Utilization of landfill leachate for the production of oleaginous yeast *Y. lipolytica*

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Abstract

In this study, the potential use of landfill leachate for the production of oleaginous yeast *Yarrowia lipolytica* was analyzed. A young landfill leachate source was utilized in flask tests for the concentration and nutrient optimization required to obtain maximum biomass growth. Lipid production by *Y. lipolytica* grown in landfill leachate was analyzed to evaluate the lipid yields at the studied conditons. In addition, compositional analysis of the yeast cell in terms of lipid, protein and carbohydrate content were determined in dry biomass obtained from each growth condition. The maximum biomass concentration produced was 15.1 g/L using 100% of landfill leachate with C/N ratio of 50 (supplement of 114 g/L glucose) and the highest lipid content (36%) was obtained from the leachate media with C/N of 100. A COD removal of 50% was obtained in raw leachate and a maximum COD removal was achieved by 70% in yeast extract-added leachate. The results of the study revealed that the oleaginous yeast *Y. lipolytica* can be a suitable species resistant to inhibitory effects of complex organic constituents of wastewater streams that can be harvested in landfill leachate for the valorization of nutrient capacity for lipid production which also contribute to the removal of contaminants.

1. Introduction

Management of landfill leachate containing a wide range of recalcitrant and toxic compounds remains a challenge of environmental pollution (Hu et al., 2016). Recent studies related to landfill leachate (LL) focus on biotechnological approaches for treatment and valorization of organic and nutrient content in order to enhance their removal and conversion to valuable microbial products. Phytotreatment of landfill leachate using energy crops (sunflower, soybean, rapeseeds) for biodiesel was studied to reduce the costs associated with irrigation and fertilization, and COD, N, P removal (Garbo et al. 2019). Another aspect of using specific cultures for treatment of landfill leachate involves removal of toxic or inhibitory compounds which can be biodegradable by these species. Especially various fungal species were studied for treatment of landfill leachate. Treatment of intermediate and old LLs with ascomycete fungal strain the *Lambertella* sp. resulted in 90% TOC and toxicity removal (Siracusa et al. 2020). In a bioremediation study, landfill leachate allochthonous and autochthonous fungal strain were used for treatment of landfill leachate for decolorization, COD and toxicity removal (Spina et al. 2018). Detoxifying species white-rot basidiomycete *Trametestrogii* was studied for reduction of recalcitrants, phenols, ammonia and hydrocarbons grown in landfill leachate concentration of up to 30% (Smaoui, 2019). Treatment with photosynthetic bacteria *R. palustris* was studied for the removal of TOC and TN (Wang et al., 2018).

Yarrowia lipolytica is one of the most studied oleaginous yeast due to its advantages of biotechnological characteristics and unique physiological features, such as the growth in hydrophobic substrates, has made it an important biotechnological yeast. *Y. lipolytica* production has been also proposed for the treatment and degradation of pollutants such as hydrocarbons, oils, nitro, halogenated and organophosphate compounds, for the reduction of metals and for the treatment of wastewater (Zinjarde et al., 2014). It is a dimorphic, non-pathogenic ascomycetous yeast often found in environments with the presence of hydrophobic substrates such as dairy products and oily waste, soils contaminated with oils, marine, sediments, and wastewaters (Ledesma-Amaro and Nicaud, 2016; Zinjarde et al., 2014).

Y. lipolytica attracts great attention for its potential in production for oil feedstock due to needs for alternative cheaper, safer, and sustainable fuels sources (Darvishi et al., 2017). It is a high-oil yeast and can contain up to 70% of lipids by dry cell weight (Munch et al., 2015). Lipid accumulation occurs intracellularly primarily formed of neutral lipids, particularly TAGs (85%) and some steryl esters (8%). Due to the absence of polyunsaturated fatty acids, the fatty acids produced by *Y. lipolytica* indeed are suitable for biodiesel production. *Y. lipolytica* shows great compatibility in large-scale fermentations in terms of biotechnological production. The application of these oleaginous yeasts in industrial processes first requires the optimization of culture conditions such as carbon, nutrient, or oxygen concentration, temperature, pH, to maximize the yeast growth under appropriate cultivation conditions.

The aim of the present study is to assess the potential utilization of landfill leachate for the biomass and lipid production of oleaginous yeast *Y. lipolytica* and to determine the effect on the reduction of contaminants based on treatment for COD, N and P removal. The outcome of the study contributes to evaluate the valorization and integrability of yeast production as a part of the treatment i.e. pre/post treatment of landfill leachate as a valuable process.

2. Methods

2.1. Growth Conditions

Landfill leachate taken from solid waste deposition site was used as growth media for *Y. lipolytica* (MUCL 28849) after autoclaving. 25 mL of leachate samples in conical 100 mL flasks that changes in the concentration of landfill leachate and supplied nutrients were used to analyze their effect on yeast growth. Supplementary nutrients of phosphorus (phosphoric acid), yeast extract, and glucose (D-glucose) were added to the LL to find the optimum doses that were determined according to the characterization of the landfill leachate in Table 1.

Samples were inoculated with the seed culture of *Y. lipolytica* that was grown in YPD medium. All media were inoculated to start with an initial OD600 of 0.9. In these studies, the initial pH value of the culture media was adjusted to be 5.8. Culture media were studied in duplicate. The flasks were incubated in a shaker incubator at a speed of 300 rpm and temperature of 28±2 °C operated with natural ventilation. *Y. lipolytica* cultures were grown to the stationary phase. Yeast growth analysis is performed by taking a sample of 100 μL in a daily period for OD600 measurement. Optical density was assessed in the samples as net OD600. The pH was daily measured in the LL samples. At the end of fermentation, biomass growth was analyzed as SS, VSS and effluent samples were analyzed after filtered through 0.45 μm to assess the changes in COD, nitrogen, and phosphorus content in the growth media.

Biochemical composition analysis of the yeast biomass samples taken were carried out by lipid, protein, carbohydrate component analysis to investigate the efficiency of lipid production and the effect of growing conditions on biochemical composition. First, yeast biomass was dried using a freeze dryer (ThermoSavantModulyo D) for 48 hours at -45°C, below 0.07 millibar. Cell lysis is required for the analysis of biomolecules. In this study, mechanical homogenization and mechanical cell disruption method were preferred. Briefly, cell fragmentation was performed in plastic tubes containing 1 mm diameter beads with a vortex device.

2.2. Analytical Methods

Lipid analysis was performed by colorimetric Sulfo-Phospho-Vanillin (SPV) method (Hao et al., 2013) and by extraction with Chloroform-Methanol using gravimetric Bligh-Dyer (1959) method. Carbohydrate analysis was carried out by the Anthrone method by reading the absorbance at 630 nm against distilled water. When using this method, Gerhard et al. (1994), Dulekgurgen (2006), and Waghmare et al. (2016) resources were used. For protein analysis Gerhard (1994) et al. and Lowry et al. (1951) method was modified, and protein analysis was performed spectrophotometrically at 750 nm.

"Standard Methods" SM 5220 B, SM 2540 E, SM 4500-H+ B, 4500-NH_3 nitrogen (ammonia) C, Makro-Kjeldahl, 4600-P Phosphorus methods were used for COD, SS, VSS, pH, NH_3-N, TKN,TP and ortho-P analysis (APHA, 2005).

3. Results and Discussion

3.1. Landfill Leachate

The characterization of the leachate is presented in Table 1 and its elemental analysis is presented in Table 2. According to the characterization results, the leachate can provide about 11 g/L organic carbon as COD, adequate nitrogen and some of the trace elements required for the microbial growth and contains low density of phosphorus and suspended solids. The pH and the alkalinity values of the raw leachate taken from the solid waste landfill are quite high. Since the pH of the raw leachate, which is near 9, rises to 10 after autoclaving and is unsuitable for culturing, it was adjusted to 5.8 which is the optimum pH for growth of *Y. lipolytica* (Fontanille et al., 2012) in leachate samples at the beginning of all studies.

Table 1. Characterization of the landfill leachate used in the study.

	Before Autoclave	After Autoclave
TCOD(mg/L)	11200	11232
SCOD (mg/L)	109032	11193
TKN (mg/L)	3634	2470
NH_3-N (mg/L)	2920	1960
TP (mg/L)	100	100
Ortho-P (mg/L)	3.7	3.9
SS-VSS (mg/L)	852-751	791-713
pH	8.3	9.8
Conductivity (mS/cm)	35.5	23.9
Alkalinity (g$CaCO_3$/L)	10.4	-

Table 2. Elemental analysis of the landfill leachate used in the study (mg/L).

Fe	Ca	Al	Mn	Zn	Li	Mg	K
7.785	66.04	0.343	0.127	0.805	0.613	77.5	667.2

3.2. Growth Optimization of Yarrowia Lipolytica in Landfill Leachate

3.2.1. Optimum Concentration of Landfill Leachate

Although the COD of the raw leachate is at a low concentration of 11.2 g/L, the growth of *Y. lipolytica* in leachate at different concentration values was first observed due to the possibility of inhibition of microbial growth in 100% LL.

The mean net optical density change over time in media containing 50, 60, 70, 80, 90 and 100% leachate is presented in Figure 1. Cultures grown for 120 hours passed from the exponential phase to the stationary phase when samples were taken approximately at each 24 hours for all of the concentration samples of LL. *Y. lipolytica* could grow in 100% of landfill leachate without any requirement of dilution in case of inhibitory effect of toxic compounds. While a decrease started in optical density on the 2nd day at 50% and 60%, on day 3 at 70%, while the growth in 80%, 90% and 100% remained in the stationary phase for a longer time. As can be seen from the low inlet COD values at dilute leachate concentrations such as 50% and 60%, the transition to the death phase began after 24 hours due to the depletion of the limited carbon source. In 80%, 90%, and 100% of leachate, approximately the same optical density was observed during the growth. The highest value is 7.3 as optical density at 100% and 90% leachate after 48 hours. In order to get as high biomass yield as possible from the oleaginous yeast, LL was not diluted to decrease the carbon source and 100% of LL was used in the next stages for growth of *Y. lipolytica.*

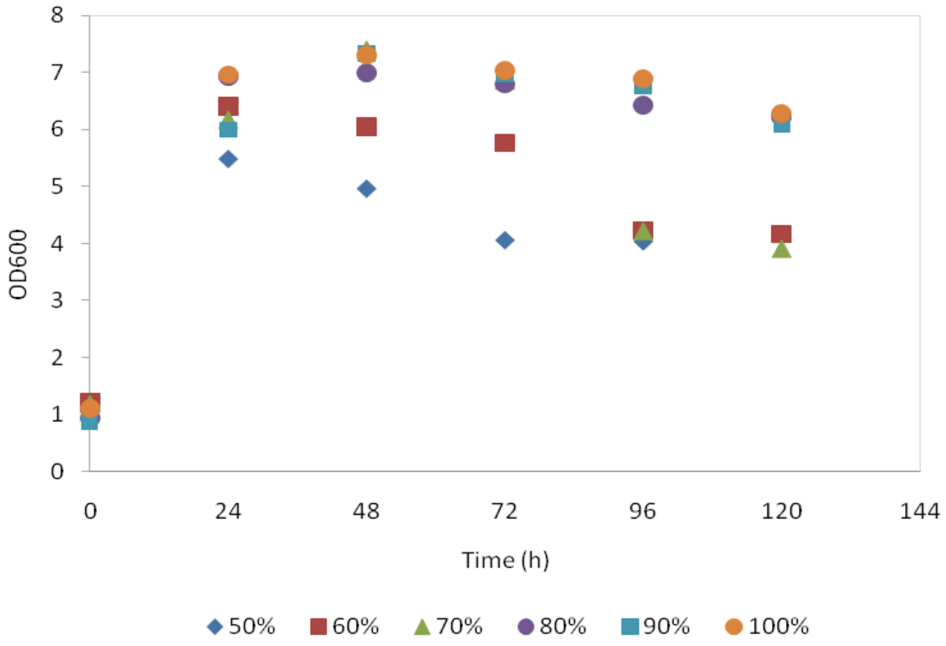

Figure 1. Change in optical density due to the growth of *Y. lipolytica* grown in different LL concentration between 50-100%.

3.2.2. Supplement of Phosphorus and Yeast Extract

After the leachate concentration was determined as 100%, the characterization in Table 1 was used to determine the optimization requirement of the nutrients. Accordingly, carbon source (11.2 gCOD/L) and total phosphorus (100 mg/L) values were at low levels as limiting nutrients in the landfill leachate.

First, the microbial growth that phosphorus can limit was investigated. Phosphorus-optimized growth rates in as a source of phosphorus phosphoric acid-supplemented leachate samples were investigated. In Figure 2, where the microbial growth is shown by average optical density of parallel samples, the phosphorus concentrations added to the leachate are 125, 250, 375, 500 and 1000 mg/L as ortho-P. Since the maximum optical density was reached after 48 hours in Figure 1, cultures were grown for 48 hours until the highest optical density was observed. It is seen that the highest growth rates occurred approximately at the same point (OD: 6.8) with the addition of 375, 500 and 1000 mg/L phosphorus.

In addition to phosphorus, the effect of yeast extract supplementation on the growth of *Y. lipolytica* in LL was investigated which was often used as a stimulant in microbial growth studies. The average optical density of the parallel LL samples that change in the concentration of yeast extract was shown in Figure 3. Yeast extract had a greater effect on the increase of microbial growth than the addition of phosphorus. While there was less increase in the raw LL, and 100 and 1000 mg/L Y.E. added LL, there was a significant increase in the optical density until 15.6 in the leachate containing 10.000 mg/L of yeast extract. It was observed that during microbial growth the pH generally rises to around 7.5-9 (Figure 2 and Figure 3).

SS and VSS were measured at the end of the culture period of the phosphorus and yeast extract added LL samples were shown as average values of parallel LL samples in Figure 4A. Supplement of phosphorus and yeast extract to the leachate increased the amount of biomass produced. A net VSS increase of 400 mg/L was obtained in the leachate with the addition of 125 mg/L phosphorus from 2200 mgVSS/L measured in raw LL to 2600 mg/L. The same VSS increase was seen approximately with the increasing P addition of 250, 375, 500, 1000 mg/L above 125 mg/L. With the supplement of yeast extract of 100, 1000 and 10,000 mg/L, the biomass yields obtained were as a net VSS of 2400, 2700, and 5500 mg/L, respectively. In the COD removal rates given in Figure 4B), the rate of removal in raw leachate increased from 50% to 60% with the addition of 125 mg/L phosphorus, and the increase in removal efficiency did not continue at subsequent concentrations. The highest COD removal efficiency was achieved in LL with 10,000 mg/L Y.E. approximately by 70%.

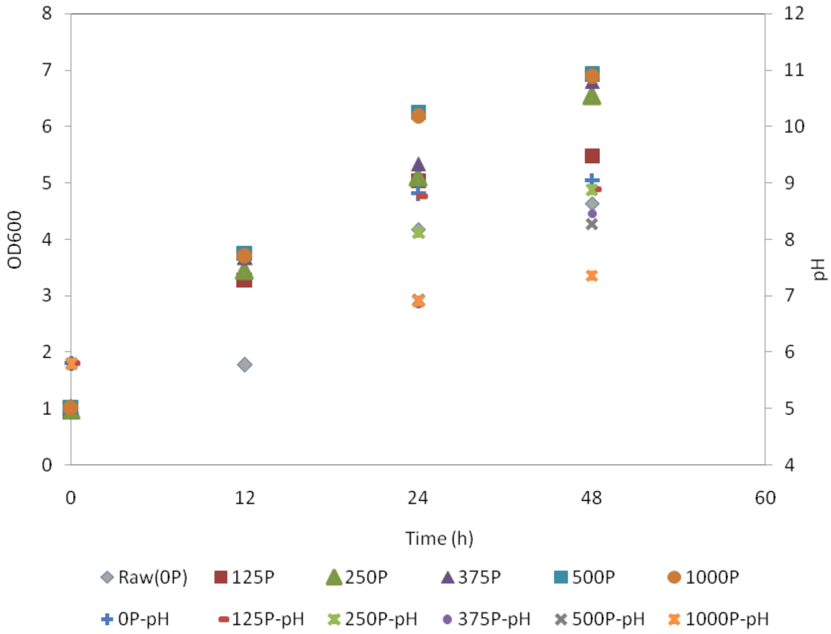

Figure 2. Change in optical density due to the growth of *Y. lipolytica* in raw and phosphorus added landfill leachate at the concentration of 125, 250, 375, 500, 1000 mg/L of ortho-P.

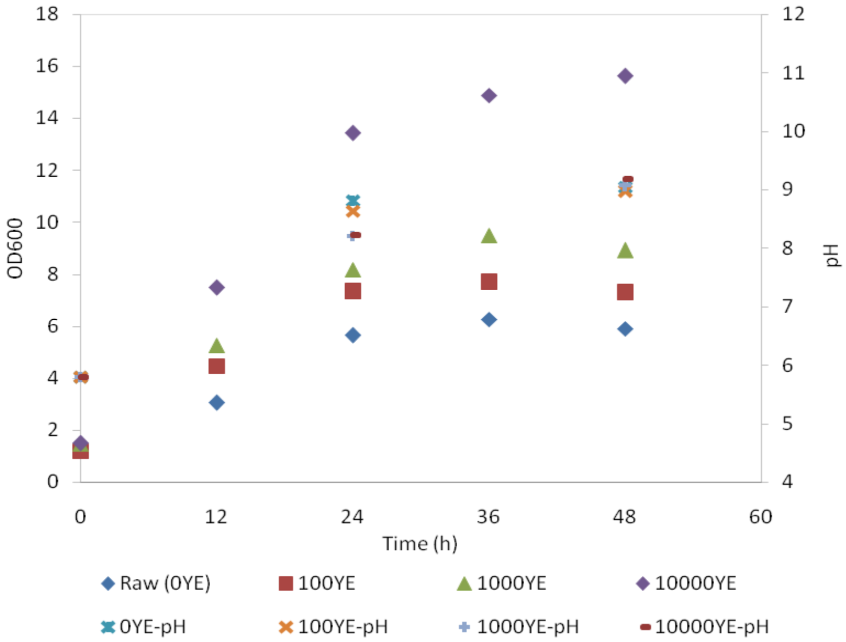

Figure 3. Change in optical density due to the growth of *Y. lipolytica* in raw and landfill leachate supplied with 100, 1000, 10,000 mg/L of yeast extract.

B

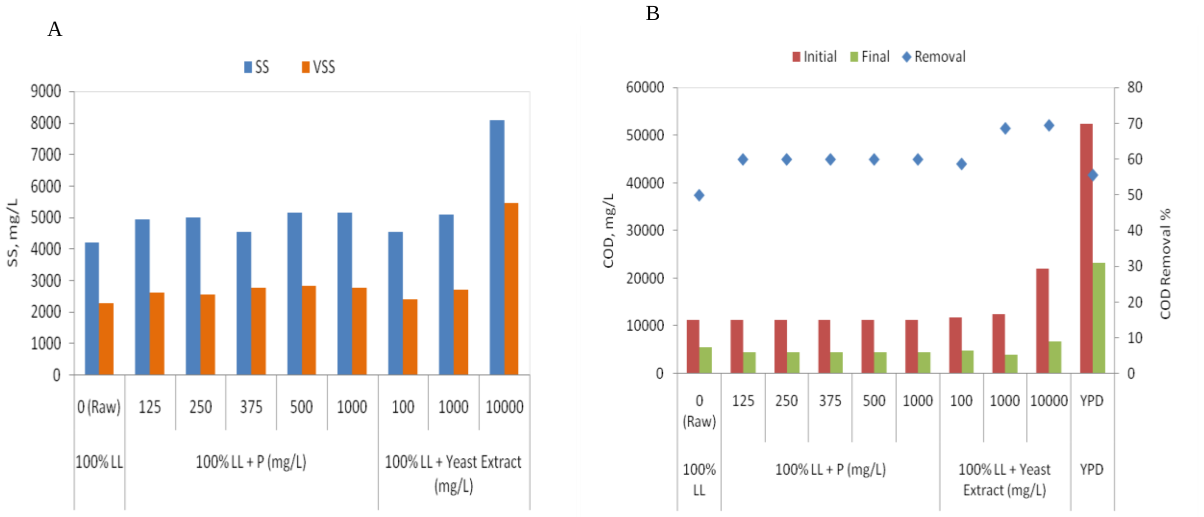

Figure 4. Net growth of *Y. lipolytica* as SS-VSS (4A) and change in COD (4B) in raw, phosphorus, and yeast extract supplemented landfill leachate.

In Figure 5, total phosphorus (5A) and NH_3-N (5B) during 48 hours of growth in landfill leachate samples were shown. The total phosphorus values represent the change in phosphorus in landfill leachate before and after microbial growth that was consumed by yeast cells for growth or by precipitation of phosphorus during biomass separation in the effluent. In the ammonia nitrogen change shown in Figure (5B), yeast extract added leachate samples showed greater reduction in ammonia nitrogen as compared to the phosphorus added leachate samples. In parallel, the highest biomass (5.5 g/L) production was observed with the addition of yeast extract.

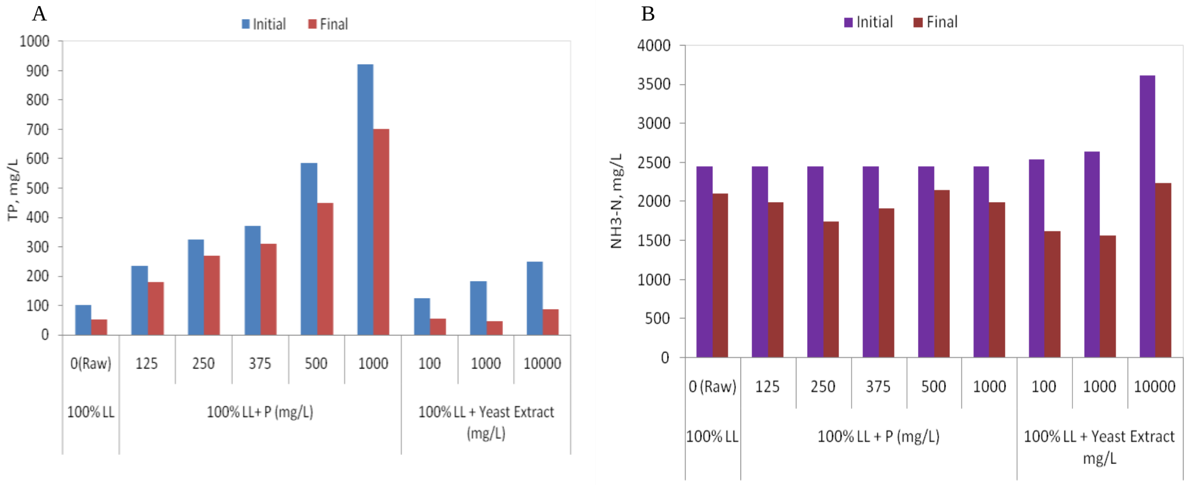

Figure 5. Change in TP (5A) and NH_3-N (5B) in landfill leachate media supplemented with phosphorus and yeast extract.

3.2.3. Carbon Source Addition

The carbon content of the landfill leachate (11.2 gCOD/L) was not sufficient to produce an enriched biomass yield and lower than the amount of carbon source in YPD media used to grow the seed culture. Therefore, the carbon of LL was increased by adding glucose to get higher biomass and lipid yields from the oleaginous yeast. The effect of carbon/nitrogen ratios on the growth and lipid production of oily yeast has been generally shown to influence the yield of microbial lipids in the literature (Hassan et al., 1996). The growth of *Y. lipolytica* was investigated with the addition of glucose to leachate as carbon source based on four different C/N ratio and under previously determined optimum landfill leachate concentration and optimum phosphorus conditions as 100% LL and 125 mgP/L respectively.

The carbon/nitrogen ratio of the raw leachate is approximately 5 in terms of COD/TKN. In the C/N ratio study of the leachate, microbial growth was analyzed at C/N ratios of 5 (raw), 50, 100, and 125. Due to the low carbon content of the leachate, the amount of glucose required to reach these ratios were 114, 239, 301.5 g/L respectively.

In Figure 6, the growth of *Y. lipolytica* in terms of optical density in different C/N ratios of parallel leachate samples and pH change were presented. All of the LL samples were added with 125 mg/L phosphorus before seeding. In C/N 5, the optical density started to decrease after reaching up to OD of 5, which showed a similar growth observed in the raw LL in Figure 2. With glucose addition, in leachate samples with C/N 50, 100 and 125 the optical density continued to increase up to OD of 17, 15, and 13 respectively. LL was the most productive in C/N 50 than C/N 100 and 125 in which the highest optical density of *Y. lipolytica* was measured after 60 hours of the incubation. The change of pH during microbial growth was followed in different C/N conditions. The same situation of pH increase to 8-9 levels that was observed in the phosphorus addition in to raw leachate was also seen here (raw LL: C/N 5). However in carbon-added leachate samples, the pH increased until the exponential growth phase, then declined until pH of 3.5-4 after 72 hours of growth due to the effect of higher glucose content.

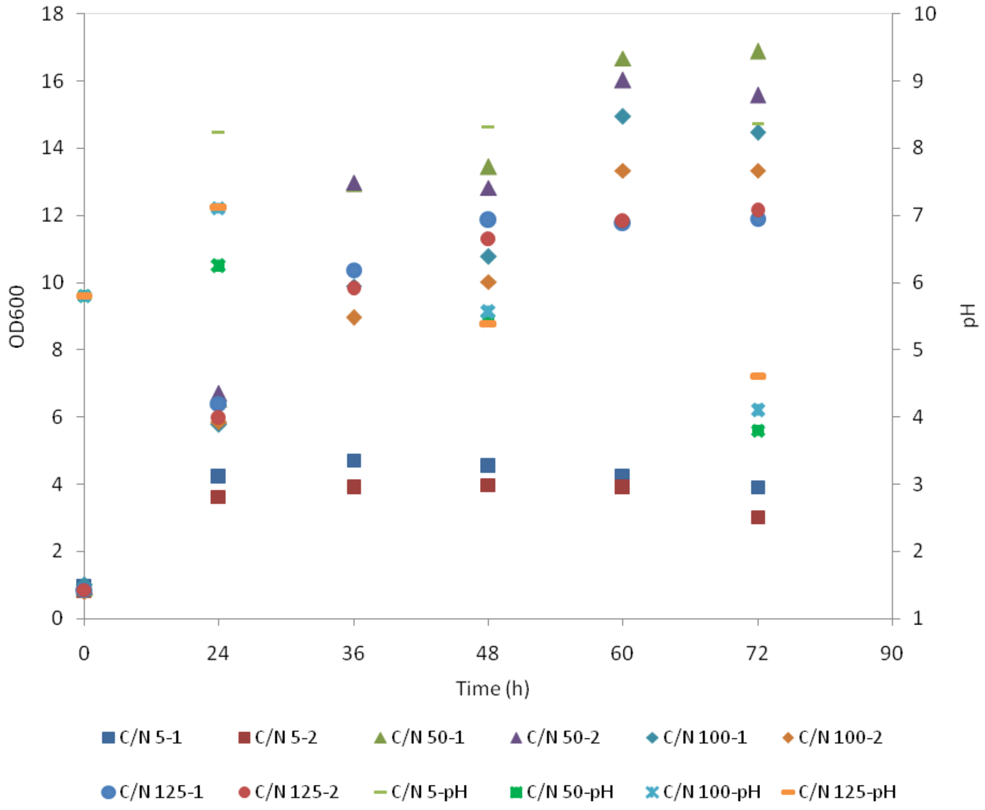

Figure 6. Change in optical density and pH due to the growth of *Y. lipolytica* at different C/N ratios of raw and LL with glucose.

The net SS-VSS results of the *Y. lipolytica* grown in raw and glucose added different C/N ratios of LL were shown in Figure 7A and the initial and final COD values, % COD removal rates in the leachate samples in Figure 7B. The highest amount of biomass obtained was 15 g/L as VSS in leachate with C/N 50. The growth in C/N 5 which was the raw leachate was 2.2 g/L as VSS, while a biomass production of 9.3 and 9.6 g/L as VSS was measured in LL with C/N ratio of 100 and 125. The biomass first increased with the increase of C/N with carbon addition, and a slight decrease at higher C/Ns shows parallelism with the literature findings. It is known that oleaginous yeast converts its biochemical mechanisms from biomass production to lipid production in the presence of excess carbon source in growth environments where nitrogen is a stress condition. In carbon addition, the highest COD removal was obtained with 48% in C/N ratio of 50 leachate. In general, the optimum glucose addition was determined as C/N of 50 at which the highest biomass yield was obtained.

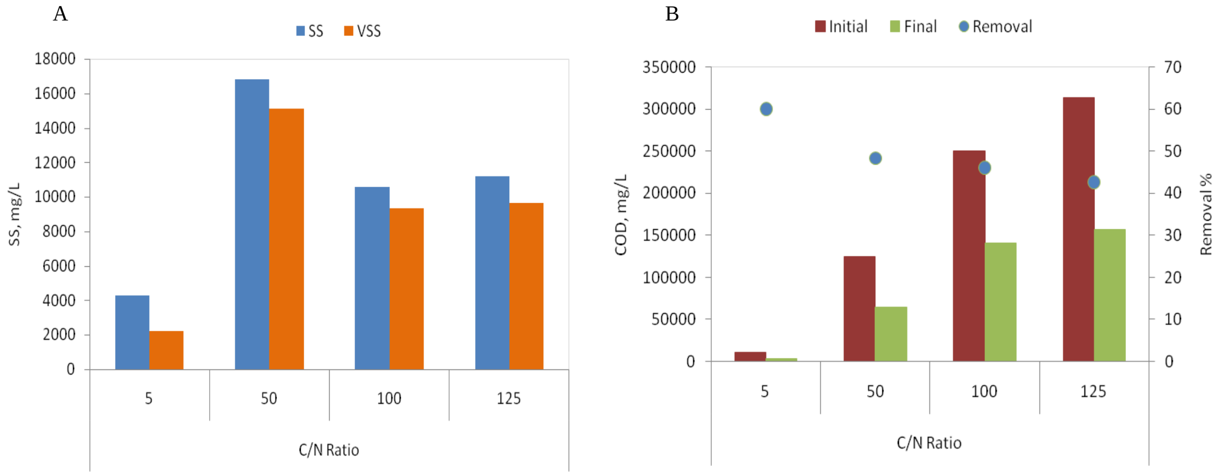

Figure 7. Net growth of *Y. lipolytica* as SS-VSS in landfill leachate with different C/N ratios (7A),change in COD in landfill leachate with different C/N ratios (7B).

3.3 The Lipid, Protein and Carbohydrate Composition of Y. Lipolytica Grown in Landfill Leachate

The lipid, protein, and carbohydrate values of *Yarrowia lipolytica* biomass grown in leachate samples were shown in Figure 8 and 9. The lipid variation due to different leachate concentration is in the range of 10.3-18.2% (Figure 8A). Highest lipid content measured was 18% in biomass grown in 90% LL considering the LL concentration optimization study. Considering the biomass content in glucose added LL samples as the C/N ratio of the leachate changed, the amount of lipids also showed an increase up to the C/N ratio of 100, while the lipid value at the C/N ratio of 125 value decreased by 44% of the lipid of C/N ratio of 100 (Figure 8B). Although the C/N ratio of 100 resulted in the highest lipid content (36%), the biomass production at C/N ratio of 50 is 1.6 times higher than the biomass produced at C/N ratio of 100, and therefore the lipid productivity efficiency of the C/N of 50 stress condition is approximately 1.4 times higher compared to the C/N ratio of 100.

With the addition of phosphorus, lipids in the range of 20-25.4% were observed (Figure 9A). It is seen that the highest lipid amount is 25.4% as a result of 375 mg/L phosphorus addition. With the addition of yeast extract, lipid values first decreased by 40% and reached 19.8% under the condition of 10000 mg/L yeast extract (Figure 9B). These lipid values are also proportional to lipid productivity. While phosphorus addition showed similar lipid productivity, 10000 mg/L yeast extract did not give the highest lipid value, but it had high lipid productivity due to high biomass (5475 mg/L) in the growth medium.

Studies with landfill leachate in the literature have been carried out with algae rather than oleaginous yeast. Therefore, the results of *Y. lipolytica* grown in landfill leachate in the present study make a contribution for future studies. Although microalgal biodiesel production has some advantages, compared to *Y. lipolytica* microalgal biodiesel production is not the best alternative to petroleum diesel. *Y. lipolytica* has higher growth rates and lipid content, lower doubling times than microalgae (Beopolous et al., 2009; Papanikolaou et al., 2001). Algal biomass productivity was set at 4 g/L for photobioreactors (Sitepu et al, 2014) while *Y. lipolytica* can grow rapidly up to 41 g/L (Fontanille et al., 2012) and accumulate lipids over 50% by dry weight (Bati et al., 1984). Another superiority of the oleaginous yeast over the microalgae is the potentially protection from bacterial contamination due to faster growth and growth at low pH conditions (Sitepu et al., 2014). On the other hand, oleaginous yeast feeds on organic carbon and requires cost calculation of substrates.

The effect of phosphorus to the landfill leachate which had low P content was investigated to increase the biomass and lipid production of *Y. lipolytica* and it was observed that it improved the production of larger amount of biomass. Besides its benefits, it was reported that excess P in microalgae culture causes excessive accumulation of polyphosphates within microalgae cells and also causes P to bind to intracellular components (Chang et al., 2019).

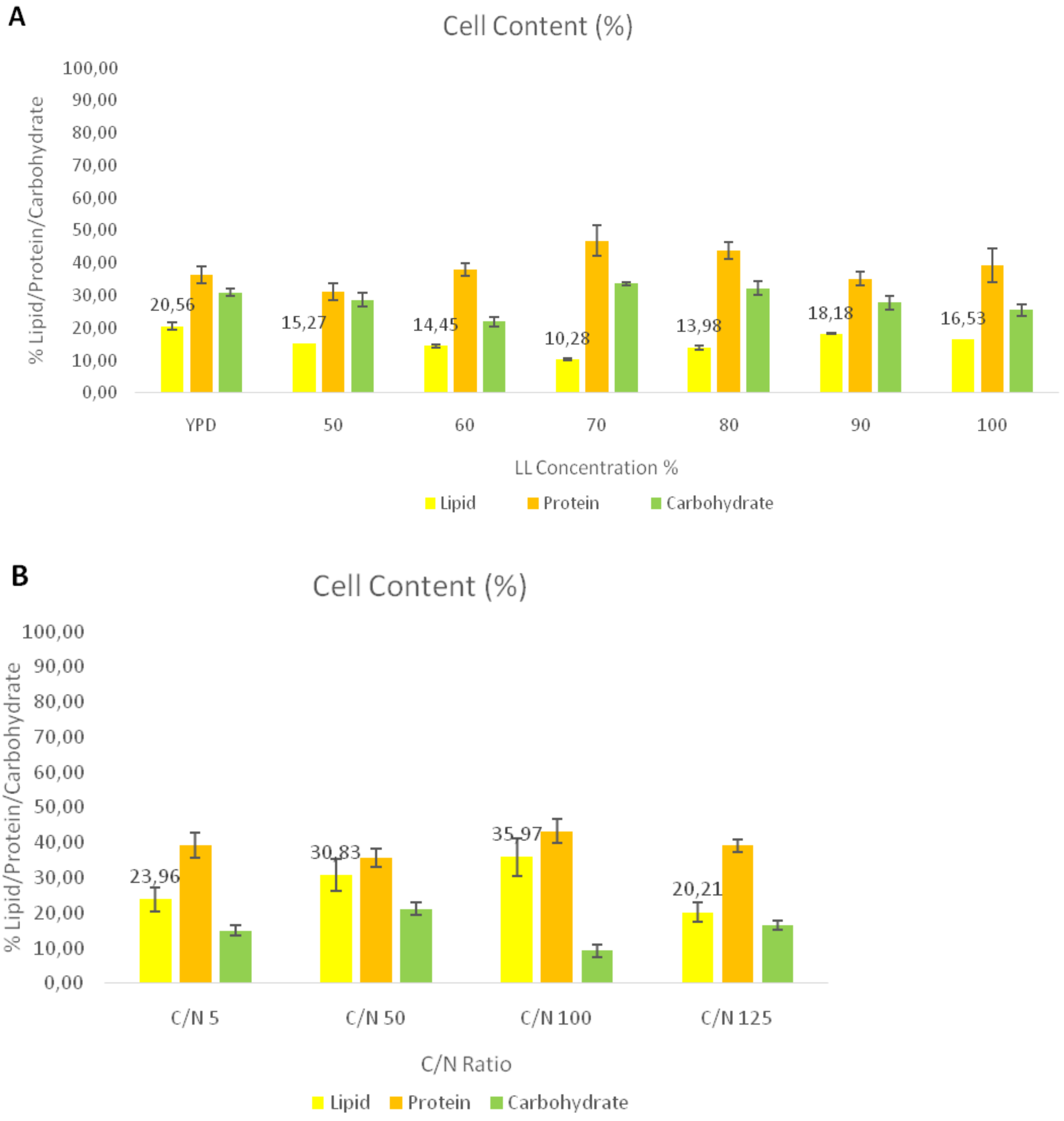

Figure 8. Lipid, protein, and carbohydrate values of *Y. lipolytica* grown on leachate A) different leachate concentration B) different C/N ratios.

Considering the waste treatment ability of *Y. lipolytica*, 80% reduction in COD value in olive oil mill wastewater (Scioli et al., 1997) and a 90% reduction in COD in palm oil factory wastewater was observed (Oswal et al., 2002). Similarly, up to 60-70 % of COD removal was achieved in landfill leachate using *Y. lipolytica* in the present study which can increase the treatment efficiency of landfill leachate in terms of waste valorization and management. On account of these benefits *Y. lipolytica* can also be used to treat wastewater within the framework of waste recycling.

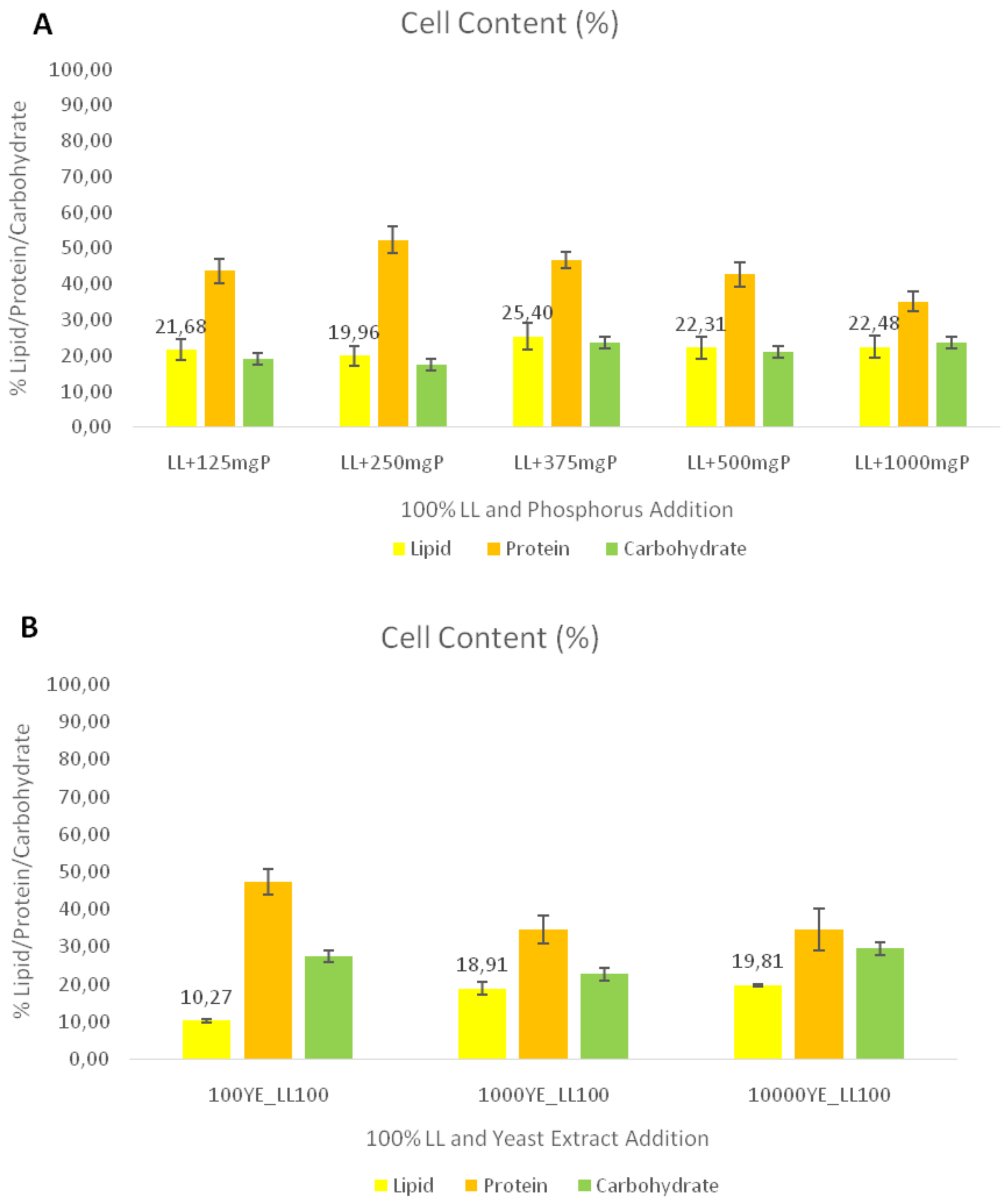

Figure 9. Lipid, protein, and carbohydrate values of the *Y. lipolytica* grown in leachate A) phosphorus addition B) yeast extract addition.

Nitrogen-limited conditions are known to stimulate lipid productivity of the yeast cells that is controlled with C/N ratio. Landfill leachate lacks the necessary conditions to increase the lipid content, since the C/N ratio is lower than wastewater with high oil content, such as olive black water. For this reason, there are few studies in the literature on the production of biodiesel from leachate using *Y. lipolytica* (Katre et al., 2012). The cultivation of *Y. lipolytica* on glucose in a fed batch bioreactor was carried out with a C/N ratio of 62 and an overall yield of biomass (43g/L), and lipid content near to 37% were obtained (Fontanille et al., 2012). Similarly, the highest lipid content of *Y. lipolytica* achieved in batch culture of landfill leachate was obtained at a C/N ratio of 125 by 36% using glucose as additional carbon source. When higher biomass productivity rates were considered a C/N ratio of 50 could sustain higher lipid production by a lipid content of 31 %.

4. Conclusion

Y. lipolytica, which has good extracellular hydrolysis activity, can be an alternative microorganism to treat landfill leachate. It is very promising regarding to the results of the present study to conclude that landfill leachate can provide potent growth of *Y. lipolytica* despite its different pollutants and macromolecules likely resistant to degradation/decomposition. 100% LL as the optimum concentration, the growth and lipid production of *Y. lipolytica* were increased by addition of phosphorous and yeast extract resulted in increased biomass production with respect to raw leachate (2.6 mg/L and 5.5 mg/L as VSS respectively). Then, stress conditions with C/N 50 found to be the best conditon in terms of a biomass productivity of 15.1 gVSS/L with a lipid content of 31%. These results give promising results in terms of revaluation of landfill leachate and obtaining valuable product.

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