Study on the Effects of Initial pH, Temperature and Agitation Speed on Lipid Production by *Yarrowia lipolytica* and *Chlorella vulgaris* using Sago Wastewater as a Substrate

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Abstract

Oleaginous microorganisms acts as a promising source for biofuels. They can also be used for treatment of polluted water if oleaginous microorganisms are grown in wastewater. This paper focuses on harnessing oleaginous microbes namely, *Yarrowia lipolytica* (yeast strain) and *Chlorella vulgaris* (microalga) for lipid production using untreated sago wastewater as the medium. The wastewater was characterized and the growth parameters such as initial pH, temperature and agitation speed for both the organisms were evaluated. The lipid produced at optimum conditions by *C. vulgaris* is 2.5 times higher when compared to *Y. lipolytica*. This is due to the better adaptability of *C. vulgaris* to sago wastewater as a substrate and its ability to utilize the medium optimally. This research has also evaluated the treatment efficiencies of both organisms after growth and harvest.
Key words: *Oleaginous microbes, Sago wastewater, pH, Temperature, Agitation speed, Treatment*

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1. Introduction

Over the past two decades, the use of biodiesel has increased due to the awareness and concern about the environmental pollution caused by the combustion of fossil fuels. The term biofuel refers to fuels generated from biological sources. Biodiesel, one of the biofuels, is generally referred to as a mixture of fatty acid methyl esters derived from vegetable oils and animal fats that are used as a substitute for petroleum diesel [24]. Based on the feedstock, biodiesel production is classified into three generations *viz.*, first-generation derived from food crops, second-generation derived from non-food crops and third-generation from an oleaginous microorganism [18], [14]. Oleaginous microorganisms are a renewable and sustainable means to produce lipids that are reliable feedstock for biodiesel production. The accumulated lipids in the form of triglycerides are a prominent biodiesel feedstock [25], [31], [30]. Oleaginous microorganisms produce intra and extracellular lipids in their cells that can be economically extracted from their biomass. Microbial oils are produced by microorganisms such as yeast, fungi, mold, bacteria, microalgae, etc. [22].

Abdel-Raouf et al. (2012) reported that many species of microalgae can grow profusely in different wastewaters through their ability to utilize the abundant organic carbon, inorganic nitrogen and phosphorus present in the wastewater. Chinnasamy et al. (2010) has conducted a similar study on carpet industry effluents along with municipal sewage to evaluate the feasibility of algal biomass and biodiesel production and has reported a significant result [6].

In this context, microalgae have shown great promises and intensive research in treating wastewater by using high-rate algal treatment by growing and consequent harvesting of the biomasses as a biodiesel feedstock has gained more importance. Further, this research is extended by identifying the microalgae strains that are capable of synthesising significant quantities of biomass and lipids and the cultivation conditions that will increase lipid productivity [16], [19]. This is due to the use of microalgae for biodiesel production, which has garnered a significant amount of interest.

Similarly, another potent oleaginous microorganism is yeast which has the capacity to accumulate intracellular lipids greater than 20% of cell dry weight [27]. Some of the yeast species have reported to accumulate lipid up to 80% of their dry cell weight [3]. A study carried out by Thirulogachandar et al. (2015) has found that *Y. lipolytica* has the ability grow in slaughter house wastewater and accumulate lipids. These studies have demonstrated that both, microalgae and yeasts, are very effective in terms of lipid accumulation and are inherently capable of growing in different wastewaters [30].

Sago is a processed edible starch, which is produced from tapioca roots. Sago industries are agro-based that produce 40000 L of wastewater for the production of one ton of sago. Conventional treatment technologies like activated sludge processes and anaerobic wastewater treatment processes are employed for treating this wastewater. These processes generate sludge and debris which again raise the question about its disposal. Hence, there is a need for a new treatment process or an integrated treatment process that would bring out a value-added product instead of sludge and debris.

Assessing the magnitude of the pollution caused by the sago effluent discharge into the environment, the present work studies the utilization of these organic carbon and nutrients present in the wastewater for growing oleaginous microorganisms (yeast and algae). Their lipid production capacities are assessed under varying process parameters after which the intracellular lipids from the organisms are extracted and characterised. The reduction in the organic pollutant in the wastewater after the cultivation of these organisms was also studied.

2. Materials and Methods

2.1 Sago Wastewater

Sago wastewater was collected from Raja Sago Factory located in Salem, India. The cumulative wastewater sampling were made between June and December 2018. The samples were collected before it enters to the primary settling tank in an airtight container, sealed for transportation and preserved at a temperature below -4 °C to prevent degradation due to microbial action. The sago wastewater was characterized to determine its composition and it was performed as per the standard methods for the analysis of water and wastewater

examination [2]. All the chemicals used in this experiment were purchased from Sigma Aldrich, Mumbai, India.

2.2 Selection and Culturing of Microbes in Wastewater

The yeast and algae strain, namely *Y. lipolytica* and *C. vulgaris* were selected for the studies as they have the ability to grow in different medium compositions [5], [15]. Both the yeast and algal species were subjected to studies under different growth conditions. The cultures were purchased from MTCC, Chandigarh, India.

2.3 Inoculum Preparation

YPD Medium and Bold's Basal Medium were used as a pre-culture medium to cultivate *Y. lipolytica* and *C. vulgaris* respectively [9]. The broth mediums were sterilized before culturing. 2% inoculum concentration was used for inoculation and the mediums were incubated at 25 °C. The growth of the organisms can be observed by the appearance of a turbid nature in the medium. The pre-culture of *C. vulgaris* was grown under aeration with 0.03% CO_2, and a 16 h light and 8 h dark cycle [4], [7] whereas *Y. lipolytica* was grown in the dark [30].

2.4 Growth Studies

The feasibility of the *Y. lipolytica* and *C. vulgaris* strain to grow under the native characteristics of sago wastewater as a substrate was carried out in an Erlenmeyer flask. 2% inoculum was used for inoculation and the flasks were incubated at an rpm of 120. Both the strains were incubated at room temperature.

2.5 Optimization of Process Parameters

The growth parameters such as pH, temperature and rpm were varied for obtaining higher biomass growth and lipid accumulation. pH, one of the most important parameters for the growth of microbes, was varied between 2.0 and 10.0 (with an increase of 2.0) with the help of 0.1 N NaOH and HCl. Five flasks were used to carry out the experiment to which 2% inoculum was added and incubated to observe the microbial growth and lipid accumulation.

The temperature varied from 20 to 40 °C with an increase of 5 °C. Five flasks were used to carry out the experiment, and to all the flasks 2% inoculum was added and incubated at 120 rpm to observe the microbial growth and lipid accumulation. The optimized pH was used in this study.

Constant agitation which is required for the uniform supply of nutrients for the growth of the culture was provided by an orbital shaker with the incubator. The stirring speeds were varied as 90, 120, 150 and 180 rpm and the parameters of pH and temperature were maintained at the optimum value and analyzed for the biomass growth and lipid accumulation.

2.7 Biomass Concentration

The samples were collected in every 6 h interval and the concentrations were measured by UV-Visible Spectrophotometer at the wavelength of 600 nm. It was then filtered to measure the concentration of biomass in terms of gram using Whattmann filter paper no.42.

2.8 Extraction of Lipid

Lipid was extracted from the dried biomass by Folch's method [12]. The 5 mL samples collected after 6 h intervals were centrifuged using table top centrifuge at an rpm of 5000 for 10 min. The homogenization of biomass was performed in 4 mL of the solvent mixture, 2:1 chloroform-methanol (v/v) following a rinse with the same amount. Post-treatment extracts were washed with 2 mL of 0.73% NaCl water solution. The bilayer separation was observed in which the organic layer was subjected to further qualitative and quantitative analysis.

2.9 Lipid Confirmation Test

The confirmation of lipid was performed by an ethanol Emulsion Test by the following procedure: the obtained lipid was separated in a test tube. 2 mL of ethanol was added to the test tube containing lipid with distilled water in it, after which the sample was allowed to centrifuge. The appearance of a milky white colour confirms the presence of lipid.

2.10 Determination of Fatty Acid Profile

The fatty acid profile of the extracted bio oil was analyzed using a gas chromatography coupled with mass spectrometry (Shimadzu Model GCMS-QP2010 Japan). This system has a capillary column coated with dimethyl-polysiloxane (SH-RxiTM-1MS). The column temperature was maintained at 90 °C for 5 min and later increased at the rate of 20 °C min^{-1} till it gradually reached 260 °C which was then kept constantly for 10 min. Helium was used as a carrier gas with a flow rate of 1 mL min^{-1} and the sample injection volume was 1 µL. Qualitative and quantitative analysis of fatty acid was carried out based on the calibrated data from methyl ester standards.

3. Results and Discussion

3.1 Characterization of Wastewater

Characteristics of the wastewater are tabulated in Table 1. The effluent comprises soluble starch, cellulose and gritty matter giving a cloudy white colour to the effluent. Even though these effluents do not comprise any toxic material, they need to be treated before being discharged into water bodies because of their high total organic carbon content that increases COD and BOD and gives out an unpleasent odour due to putrefaction. If disposed on land, the soluble solids present in the effluent can clog soil pores and have detrimental effects on soil fertility. The 5.5 pH falls within the acidic range which is similar to the findings of Nandy et al. [23]. The BOD:COD ratio was 0.4501 which is within the range of 0.45-0.48 as observed by Doraisamy et al., who analyzed the seasonal variation in wastewater from sago industries [11]. The characterization of wastewater indicates its biodegradability and is suggestive of the raw wastewater to be used directly for the growth of oleaginous microorganisms.

Table 1. Characteristics of sago wastewater

Parameters	Values
pH	5.5
Colour	Milky white
BOD (mg L-1)	2350
COD (mg L-1)	5220
BOD/COD	0.4501
Total Solids (mg L-1)	3060
Total Suspended Solids (mg L-1)	120
TDS (mg L-1)	2880
Chlorides (mg L-1)	460
Total Organic Carbon (mg L-1)	7130
Total Carbon (mg L-1)	7424
Inorganic Carbon (mg L-1)	293
Total Nitrogen (mg L-1)	296.1
Phosphorus (mg L-1)	34

3.2 Growth Studies

The ability of yeast and algae to grow in sago wastewater as substrate was studied and both the species have shown significant growth. *Y. lipolytica* and *C. vulgaris* grown in raw wastewater produced 1.39 and 1.99 mg L^{-1} biomass and 0.1681 and 0.1777 mg L^{-1} lipid without

optimization at room temperature. The observed result shows that sago wastewater can be used as a suitable and sustainable growth medium for biofuel feedstock. Hence, these oleaginous microorganisms can be used for the remediation of sago wastewaters for the removal of the organic load.

Figure 1: Wastewater Medium and Growth of Microbes in Wastewater

3.3 Optimization of Process Parameters

3.3.1 Initial pH

The growth of *Y. lipolytica* and *C. vulgaris* was significantly affected by the change in pH which was evident from the biomass production and lipid accumulation as shown in Table 2. The growth of *Y. lipolytica* was higher at pH 6 with higher lipid accumulation (ie. 1.42 and 0.1980 g L^{-1} respectively). A similar result was obtained in the study carried out by Gropoșila-Constantinescu et al. [17]. But for *C. vulgaris*, maximum biomass growth and lipid accumulation were achieved at pH 8. This may be because of the reduction in cell release from auto spore, which was evident in previous studies in literatures [32]. The growth of *C. vulgaris* and the lipid accumulation were higher at pH 8.0 (i.e. 2.38 and 0.4605 g L^{-1} respectively). This is due to the control in cell proliferation which increases lipid accumulation in biomass. This observation was further supported by the studies carried out by Shah et al. (2014) and Rai et al. (2015)[26], [28].

Table 2. Effect of pH (30 °C and 120 rpm)

pH	*Y. lipolytica*			*C. vulgaris*		
	Biomass (g L^{-1})	Lipid (g L^{-1})	Lipid (wt%)	Biomass (g L^{-1})	Lipid (g L^{-1})	Lipid (wt%)
2	0.22	0.0065	2.95	0.21	0.0065	3.1
4	0.63	0.0560	8.88	1.35	0.1072	7.9
6	1.42	0.1980	13.94	2.03	0.2103	10.3
8	1.35	0.1404	13.50	2.38	0.4605	19.3
10	0.19	0.0039	9.90	1.85	0.2220	12.0

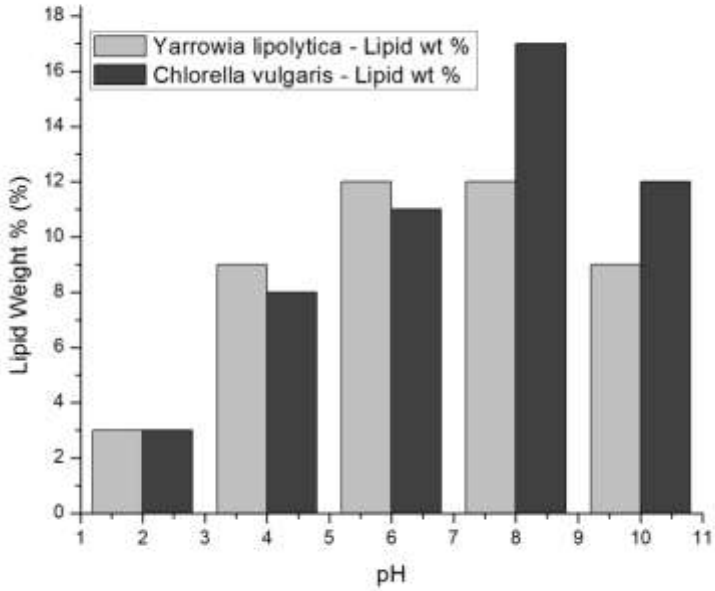

Figure 2: Effect of pH on Lipid Weight

3.3.2 Temperature

From the observations as shown in Table 3, it was noted that the change in temperature resulted in only a slight difference in the growth of *Y. lipolytica*. But lipid accumulation has a significant change with respect to temperature. For *C. vulgaris*, lipid accumulation was found to be higher at 35 °C which implies that an increase in temperature enhances the cell proliferation by utilizing the accumulated lipids. A similar study conducted by Crolla (2004) showed that the biomass growth of *C. vulgaris* was higher in temperatures between 25 and 30 °C with a maximum growth of 1.76 g L^{-1} at 30 °C [8]. It is because of the difference in the characteristics of wastewater. In the present work it was observed that *Y. lipolytica* accumulated maximum lipid at 30 °C. In a similar study conducted by Groposila (2015) the lipid accumulation of *Y. lipolytica* was higher at 28 °C [17]. Lower (20 °C) and higher temperatures (40 °C) were not favourable for the growth of both the microbes.

Table 3. Effect of temperature

Temp (°C)	*Y. lipolytica* (6 pH and 120 rpm)			*C. vulgaris* (8 pH and 120 rpm)		
	Biomass (g L^{-1})	Lipid (g L^{-1})	Lipid (wt%)	Biomass (g L^{-1})	Lipid (g L^{-1})	Lipid (wt%)
20	1.15	0.118	10.26	1.82	0.218	12.00
25	1.31	0.148	11.29	2.05	0.336	16.39
30	1.42	0.198	13.94	2.38	0.461	19.34
35	1.20	0.162	13.50	2.42	0.499	20.62
40	1.08	0.107	9.90	1.94	0.285	14.70

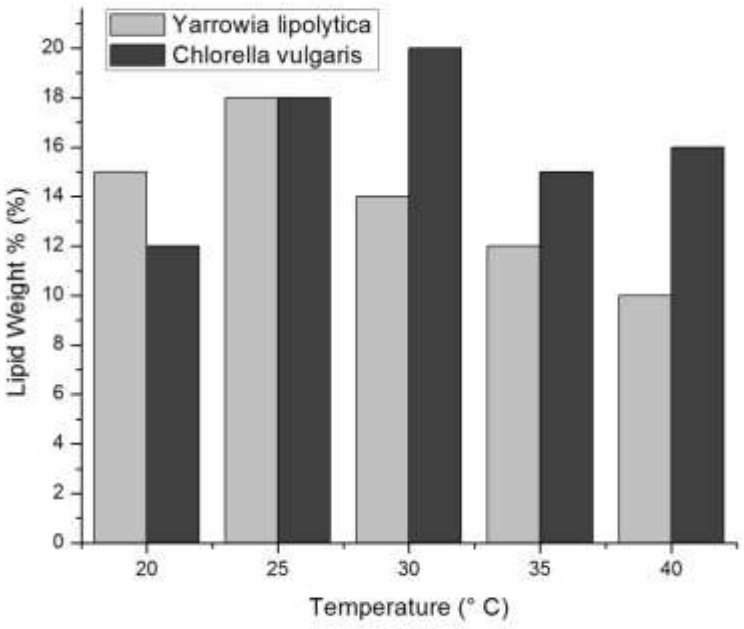

Figure 3: Effect of Temperature on Lipid Weight

3.3.3 Agitation or Stirring speed

The stirring speed of the media influenced the biomass growth of both the microbes. Stirring increases the contact of cells with the substrate though higher speed causes the formation of vortex resulting in the disturbance of the contact between cells and substrates. This causes a decrease in biomass and lipid production at a higher rpm of 180. Yeasts required comparatively a lower stirring rate (120 rpm) when compared to algae (150 rpm) because it was propagated through budding. The budding yeast cells can be damaged by mechanical stress caused by agitation of the culture [31]. **A**gitation facilitates transport efficiency by preventing sedimentation and adhesion on to the flask wall in *C. vulgaris*. It also facilitates proper distribution of light. Therefore, an optimal agitation of 150 rpm was required for maximum growth as observed from Table 4.

Table 4. Effect of stirring speed

Stirring speed (rpm)	*Y. lipolytica*			*C. vulgaris*		
	Biomass ($g\ L^{-1}$)	Lipid ($g\ L^{-1}$)	Lipid (wt%)	Biomass ($g\ L^{-1}$)	Lipid ($g\ L^{-1}$)	Lipid (wt%)
0	0	0	0	0	0	0
90	1.29	0.115	8.9	2.33	0.283	12.14
120	1.42	0.198	14.1	2.42	0.467	19.30
150	1.38	0.126	9.1	2.51	0.499	19.88
180	1.37	0.111	8.1	2.49	0.398	15.98

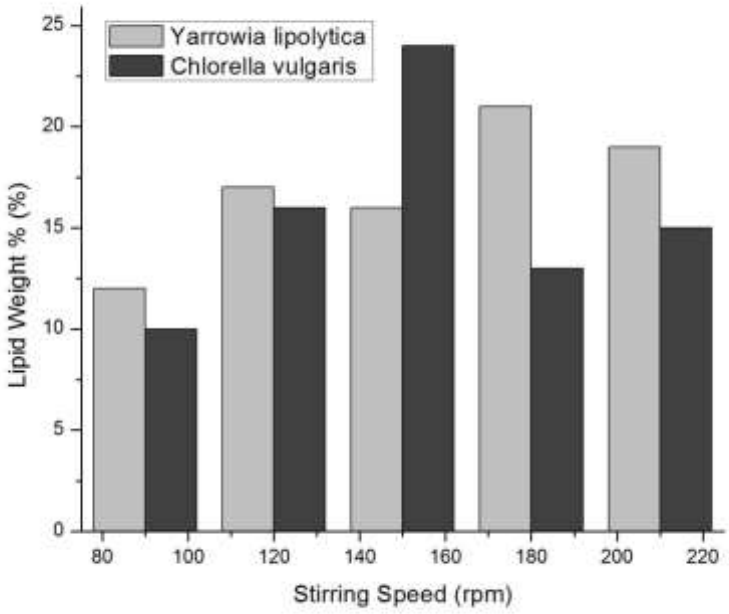

Figure 4: Effect of Stirring Speed on Lipid Weight

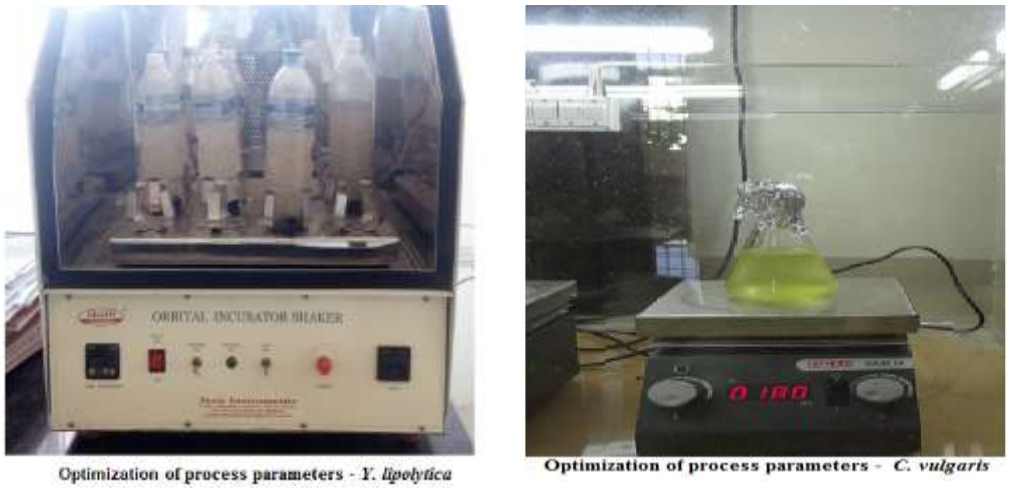

Figure 5: Optimization of Process Parameters

3.4 Lipid Identification Test

The lipid content was higher in *C. vulgaris* when compared with *Y. lipolytica* in all cases. This may be due to the presence of additional nutrients such as phosphates and chemicals that may suppress the growth and activity of yeast when compared to algae. The obtained lipid was confirmed by an ethanol emulsion test.

Yeast - Wet Biomass Algae – Wet Biomass Lipid Confirmation – Ethanol EmulsionTest

Figure 6: Biomass and Lipid

3.5 Lipid Profile Analysis

Even though the substrate is sago wastewater, the lipid profile is found to be similar to other sources reported in literature [4], [10], [13]. The lipid produced by *Y. lipolytica* and *C. vulgaris* in the control study (without sago wastewater) was 4.42 and 8.97 wt% respectively and in sago wastewater as substrate was 14.14 and 19.88 wt% respectively. It was observed that total lipid accumulated and biomasses produced by both of these organisms were relatively higher when compared to controls.

Table 5. Fatty acid profile

Fatty acid	Carbon number	*Y. lipolytica*	*C. vulgaris*
Myristic acid	C14:0	0.18	1.15
Myristoleic acid	C14:1	0.05	*ND**
Pentadecenoic acid	C 15:1	0.02	*ND**
Palmitic acid	C16:0	8.07	47.21
Palmitoleic acid	C16:1	10.22	*ND**
Steric acid	C18:0	1.23	5.22
Oleic acid	C 18:1	72.76	0.91
Linoleic acid	C18:2	6.30	7.96
Linolenic acid	C 18:3	1.07	35.31
Arachidic acid	C20:0	0.10	6.10
Paullinic acid	C 20:1	0.73	*ND**

* ND - Not Detectable

Biodiesel quality and its performance during combustion depend only on fatty acid composition. The fatty acid profile of the extracted bio oil of *Y. lipolytica* and *C. vulgaris* is presented in Table 5. Oleic and palmitoleic are major fatty acids present in *Y. lipolytica* bio oil. Similarly, palmitic and linolenic are the major fatty acids in *C. vulgaris*.

Cetane number is the ability of the fuel to ignite quickly after injection; the higher the value the better emission of fuel. This is an important parameter considered for the selection of feedstock to produce biodiesel [20]. The cetane number of biodiesel produced from *C. vulgaris* bio oil will be higher than that of *Y. lipolytica* bio oil, which is because of its rich saturated fatty acids (55.22%). But the same composition will affect the cold flow property and increase the viscosity of biodiesel due to the presence of the high melting saturated fatty acids [21]. The higher amount of unsaturated fatty acid present in bio oil influences the oxidation stability and the formation of deposits in diesel engine's injectors. Therefore, fatty acid profile suggests that biodiesel produced from *Y. lipolytica* bio oil requires oxidation inhibitors to increase the resistance against auto-oxidation. In general, the fatty acid profiles of bio oil extracted from both *Y. lipolytica* and *C. vulgaris* biomass suggest that it can be used as a biodiesel feedstock.

3.6 Effluent Studies

From the comparison Table. 6, it is clear that all the nutrients were utilized by the organisms for their growth and lipid accumulation. For biomass growth and lipid accumulation around 32.56% of COD was utilized by the yeast strain, whereas, algae consumed around 43.48% of initial COD as substrate. The study conducted by Chu et al. [7], shows that phosphorus under nitrogen limited condition will be assimilated by the algae at a higher rate. In this study, around 3.8 times increase in assimilation efficiency was observed with 80% removal of phosphates.

In the present study around 70% of phosphate was removed. It was observed that when raw wastewater without any pre-treatment was utilized, around 30 to 50% of nitrogen and phosphorus was utilized by the organisms. This may be because of the elemental form in which they exist in wastewater.

Table 6. Wastewater characteristics before and after biomass culturing

Parameters	Raw wastewater	*After biomass separation*	
		Y. lipolytica	***C. vulgaris***
pH	5.5	5.8	7.3
Colour	Milky white	Pale yellow	Pale green
COD (mg L^{-1})	5220	3520	2950
TDS (mg L^{-1})	2880	2205	1901
Total Nitrogen (mg L^{-1})	296.1	180	127
Phosphorus (mg L^{-1})	30	12	10

The study concludes that wastewater can be used as a source for yeast cultivation and it reduces the cost of wastewater treatment. In literature, wastewater with added nutrients resulted in a promising yield of biomass and lipid. However, in this present work raw wastewater without any supplement was used as a substrate and a significant result was obtained. It shows sago wastewater can be used as a substrate for lipid production. However, both the organisms cultivated on sago wastewater showed robust growth under natural condition. This leads us to the conclusion that without adding any nutrients or using any controlled growth environment, the microorganism has the ability to grow and sustain in the wastewater leading to significant biomass production.

4. Conclusion

Carbon, nitrogen and other nutrients present in sago wastewater were successfully used as a low-cost nutrient for lipid production from yeast and microalgae. Lipid production by the growth of *Y. lipolytica* and *C. vulgaris* using wastewater was proven to be largely dependent on environmental conditions such as pH, temperature and stirring speed. A maximum lipid production of 0.198 g L^{-1} for *Y. lipolytica* and 0.499 g L^{-1} for *C. vulgaris* were obtained in this study. During this observation, a maximum lipid yield of 21% from *Chlorella vulgaris* was noticed. From the comparison, *C. vulgaris* was found to be 40 % more efficient than *Y. lipolytica* in production of lipid using wastewater as the source for nutrients. The study concludes that sago wastewater can be used as a substrate for culturing *Y. lipolytica* and *C. vulgaris* and it also reduces the cost of treatment of wastewater and for producing biodiesel feedstock.

Declaration

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