 5. Surface response methodology results of *Yarrowia lipolytica*: Contour plots for optimal condition for the highest biomass with highest lipid productivity for combined variables (a), (b) K_2HPO_4 with $MgSO_4$ and (c), (d) K_2HPO_4 with yeast extract.

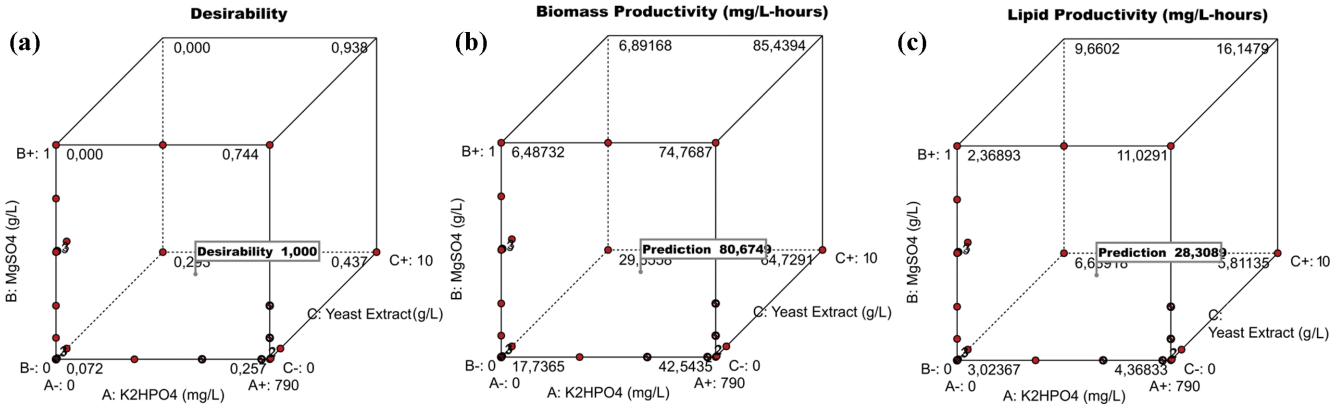

Fig. 6. Surface response methodology results of *Yarrowia lipolytica*: Cube representation for highest biomass with highest lipid productivity (a) desirability, (b) biomass productivity and (c) lipid productivity.

Table 6. The validation of predicted cultivation performance

Yarrowia lipolytica cultivation performance	Optimum points			Before optimization		Percentage of enhancement (%)	
	Predicted	Experimental	RMSD	M20	YPD	M20	YPD
K_2HPO_4 (mg/L)	336	336		-	-		
$MgSO_4$ (g/L)	0.17	0.17		-	-		
Yeast extract (g/L)	4.54	4.54		-	-		
Biomass productivity (mg/L/hour)	80.7	78.6±6	%2.75	36.2±1.4	56.2±4.3	110±8	71±6
Lipid productivity (mg/L/hour)	28.3	26.2±1.1	%0.71	7.6±0.8	11.7±1.7	179±21	118±18

*Data are expressed as mean±SD (standard deviation)

the amount of H_2 production will vary depending on substrate type and concentration. The H_2 and/or CH_4 produced by this technology can be used to generate electricity in waste biorefineries [16]. On the other hand, MFC is unable to compete with other energy sources such as ethanol, lipids and other value-added chemical products that can be obtained from fermentation [53,54].

CONCLUSION

This study demonstrates that the lipid and biomass productivity of an industrially important strain, *Yarrowia lipolytica*, can be increased by combining molasses substrate with other nutrient sources. The studies of single and combined nutrient supplementation to molasses showed that phosphorus supplementation as a single factor resulted in the highest biomass productivity and lipid productivity, while the combination of phosphorus and magnesium maintained this high lipid productivity. The surface response methodology demonstrated the optimal condition for the highest biomass and lipid productivities, which is also higher than the YPD broth growth condition. The result overcomes the need for time-consuming, labor-intensive batch experiments and could be applied to large-scale yeast cultivation with molasses substrate.

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DECLARATION OF COMPETING INTEREST

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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