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OPTIMIZATION OF METSCHNIKOWIA PULCHERRIMA GROWTH IN DISTILLERY SPENT WASH FOR BIODIESEL PRODUCTION

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ABSTRACT

Biodiesel is a renewable fuel which synthesis from wastewater is a promising alternative to fossil fuels that are limited in availability. Organic molecules of wastewater contain a lot of chemical energy. Waste to energy is the most preferred technique in which wastewater used as a substrate for some microorganisms that can store lipids within their cells. Microbial oil is a renewable alternative to petroleum and diesel fuels as well as microorganisms utilize waste water instead of food crops or other crops for biodiesel production. Distillery spent wash, high organic load wastewater is used for growing *Metschnikowia pulcherrima* oleaginous yeast which accumulates lipids of 40% of their dry weight and the lipids converted to non-toxic and biodegradable biodiesel (Fatty Acid Alkyl Esters) by trans-esterification. It is beneficial to environment by reducing the waste and converting it into energy source. In this paper, optimization of growth condition of *M. pulcherrima* was analyzed for static and shaking condition, different pH values and incubation periods. 0.466 g/L lipid yield obtained from distillery spent wash in the optimum condition of pH-6.0, 25^{O}C temperature and shaking at 160rpm.

KEYWORDS: biodiesel, distillery spent wash, anaerobically, trans-esterification, oleaginous yeast, biodegradable, Fatty Acid Alkyl Esters.

INTRODUCTION

Biodiesel (Fatty Acid Alkyl Esters), an alternative diesel fuel which is made from renewable biological sources such as vegetable oils, animal fats and other industrial wastes. It is biodegradable and nontoxic and it has low emission profiles. Therefore it is environmentally beneficial.

70% of the total global energy requirements are full filled by fossil fuels which used for transportation, manufacturing and domestic heating. The drastic increase in the demand of transportation fuel has led to the search of an alternative source for fossil fuels since they are getting exhausted. Biodiesel serves as a promising alternate for this and also had an added advantage such as its production is economic, eco-friendly and has many resources. Biodiesel fuel has the potential to reduce the level of pollutants and also the probable carcinogens.

Now-a-days, waste to energy conversion is the novel technique because of waste minimization and revenue generation through byproduct recovery. Production of biodiesel from wastes is a suitable technique since it results in generation of fuel on par with waste stabilization. Wastes can be used as a feedstock for specified microorganisms which can store intracellular lipids called oleaginous microorganism. These lipids are converted to biodiesel. Therefore pollution is avoided by waste reduction and converting it into biodiesel which is a potential alternative to fossil fuels like petroleum based fuels and plant or animal fat derived fuels. However, the cost of biodiesel is the main hurdle to commercialization of the product. The cost of biodiesel lowered by using waste materials as a raw material and recovery of high quality glycerol as a byproduct are considered as the primary option for biodiesel production.

MATERIALS

Distillery spent wash

In a developing country like India, distillery industry is one of the most polluting industries which generates large volumes of high strength wastewater. Molasses is most common raw material used in distilleries for bioethanol production. After alcohol distillation, large volumes of highly colored spent wash is generated which has high organic load and dark brown in color and it has low pH and high temperature. It is highly putrifiable because of high concentration of organic solids (pH: 4.3-4.5; COD: 100-130 g/l; BOD: 60-75 g/l).

Distilleries produce 88% of the total raw materials as a waste which has high organic load. The treatment technique should be employed not only reduce the pollution load but also generate some useful by-products and energy. Biological treatments are mostly preferred because of the production of useful byproducts which may be in large demand. According to the Central Pollution Control Board (CPCB) of India, the Minimal National Standards (MINAS) for discharge of industrial effluents into water bodies are, BOD less than 30 mg/l, COD less than 250 mg/l and pH in the range of 6.0-8.0, Total Suspended Solids less than 100 mg/l. This demands very efficient DSW treatment method, so as to meet these MINAS values. Suitability of distillery spent wash for biodiesel production are following,

- Presence of large amount of effluent.
- Distillery spent wash contains more carbohydrate content.
- Easily biodegradable
- Do not contain toxic elements

Metschnikowia pulcherrima

Metschnikowia pulcherrima is oleaginous yeast which can accumulate 40% of their dry weight as lipids. Oleaginous micro-organisms have the capability to synthesize and accumulate high amounts of Triacylglyceride (TAG) within their cells, up to 70% of their biomass weight such as yeasts and filamentous fungi. These lipids have similar composition and energy value to plant and animal oils, but their production do not affect the food resources. The organisms are based on inexpensive carbon sources, such as raw materials, by-products, and surplus. Furthermore, fungal Single cell oils have short life cycles, display rapid growth rates, unaffected by space, light or climatic variations, easier to scale up and have the ability to utilize a wide range of inexpensive renewable carbon sources such as lingo-cellulosic biomass and agro-industrial residues and wastewater.

M. pulcherrima is non-*Saccharomyces* yeast that may be involved in the vinification of wine. It is highly effective biocontrol yeast due to its pigment pulcherrimin that accumulates in the cells and in the growth medium. The yeast can tolerate low pH values, but pH over 7 is inhibitory for the growth. Maximum temperature for growth is around 39^{o}C. It grows on high glucose concentrations. The growth substrate is mainly glucose and other sugars. Sulphur dioxide Inhibits growth of yeast.

METHODS

Sample collection and Characterization

The sample of distillery spent wash was collected from Bannari Amman Sugars Limited, Coimbatore. The distillery spent wash were preserved at temperature lower than 4^{o}C but above freezing to prevent it from undergoing biodegradation due to microbial action and stored in a deep freezer.

The characteristics of distillery spent wash including pH, BOD, COD, Total suspended solids (TSS), Total dissolved solids (TDS), Alkalinity, Sulphates (SO_4^{2-}) and Chlorides (Cl^-) were analysed according to the procedure outlined in standard methods (APHA, 1990). All experiments carried out in ambient temperature.

Fig. 1 Sample collection area and Distillery spent wash sample.

Culturing of microorganism

Metschnikowia pulcherrima MTCC 632 was obtained from Microbial type culture collection and Gene bank (MTCC), Chandigarh in the form of freeze dried culture.

Culturing of *M. pulcherrima* in control medium (Malt yeast liquid medium) was done in the nitrogen limiting medium. Before inoculation, the pH of the medium adjusted to 6 by adding NaOH or HCl. The microorganism was grown under aerobic conditions at 25°C in a rotary shaker at 150 rpm.

Distillery spent wash (DSW) was pretreated by Sodium Hydroxide (NaOH) for adjusting the pH to 6.0. Before inoculation, sterilization of wastewater was done. The yeast strain (*M. pulcherrima*) inoculated in the laminar air flow chamber to prevent the entry of other microorganisms and it was grown under aerobic condition at 25°C in a rotary shaker at 150 rpm.

Growth curve analysis

Growth of microorganism was observed with the dry biomass weight basis. Growth curve analyzed for both control medium (Malt yeast liquid medium) and distillery spent wash. Every 24 hours, the biomass dry weight was taken upto 6 days (144 hours) for growth curve analysis. Both control medium and distillery spent wash, growth of microorganism increases upto 4th day (96 hours) and it reaches the stationary phase after that the microorganism reaches death phase. High growth of microorganism obtained in the 4th day.

Fig 2. *M. pulcherrima* growth

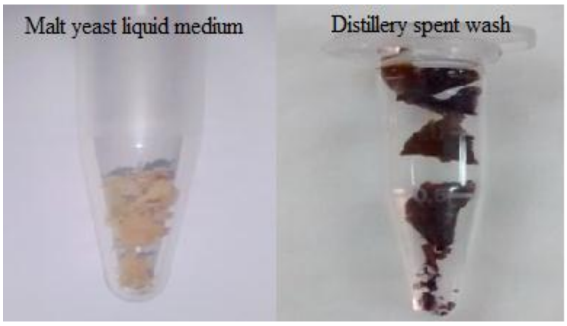

Fig 3. Dry biomass

M. pulcherrima grows like a cluster form and it is settled at the bottom which can be easily obtained by filtering the medium or wastewater. In the 4th day analysis, 33.69 g/L and 1.33 g/L of high biomass dry weight obtained in the control medium (Malt yeast liquid medium) and distillery spent wash respectively.

Lipid extraction from biomass

Dry biomass was homogenized in a pestle and mortar using acid hydrolyzed with 0.25 molL^{-1} HCl. Cell disruption was achieved by adding hexane:isopropanol (3:2) to the homogenized biomass in a bead mill. Bead mill is a metal or glass vessel contains glass beads which rotate at 200 rpm for 3 hours and the movement of glass beads at high speed makes cell wall disruption. Therefore, inter cellular things are come out by the cell disruption. After that centrifugation process was done at 5000 rpm for 10 minutes, layer separation was obtained. Top layer is the lipid layer, middle layer is hexane:isopropanol and the lipid extracted biomass was settled at the bottom. Ethanol emulsion test was done, appearance of cloudy nature confirms the presence of lipid.

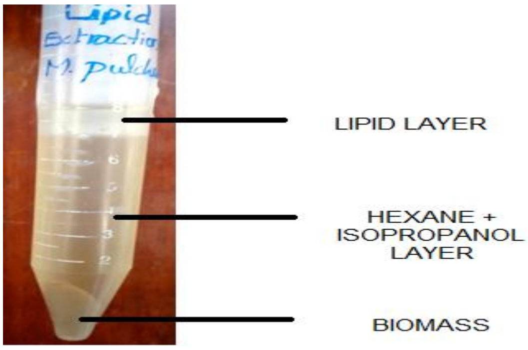

Fig 4. Layer separation after centrifugation

Fig 5. Extracted biodiesel

Transesterification

0.2 gram of NaOH catalyst was dissolved in the 11 ml of ethanol which is prepared first for transesterification of lipids. 5 times of lipids and one times of ethanol+NaOH were mixed thoroughly and it was heated for 10 minutes at 55°C. FTIR is a qualitative analysis which is used to find the types of fatty acids esters present in the sample obtained from the transesterification of lipids.

RESULTS AND DISCUSSION

Characteristics of distillery spent wash

The distillery spent wash was analyzed for its characteristics as per standard methods of analysis and the results are tabulated.

Table 1: Characteristics of distillery spent wash.

S. No.	PARAMETERS	VALUES*
1	Color	Dark brown
2	Odour	Sugar smell
3	pH	4.2
4	Conductivity	26 - 28
5	DO	Nil
6	BOD	35000 – 40000
7	COD	86000 – 110000
8	Total solids	68260
9	Total suspended solids	21360
10	Total dissolved solids	46900
11	Alkalinity	13600
12	Sulphates	5600
13	Chlorides	6000
14	Total carbon	40575
15	Total organic carbon	39868
16	Inorganic carbon	706
17	Total Nitrogen	3552.8
18	C/N ratio	11.42

*all the values are in mg/l or ppm except color, odour, pH and conductivity is in mS/cm.

Growth curve analysis

Growth curve of *M. pulcherrima* in control medium (Malt yeast liquid medium) was observed for 6 days. High growth of microorganism was observed at 4th day and 33.69 g/L dry biomass was obtained. Table shows the biomass dry weight obtained for every 24 hours upto 6 days.

Table 2: Biomass dry weights obtained from malt yeast liquid medium and distillery spent wash.

S. No.	Time (days)	Biomass dry weight (g/L)	
		Malt yeast liquid medium	Distillery Spent Wash
1	1	1.553	0.312
2	2	5.642	0.46
3	3	10.9575	0.752
4	4	33.69	1.3325
5	5	3.759	1.151
6	6	1.2765	0.321

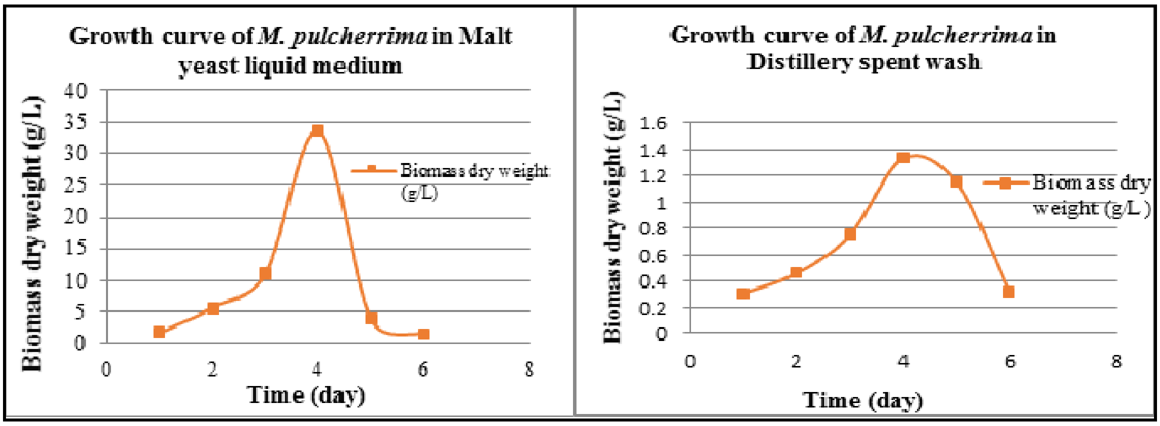

Fig 6: Growth curve of *M. pulcherrima* in malt yeast liquid medium and distillery spent wash

Lipid yield from biomass

Fourier transform infrared spectroscopy (FTIR) was used to find the presence of lipid in the sample. Highest lipid yield of 11.8 g/L from malt yeast liquid medium and 0.466 g/L from distillery spent wash obtained at 4th day growth of *M. pulcherrima*.

Table 3: Lipid yield from malt yeast liquid medium and distillery spent wash.

S. No.	Time (days)	Lipid yield (g/L)	
		Malt yeast liquid medium	Distillery spent wash
1	1	0.54	0.1092
2	2	1.97	0.161
3	3	3.84	0.2632
4	4	11.8	0.466
5	5	1.32	0.402
6	6	0.45	0.112

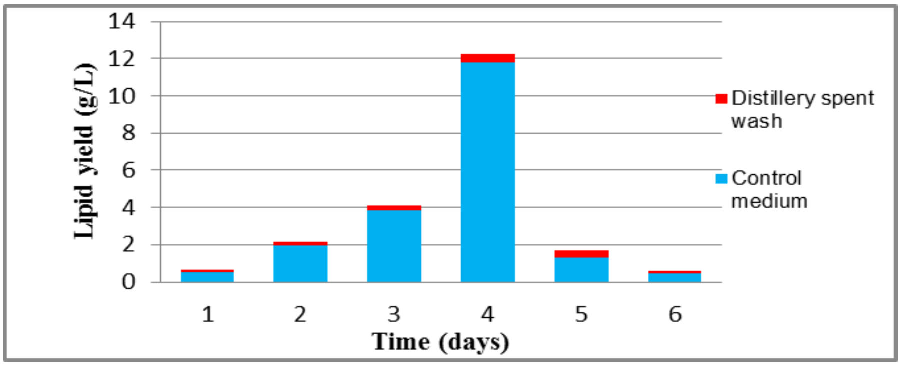

Fig 7: Lipid yields from malt yeast liquid medium and distillery spent wash.

The following FTIR reports shows the fatty acids present in a lipid sample from biomass obtained from a distillery spent wash.

Table 4: FTIR results and their fatty acid types.

S. No.	FTIR Results	Compound	Fatty Acid types
1	Cholesterol USP cryst	Lipoproteins	Triglyceride
2	Glyceroldehyde Unnatural form	Glycerol	Triglyceride
3	Methyl N- valerate	Esters of valeric acid	Glyceride volatile acid
4	Nonadecanoic Acid	Fats	Saturated fatty acid
5	Methyl methacrylate stable	Trimyristin	Triglyceride of myristic acid
6	Methyl 2 3- Dichloropropionate	Ester group	long chain caboxylic acid
7	Triethyl methane tricarboxylate	Ester group	long chain caboxylic acid

Biodiesel production

Biodiesel is produced by transesterification of lipids. Qualitative analysis of Fourier transform infrared spectroscopy was done to confirm the presence fatty acid ethyl esters groups in the obtained product. Ethanol was used for transesterification process therefore fatty acid ethyl esters are obtained. Fatty acid esters like Butyl stearate, fatty acid ethyl esters like ethyl myristate, ethyl palminate and ethyl linoleate are obtained by the FTIR analysis.

Table 5: FTIR Results and their ester groups.

S. No.	FTIR Results	Compound	Ester types
1	Butyl Stearate	Stearic acid esters of normal butyl alcohol	Fatty acid esters
2	Ethyl Myristate	Ethyl esters of myristic acid	Fatty acid ethyl esters
3	Ethyl Palmitate	Ethyl esters of palmitic acid	Fatty acid ethyl esters
4	Ethyl Linoleate	Ethyl esters of linoleic acid	Fatty acid ethyl esters

OPTIMIZATION OF GROWTH OF *Metschnikowia pulcherrima*

Effect of static and shaking condition

M. pulcherrima growth and lipid accumulation in both static and shaking condition at 160 rpm was evaluated after four days of incubation at 25ºC. High growth of M. pulcherrima occurs at shaking condition when compared to static condition. Biomass dry weight of 33.7 g/L and 1.3 g/L for malt yeast liquid medium (control medium) and distillery wash was obtained respectively.

Table 6: Biomass dry weights obtained in a static and shaking condition

Condition	Biomass dry weight	
	Malt yeast liquid medium (g/L)	Distillery spent wash (g/L)
Static	16.74	1.031
Shaking	33.69	1.3325

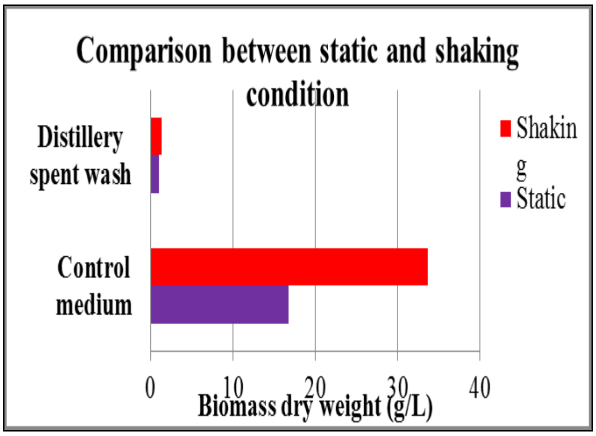

Fig 8: Comparison between static and shaking condition.

Effect of incubation period

M. pulcherrima MTCC 632 was inoculated in the malt yeast liquid medium and distillery spent wash adjusted at pH 6.0 and incubated in a static condition and shaking condition (160 rpm) at 25ºC for different periods of time i.e., 24, 48, 72, 96 and 120 hrs. During the fermentation, the conical flasks were taken at regular intervals of 24 hrs to find biomass dry weight. Both static and shaking condition, higher amount of biomass obtained at 4th day (96 hrs) for malt yeast liquid medium and distillery spent wash.

Table 7: Biomass dry weights obtained from dsw in the static and shaking condition.

Incubation period (days)	Biomass dry weight in distillery spent wash (g/L)	
	Static	Shaking
1	0.289	0.313
2	0.441	0.461
3	0.623	0.752
4	1.031	1.332
5	0.482	1.153
6	0.251	0.321

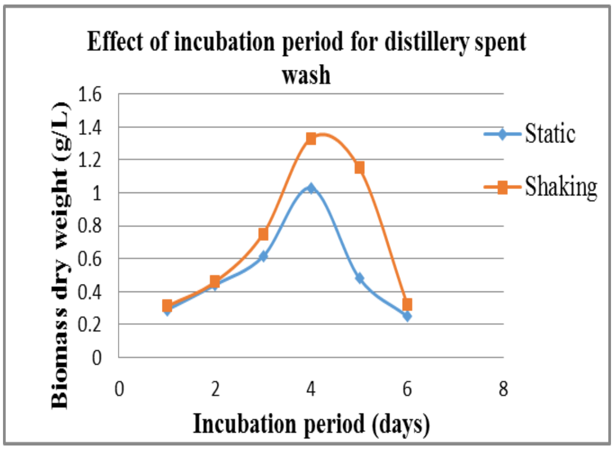

Fig 9: Effect of incubation period for distillery spent wash.

Effect of pH

This experiment was used to evaluate the effect of initial pH medium on the biomass dry weight. The pH of the samples were adjusted before autoclaving at different pH values ranging from pH 3.0 – pH 8.0 with 1 N HCl or 1 N KOH. pH- 6.0 is a optimum pH value for obtaining higher amount biomass.

Table 8: Biomass dry weight obtained for different pH values.

pH	Biomass dry weight (g/L)	
	Malt yeast liquid medium	Distillery spent wash
3	0.441	0.00435
4	0.689	0.159
5	5.676	0.495
6	16.73	1.031
7	0.682	0.276
8	0.593	0.117

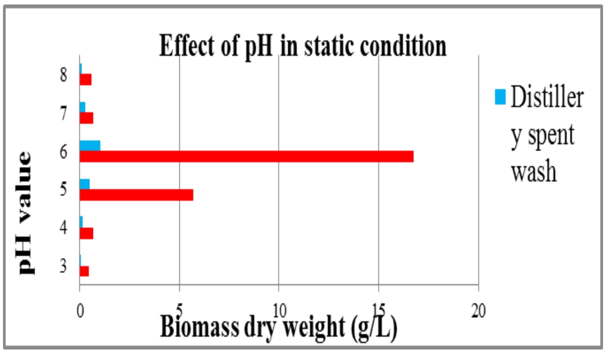

Fig 10: Effect of pH in static condition.

CONCLUSION

Biodiesel production has gained importance because of the increasing energy demand and depletion of fossil fuels. Carbohydrate rich industrial waste water can be used as starting materials for the growth of oleaginous microorganisms and for the production of microbial lipids. Due to the abundance of the industrial waste water (contains high organic load), offering a renewable feedstock for the production of biofuels.

When compared to the lipid yield from distillery spent wash, higher lipid yield achieved in a control medium (Malt Yeast liquid medium). Lipid yield in control medium is 12 g/L and distillery spent wash is 0.46g/L. The growth of *M. pulcherrima* in distillery spent wash and control medium was optimized for obtaining high lipid yield. Optimum values for high lipid yield are pH-6.0, temperature 25^{O}C and 160 rpm in a shaking condition.

It should be noted that microorganisms generate lipids for energy storage only when the ratio C/N is sufficiently high. Excess nitrogen concentrations will lead to reduced lipid yields, whereas in absence of nitrogen the biomass growth will be stopped. The lipid yield depends on C/N ratio. So the methods have to be optimized to get high C/N ratio. When the lipid yield is high, the biodiesel yield will be apparently high.

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