Evaluate the effect of C/N ratio on *Yarrowia lipolytica* grown in fermented food waste

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Abstract

Yarrowia lipolytica is an oleaginous yeast with the ability of accumulate great amount of lipid. By increasing demand of biofuel as a sustainable replacement of chemical fuel, microbial oil has gained attention as a valuable product and favorable feedstock for biodiesel production. Organic wastes generated from food industries are a major of concern in terms of environmental pollution. In order to manage fast growing food wastes (FW) problem in the world, economic and sustainable methods are required to be developed. Oleaginous microorganisms are considered as potential tools to metabolize organic wastes and convert them to valuable products such as intracellular lipid. Carbon rich and nitrogen limited medium is a key factor that leads to improve lipid production by oleaginous yeast. The aim of this study is evaluating the effect of three different COD/TKN ratios as 75, 100 and 125 on lipid accumulation of *Y.lipolytica*. Three different carbon sources as glucose, glycerol and potassium acetate were applied to increase COD concentration of the medium and effect of each one on lipid accumulation was assessed. In terms of biomass generation, medium with highest carbon concentration (COD/TKN 125) leads to generate highest biomass of 12.17±0.87 g/L in presence of glycerol. In terms of lipid accumulation, the optimum COD/TKN concentration was identified as 100 and the highest lipid content was achieved as 38.15±1.03% in presence of glycerol.

1. Introduction

Nowadays, due to global population growth and also rise of economy, amount of generated municipal solid waste is enhancing day by day. Accumulation of Food Waste (FW) become a major cause of concern as the amount of global FW is estimated around 1.6 billion tons (Yaashikaa et al., 2019; Dahiya et al., 2018). With the aim of develop a FW management strategy, different microorganisms are exploited as renewable source to consume complex organic matters and also recovery of some valuable products as biofuel besides treatment of waste. Oleochemicals are valuable products derived from microorganisms, animal fats and plant oils, which are considered as favorable feedstock in petrochemical industry. They can be used as biodiesel, plastics, surfactants, surface coating and paints. It is expected to reach global demand of fatty acid and fatty alcohol to more than 10 Mt in year 2020, that needs the increase in generation of fatty acid methyl esters (Yang et al. 2018). Microbial lipid generally composed of triacylglycerides (TAGs) are generated by oleaginous yeasts, bacteria and algae. These organisms have the ability of accumulate lipid in their body as more than 20% of dry mass (Ratledge and Wynn 2002), with fast growth rate and no land use in comparison with animal or plant oils. Biodiesels derive from microbial lipid as a renewable source has received attention due to its non-toxicity, low aromatic compound, biodegradability, high energy content and no interfere in human and animal food chain (Wang et al., 2020, Ma et al., 2018).

Among the oleaginous yeasts, *Yarrowia lipolytica* draws attention in biotechnology techniques. This yeast is generally found in hydrophobic environments like oily wastes, oil contaminated soils, dairy products, soy source and marine environment. It is classified as Generally Regarded As Safe (GRAS) and nonpathogenic yeast by American Food and Drug Administration (FDA) with the ability of accumulate lipid more than 40% dry mass (Madzak, 2015). There are some different factors such as carbon/nitrogen ratio, source of carbon and nitrogen, pH and temperature which directly affect the amount of accumulated lipid in oleaginous yeast. Based on the literature, lipid accumulation is induced at molar C/N ratio of higher than 20. Therefore, to achieve highest lipid accumulation, this ratio needs to be optimized. In some different studies implicating oleaginous yeasts, microbial lipid accumulation enhanced constantly by increasing the initial C/N ratio (Zikou at al., 2013). In this study COD/TKN ratio was applied and three different ratios as 75, 100 and 125 was selected. On the other hands, three different carbon sources as glucose, glycerol and potassium acetate were applied with the aim of increase the COD concentration in the medium.

Yarrowia lipolytica naturally has the ability to use glucose, glycerol and organic acids. In medium contain glucose, it can generate high amount of biomass while by increasing glucose concentration, growth decrease (Carsabna et al., 2018). Glycerol is byproduct of biodiesel production and a low-cost carbon source that is available nowadays due to growing of biodiesel industry. It is considered as a favorable feedstock for microbial lipid production (Dobrowolski et al., 2016).

2. Materials & Methods

2.1 Inoculum, growth and culture condition

Y. lipolytica strain MUCL 28849 was obtained from BCCM/MUCL (Agro) Industrial Fungi & Yeasts Collection, Belgium. It was kept on potato dextrose agar at 4°C and was sub cultured weekly on YPD medium. The medium contained 10 gr/L yeast extract, 20 gr/L D-glucose and 20 gr/L peptone. YPD medium was autoclaved at 121 °C and 15 psi for 20 min. All chemicals used in this study were analytical grade and obtained from Sigma– Aldrich or Merck. 1 L Erlenmeyer flask with active volume of 500 mL was used for inoculum growth. Applied temperature was 28± 1°C and a rotary shaker was applied at 100 rpm for 3 days. All glassware and equipment were autoclaved at 121°C for 20 minutes.

2.2 Food Waste preparation and hydrolysis

Food waste was collected from dining hall of Istanbul Technical university, Istanbul, Turkey. Firstly, food waste was grinded with food processor, then dried at 70 ºC and passed through 3mm filter. The parts less than 3 mm was collected and used as feedstock in fermentation step for hydrolysis. Collected FW was containing cow meat, chicken meat, rice, beans, bread, pasta, vegetables and cooked oil. Fermentation was applied on dry FW with Rumen microorganisms for hydrolyses. Rumen was obtained directly from stomach of sheep. In fermentation step, batch system was conducted. 1L bottles with working volume of 750ml were used with rubber stopper and aluminum cap to close bottles and kept at thermophilic condition (55±1°C) for 20 days. The medium contains 10% dried FW and 1% Rumen. SRT was selected as 5 days and pH was adjusted every day at 7 to have maximum VFA generation. Fermented FW was collected, passed through paper filter to get rid of suspended particles, and was used for inoculum cultivation. The medium was sterilized by autoclaving at 121°C and 15 psi for 20 min before cultivation.

2.3 Analytical method

Growth of *Y.lipolytica* was monitored by measuring optical density at 600 nm (OD600). Ammonia, phosphorus COD, TKN and Volatile Suspended Solids (VSS) were measured according to Standard Methods. Medium was passed through Millipore PVDF filter (0.45 μm) for VFA and soluble COD measurements. Lipid content of biomass were determined by applying modified Blight & Dyer method (Blight & Dyer, 1959). The biomass was subjected to transesterification applying chloroform, methanol, mixture of hydrochloric acid and methanol as catalyst and also methyl tridecanoate (C13:0) as internal standard. Hexane was added for phase separation and achieve FMAE containing part (Bagul et al., 2017).

2.4 Evaluate of COD/TKN ratio and set ups

Nine different sets were conducted includes 75, 100 and 175 COD/TKN ratios and three different carbon sources for each ratio. 250ml erlens with active volume of 150ml fermented FW was applied. All erlens were supplemented with 5% v/v inoculum for cultivation. Orbital shaker (N-BIOTEK NB 205) was used at 100 rpm and the flasks were incubated at 28±1°C till the growth was completed and microorganism enters stationary phase. All sets were run as duplicate. The growth was monitored every day by spectrophotometer as optical density. COD and TKN concentration were measured. This ratio was adjusted from 75 to 125 before cultivation by adding different carbon sources. TKN concentration remained the same. Samples were collected at early stationary phase for measure biomass concentration and lipid content.

3. Results

3.1 Food Waste characterization

The characterization of FW after fermentation process, is presented at Table 1. The fermented FW was analyzed to determine the organic matter, nitrogen and phosphorus composition. The characterization showed total COD of 51255±500 mg/L, 94.42±0.03 mg/L of it is soluble COD which is readily used organic carbon for microorganism. There was sufficient phosphorus in the medium, but deficiency of ammonia with concentration of 28.5±3.5 mg/L N makes the medium as nitrogen limited one. TKN concentration of fermented FW was measured as 907±14 mg/L N. Ammonia concentration was 28.5±3.5 mg/L N, indicate that most of nitrogen in the medium consist organic nitrogen. Natural COD/TKN ratio was 53.38±1.36. pH of medium was acidic and measured as 5.44±0.05. Medium pH is considered as an important parameter in lipid production by *Y.lipolytica* a key. As favorable cultivation pH range is 5-7, all the sets were conducted with natural pH and no pH adjustment was applied (Fontanille et al., 2012).

Table 1: Fermented food waste characterization

Parameters	*Values*
Total COD	51,255±500 mg/L
Soluble COD	48,400±492 mg/l
TKN	907±14 mg /L N
Ammonia	28.5±3.5 mg/L N
Total phosphorus	242±8 mg/L PO_4^{3-}
ORTO phosphate	143±23 mg/L PO_4^{3-}
pH	5.44±0.05

Note: Data are expressed as mean±SD, n = 5.

3.2 Biomass Generation

Fermented FW with natural COD/TKN ratio of 53.38±1.36 was applied as control to be able to assess the effect of different ratios on biomass and lipid.

- COD/TKN 75

To evaluate the effect of COD/TKN ratio of 75, glucose, glycerol, and potassium acetate were added to the medium separately in different experimental conditions. With the aim of increase ratio, equivalent to 19,625 mg/L COD from each carbon source was added.

Biomass growth in three different carbon sources is illustrated in Figure 1a. The biomass concentration was in its lowest amount of 3.9±0.296 gr/L when glycerol was applied. Maximum biomass concentration was obtained in the medium contain potassium acetate as 7.5±0.1 gr/L. In terms of glucose, generated biomass concentration was 6.6±0.634 gr/L. Biomass concentration of medium contain glucose and potassium are about the same with small difference when compared, but in glycerol there is significant decrease.

- COD/TKN 100

In order to increase the COD/TKN ratio to 100, equivalent to 42,300 mg/L COD glucose, glycerol and potassium acetate was added to the medium. As it is indicated in Figure 1b, after more than 600 hours no biomass growth was observed in medium with glucose. This is attributed to inhibition induced by high glucose concentration in the medium (Papanikolau et al., 2009). Biomass concentrations were measured as 7.02±0.20 and 7.49±0.45 g/L for mediums contain glycerol and potassium acetate, consequently. Results indicates slightly higher biomass concentration while potassium acetate is used. On the other hands, when this results compared with COD/TKN 75, there is approximately same biomass concentration for potassium acetate, but significantly higher concentration for glycerol.

- COD/TKN 125

64,975 mg/L COD was added to the medium using glucose, glycerol and potassium acetate to obtain COD/TKN 125. According to Figure 1c, high initial amount of glucose and glycerol inhibited the growth of microorganism and after 600 hours, there is no biomass growth in medium. When glycerol is used, the biomass growth shows significant increase and reaches to its highest concentration of 12.17±0.87 g/L, in comparison with COD/TKN of 75 and 100. It indicates that enhancing carbon source in medium has direct effect on biomass concentration. High glycerol concentration causes high biomass generation without any inhibition effect on growth.

Biomass growth in fermented food waste is used as control. COD/TKN ration keep as unchanged, and no carbon source was added. As it is illustrated in Figure 1c, biomass growth reaches to 8.31± 0.47. In medium with COD/TKN 75, the biomass growth under glycerol is significantly lower, but when glucose and potassium acetate applied the biomass increased but still less than medium with no additional carbon source. In medium with COD/TKN 100, the biomass concentration was higher than ratio 75 but again less than medium with no additional carbon source. However, when COD/TKN increased to 125, serious biomass increase was observed and reached to around one and half times greater than the one with unchanged ratio.

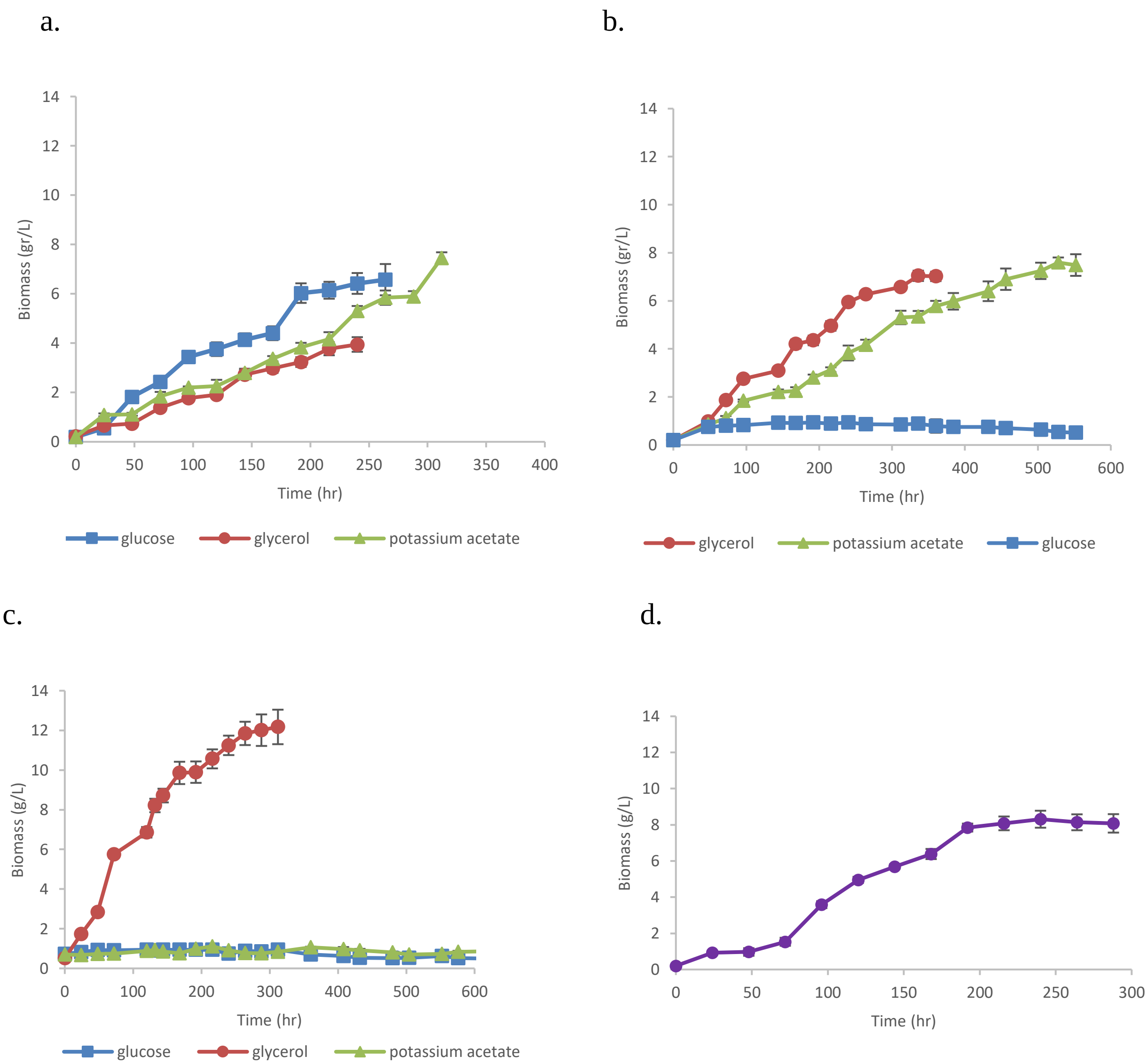


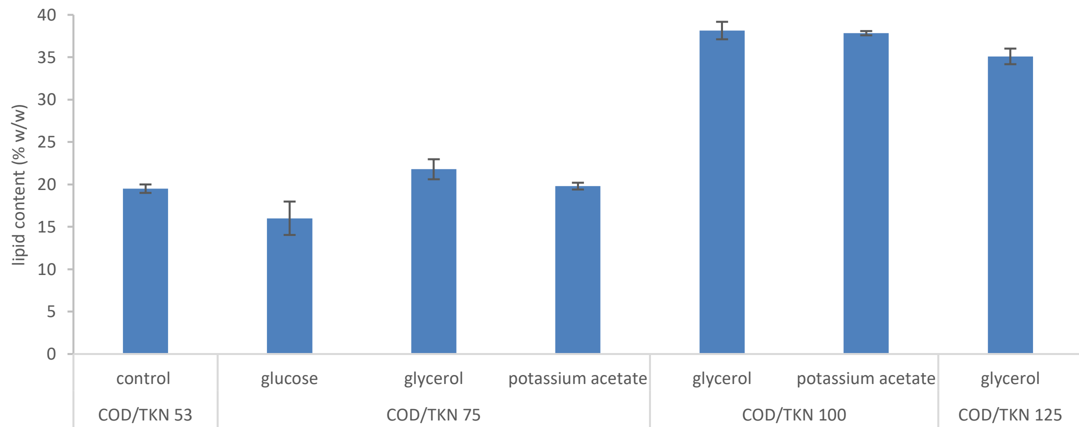
Fig. 1 Biomass concentration in (**a)** Different carbon sources at COD/TKN 75. **(b)** Different carbon sources at COD/TKN 100. (**c)** Different carbon sources at COD/TKN 125. (**d)** Control **(**Fermented food waste with unchanged COD/TKN).

3.2 Lipid accumulation

Figure 2b compares the differences in lipid accumulation in three experimental conditions. Lipid accumulation by *Y.lipolytica* growing on Fermented FW was considered as control whereas the natural COD/TKN ratio remains unchanged as 53. Figure 2a represents the lipid accumulated on each condition. In control, lipid accumulation was 19.5±0.5% of dry mass weight. By increasing the COD/TKN ratio to 75, stored lipid in glucose contain medium was a bit lower than control that can be attributed to shifting the metabolic pathway to generation of organic acids instead of lipid storage (Papanikolau et al., 2009). Applying potassium acetate doesn't alter the lipid content and shows the value of 19.8±0.4%. There was small increase when glycerol applied, and the lipid content enhanced to 21.79±1.18%. Conversely, COD/TKN 100 and 125 have notable effect on lipid generation. At COD/TKN 100 by using glycerol, lipid reached to 38.15±1.03% that is the highest value among conducted conditions. Potassium acetate shows approximately the same value as well, with lipid storage of 37.84±0.25%. In the case of COD/TKN 125, lipid content was a little lower that ratio 100 and was equal to 35.1±0.92%. The results confirm that increasing COD/TKN ratio enhance the lipid generation by changing the metabolic pathway toward lipid synthesis and storage.

Figure 2b shows the lipid concentration assessed in different COD/TKN condition and control as well. It is obvious that by increasing the organic carbon concentration, the lipid concentration increases also. Lipid concentration of control was measured as 1.62±0.04 g/L. In COD/TKN 75, medium with potassium acetate generate higher lipid concentration of 1.48±0.03 g/L than the other carbon sources. When COD/TKN ratio increased to 100, nearly the same concentration was achieved as 2.67±0.07 and 2.83±0.01 g/L for glycerol and potassium acetate, consequently. Additionally, the results indicate that in highest concentration of organic carbon, lipid concentration is in its maximum amount. In COD/TKN 125, concentration of lipid was 4.27±0.11g/L. It is clear that by decreasing biomass concentration lipid concentration was decreased also, although the lipid content of the cell was higher.

a.



b.

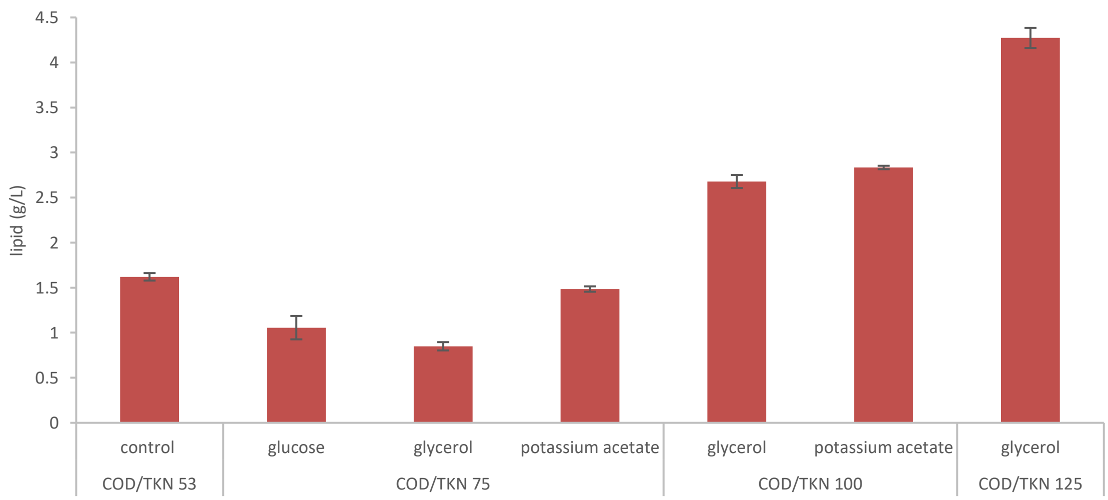


Figure 2: (a) Lipid amount (%), (**b)** Lipid concentration obtained in different initial COD/TKN ratios.

Conclusions

Yarrowia lipolytica is an oleaginous yeast with the ability of accumulate high amount of lipid inside the cell. Food waste as a cheap organic substrate, contains high amount of carbohydrate which can be converted to biofuel applying microorganisms. Nitrogen deficiency together with high amount of organic carbon are important parameters to enhance the amount of accumulated lipid by oleaginous microorganism. According to this study, FW can be applied as favorable carbon source to produce intracellular lipid. In this study, among applied organic carbon sources to increase biomass concentration, lipid content and lipid concentration, glycerol is selected as the most favorable one. By increasing COD/TKN over 75, lipid content of the cell enhanced directly. More investigations are required to optimize the condition of COD enhancement, such as adding carbon sources at the end of growth phase of microorganism.

References

Bligh E.G., Dyer W. J. A rapid method of total lipid extraction and purification, Biochemistry and physiology, 1959, 37

Celinska E, Grajek W. A novel multigene expression construct for modification of glycerol metabolism in Yarrowia lipolytica. Microb Cell Fact. 2013;12:102.

Dahiya, S., Kumar A.N., Sravan J.S., Chatterjee S., Sarkar O., Venkata Mohan S., Food wastebiorefinery: sustainable strategy for circular bioeconomy. Bioresour. Technol. 2018; 248, 2-12.

Dobrowolski A, Mitula P, Rymowicz W, et al. Efficient conversion of crude glycerol from various industrial wastes into single cell oil by yeast Yarrowia lipolytica. Bioresource Technol. 2016; 207:237–243.

E. Carsanba, S. Papanikolaou & H. Erten Production of oils and fats by oleaginous microorganisms with an emphasis given to the potential of the nonconventional yeast Yarrowia lipolytica, Critical Reviews in Biotechnology, 2018; 38:8, 1230-1243.

Fontanille P, Kumar V, Christophe G, et al. Bioconversion of volatile fatty acids into lipids by the oleaginous yeast Yarrowia lipolytica. Bioresource Technol. 2012;114:443–449.

Madzak C. Yarrowia lipolytica: recent achievements in heterologous protein expression and pathway engineering. Appl Microbiol Biotechnol. 2015;99:4559–4577.

Papanikolaou,S.*etal.*Biosynthesisoflipidsandorganicacidsby*Yarrowialipolytica*strainscultivatedonglucose.*Eur.J.LipidSci. Technol.* 2009; **111**, 1221–1232

Ratledge C, Wynn JP The biochemistry and molecular biology of lipid accumulation in oleaginous microorganisms. Adv Appl Microbiol, 2002; 51:1–51.

S.Y. Bagul, R.K. Bharti, D.W. Dhar, Assessing biodiesel quality parameters for wastewater grown Chlorella sp, Water Sci. Technol. 76, 2017; 719-727.

Wang J., Ledesma-Amaro R., Wei Y., Ji X. Metabolic engineering for increased lipid accumulation in *Yarrowia lipolytica* – A Review, Bioresource Technology, 2020; 313, 123707.

Y. Ma, Z. Gao, Q. Wang, Y. Liu, Biodiesels from microbial oils: opportunity and challenges, Bioresour. Technol., 2018; 263, pp. 631-641.

Yaashikaa, P.R., Senthil Kumar P., Varjani S.J., Saravanan. A. Advances in production and application of biochar from lignocellulosic feed stocks for remediation of environmental pollutants. Bioresour. Technol. 2019; 292, 122030.

Yang X, Sun W, Shen H, Zhang S, Jiao X, Zhao ZK Expression of phosphotransacetylase in: Rhodosporidium toruloides leading to im- proved cell growth and lipid production. RSC Adv. 2018; 8:24673–24678.

Zikou E, Chatzifragkou A, Koutinas AA, et al. Evaluating glucose and xylose as cosubstrates for lipid accumulation and gamma-linolenic acid biosynthesis of Thamnidium elegans. J. Appl Microbiol. 2013;114:1020–1032.