Production of biodiesel feedstock – microbial lipid from slaughterhouse wastewater

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Abstract: Biodiesel is an alternative diesel fuel, which can be synthesised from renewable biological sources. Lipid production using carbon source in wastewater is an emerging process as it purely depends on waste source. In the present study, the ability of *Yarrowia lipolytica* to accumulate lipids using slaughterhouse wastewater as substrate was investigated. Using raw wastewater as substrate, maximum lipid content (0.43 g/L) and biomass (1.2 g/g) were obtained. Various pre-treatment methods like acid, alkaline, heat, activated carbon and sawdust treatment were performed and two-fold increase in C/N ratio was observed in combined pre-treatment of sawdust with KOH. Using pretreated wastewater, lipid accumulation was enhanced to 32% with lipid content of 0.64 g/L. Results of this study conclude that the pre-treated slaughterhouse wastewater can be employed as a better feedstock for lipid production using *Yarrowia lipolytica*.

Keywords: oleaginous microorganisms; *Yarrowia lipolytica*; slaughterhouse wastewater; C/N ratio; pre-treatment.

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1 Introduction

Biodiesel, chemically known as fatty acid methyl ester (FAME) is an alternative energy source derived from renewable resources by transesterification of triacylglycerides, yielding fatty acid methyl or ethyl esters. It contributes to the reduction of carbon dioxide and sulphur emissions to the atmosphere and has the potential of being an environmentally benign solution for global warming, energy crisis, and depleted fossil fuel supplies. Biodiesel is compatible with current commercial diesel engines when it is added in proportionate to conventional diesel. Various sources of lipids including vegetable oils (soybean, palm and rapeseed), animal fats and waste cooking oils have been used for the production of biodiesel (Ma and Hanna, 1999). However, one of the major drawbacks is the high cost of vegetable oil contributing to about 70% of biodiesel production cost. Besides, traditional oil-rich crops are limited by land availability, influenced by the climate and are inconstant debate due to the food versus fuel issues. To overcome the limited availability of conventional resources, microbial derived feedstock can be used better alternate source for biodiesel production. The microbes can be cultured as much as need at favourable conditions and hence it is a renewable one. The conventional oils can be replaced by microbial oils obtained from safe microbes. Microbial oils or single cell oils are produced by oleaginous microorganisms such as yeast, fungi, bacteria and microalgae. Some of these microbes have the inherent ability to accumulate or store oil/lipid up to 60% of their dry weight when grown under nitrogen-limited conditions. These lipids usually consist of 80%–90% triacylglycerol with a fatty acid composition similar to vegetable oils. Microbial lipid produced by oleaginous microorganisms is reported as an ideal feedstock for biodiesel production (Thiru et al., 2011).

Oleaginous yeasts, capable of accumulating 20%–70% of their cell mass as intracellular lipids are considered an alternative strategy of producing second generation fuels including biodiesel (Ageitos et al., 2011). They are advantageous as compared to bacteria, fungi and algae due to its high growth rate and unaffected by climatic conditions. *Yarrowia lipolytica*, previously referred to as *Candida lipolytica*, is a non-conventional yeast regarded as non-pathogenic and categorised as GRAS (generally recognised as safe) by the Food and Drug Administration (FDA) and it is also considered as a good candidate for single cell oil production. The common habitats of this yeast are oil polluted environments and foods such as cheese, yogurt, kefir, shoyu, meat and poultry products. Wild-type strains accumulate up to 38% of dry weight as lipids (Papanikolaou and Aggelis, 2009).

In addition to the energy crisis, utilisation of industrial waste as substrate for biodiesel production reduces the environmental pollution. Waste management in the industries is one of the major environmental concerns since it required complex treatment methods and high cost. Wastewater characteristics and levels of pollutants vary significantly from industry to industry based on the process involved, raw material used, by-products obtained, etc. Biodiesel production using industrial wastes is a novel technique since it resulted in the fuel generation on par with waste valorisation. Few microorganisms metabolise wastes as a feedstock and store intracellular lipids which can be converted to biodiesel (Cheirsilp et al., 2012). Biodiesel cost is the main hurdle for commercialisation and it can also be minimised by utilising waste as cheap raw material, adoption of continuous transesterification process and recovery of high-quality glycerol as biodiesel by-product. According to The Ministry of Food Processing, India, as of 2010, a total of 5,616 recognised slaughterhouses slaughter over 2 million cattle and buffaloes, 50 million sheep and goats, 1.5 million pigs and 150 million poultry annually, for domestic consumption as well as for export purposes. Currently, there is no organised system for disposal of both solid and liquid waste generated in slaughterhouses in India. Largely, the solid waste is collected and dumped or disposed in the open area which is unhygienic. The liquid waste is added to the municipal sewage system, which is not desirable, as it may contain objectionable components that may affect the working of municipal sewage treatment plants. Blood is an inevitable by-product of the meat industry representing up to 4% of the live animal weight or 6% to 7% of the lean meat content of the carcass (Wismer-Pedersen, 1988). Disposal of slaughterhouse wastewater into river streams causes de-oxygenation of the river and leads to the death of all aquatic organisms present in the river; it also causes aesthetic problems because of pungent odour and appearance or when disposed of in land, it causes leaching and contaminates groundwater (Masse and Masse, 2000). Pre-treatment methods were carried out to overcome the barriers that prevent the growth of microbes in the substrate. Here, the wastewater is used as a substrate for culturing microorganisms; hence, it is necessary to treat the wastewater so that it can be effectively used as a medium for culturing microorganisms.

In the present study, the ability of *Yarrowia lipolytica* to accumulate lipid when grown in slaughterhouse wastewater was studied. Thirulogachandar et al. (2015)

produced significant quantity of lipid from slaughterhouse wastewater when used without any pretreatment. Various pre-treatment methods and their effects on enhanced lipid accumulation were reported.

2 Materials and methodology

2.1 Sample analysis

The wastewater was collected from Coimbatore Corporation Slaughterhouse, Ukkadam, Tamilnadu, India. The initial characteristics of the wastewater samples were analysed as per APHA standard methods for analysis of water and wastewater (APHA, 1992). The initial pH, chemical oxygen demand (COD), sulphates, total organic carbon (TOC) and total nitrogen (TN) of the wastewater were analysed. All the chemicals used in this study were purchased from Sigma-Aldrich.

2.2 Heat pre-treatment

Heat treatment of raw wastewater was carried out to study the effect of long chain carbohydrates hydrolysis. The major advantage of this method is zero addition of chemicals and it was performed by heating 100 mL of wastewater at 105° for 15 min (Taherzadeh and Karimi, 2008) in a fully automated autoclave purchased from Equitron. After heat treatment, the total carbon and nitrogen content were calculated using Shimadzu TOC analyser.

2.3 Acid pre-treatment method

Pre-treatment with dilute acids can result in increase of fermentable sugar concentration (Thakur et al., 2013). In this study, 3% H_2SO_4 was mixed proportionate with the wastewater (0%, 25%, 50% and 75%) for a period of 60 min under shaking. Then, the total carbon and TN values were measured.

2.4 Alkaline pre-treatment method

Alkali pretreatment was carried out with two different chemicals based on previous studies (Thakur et al., 2013). The effect of KOH and lime was studied. KOH treatment was carried out by varying the concentration from 1 g–5 g per 100 mL of slaughterhouse wastewater. Lime loading was varied from 1 g–5 g per 100 mL slaughterhouse wastewater. The samples were kept in an orbital shaker at 120 rpm for 30 min. The samples were filtered using Whatman no.1 filter paper, and corresponding C/N ratio was analysed using TOC analyser.

2.5 Sawdust pre-treatment

Sawdust acts as an additional substrate for carbon and nitrogen source (*Carbon to Nitrogen Ratios of Various Waste Materials*, 2014). Treatment was carried out by varying concentration from 1 g–5 g mixed with 100 mL of slaughterhouse wastewater. The samples were kept in an orbital shaker at 120 rpm for 60 min. Then the samples were filtered and corresponding carbon and nitrogen values were measured.

2.6 Pre-treatment with activated carbon

Activated carbon treatment was carried out by varying concentration from 0.5 g–2.0 g mixed with 100 mL of slaughterhouse wastewater. These samples were placed in shaker operated at 120 rpm for 60 min. Then the samples were filtered using filter paper and corresponding carbon and nitrogen values were measured.

2.7 Combined pre-treatment

2.7.1 Activated carbon and sawdust

This pre-treatment was carried out using 1.0 g of activated carbon and varying quantities of sawdust (1 g–5 g) and placed in an orbital shaker at 120 rpm for 60 min. The samples were then filtered using filter paper and their respective carbon and nitrogen values were measured.

2.7.2 Sawdust followed by alkaline treatment

This pre-treatment was carried out by adding 1.0 g of sawdust and 4.0 g of KOH to the wastewater of 100 mL. These samples were placed in an orbital shaker at 120 rpm for 60 min. Then the samples were filtered using filter paper, and the carbon and nitrogen values were measured.

2.8 Inoculation and yeast growth

Pre-treated wastewater was taken as a growth medium for oleaginous yeast *Yarrowia lipolytica*. Autoclaving was done before inoculation of microorganism for the purpose of preventing the growth of other microorganisms in the substrate. Sterilising the sample and growth medium will eradicate the growth of other organisms that will be harmful to the growth of *Yarrowia lipolytica*. A fresh culture of *Yarrowia lipolytica* was prepared 24 hrs prior to addition using YPG broth. 2% of inoculum was added to the flask containing raw wastewater and pre-treated growth medium. The flasks were incubated at 30° and 120 rpm in an orbital shaker for five days.

2.9 Biomass and lipid estimation

Biomass of *Yarrowia lipolytica* grown in slaughterhouse wastewater and pre-treated wastewater was obtained by centrifuging the culture suspension at 5,000 rpm for 10 min, followed by washing the pellet thrice with double distilled water. Then the washed pellet was dried in hot air oven at 80°. The biomass was then weighed gravimetrically and yield was calculated.

Folch's method was adopted to estimate the lipid content in the biomass (Folch et al., 1957). In this method, the biomass was homogenised using glass beads and extracted with chloroform/methanol mixture followed by washing and drying. The extracted lipid was quantified gravimetrically and subjected to FTIR analysis for functional group identification.

3 Result and discussions

3.1 Characteristics of slaughterhouse wastewater

Characteristics of the wastewater were evaluated by APHA (1992) standard methods for the examination of water and wastewater and the results were represented in Table 1. The wastewater exhibits higher COD range of 16,300 mg/L to 16,500 mg/L. The BOD/COD ratio of 0.55 indicates the biodegradability nature of the wastewater. The observed TSS concentration exceeds the maximum permissible limit for direct discharge into streams. Also, it contributes around 15% to 18% of total COD. The available amount of nitrogen and phosphates were good enough to meet the metabolic needs of microorganisms.

Table 1 Characteristics of slaughterhouse wastewater

Sl. no.	*Parameters*	*Values*
1	pH	7.4–7.8
2	Colour	Deep red
3	BOD	8,430–8,700
4	COD	16,330–16,500
5	Total solids	9,225–9,400
6	Total suspended solids	2,532–2,575
7	Total volatile solids	5,525–5,575
8	Alkalinity	1,652–1,720
9	Sulphates	86
10	Total organic carbon	840
11	Total carbon	1,255
12	Inorganic carbon	412
13	Total nitrogen	195
14	C/N ratio	6.41

Note: All values are in mg/L except pH and colour.

3.2 Pre-treatment

The changes in C/N ratio with respect to different pre-treatment methods were studied and tabulated in Table 2. The highest C/N ratio of each pre-treatment was shown Figure 1. Pre-treatment with dilute acid has reduced both carbon and nitrogen content which resulted in the decrease of C/N ratio. The addition of sawdust favours increase in organic carbon to a meagre amount. Saw dust mainly comprises of lignin, cellulose and hemicelluloses which on acid pre-treatment gets solubilised. It was observed that addition of sawdust resulted in 40% increase of C/N ratio and KOH resulted in 45% increase. In combined treatment method (sawdust + KOH), significant change were noted with two fold increase in C/N ratio (135%). Combined pre-treatment (Sawdust + KOH) was observed to be more effective as the lignin content in sawdust was solubilised by alkali and contributes to increase in carbon content.

Figure 1 Effect of pre-treatment methods on C-N ratio (see online version for colours)

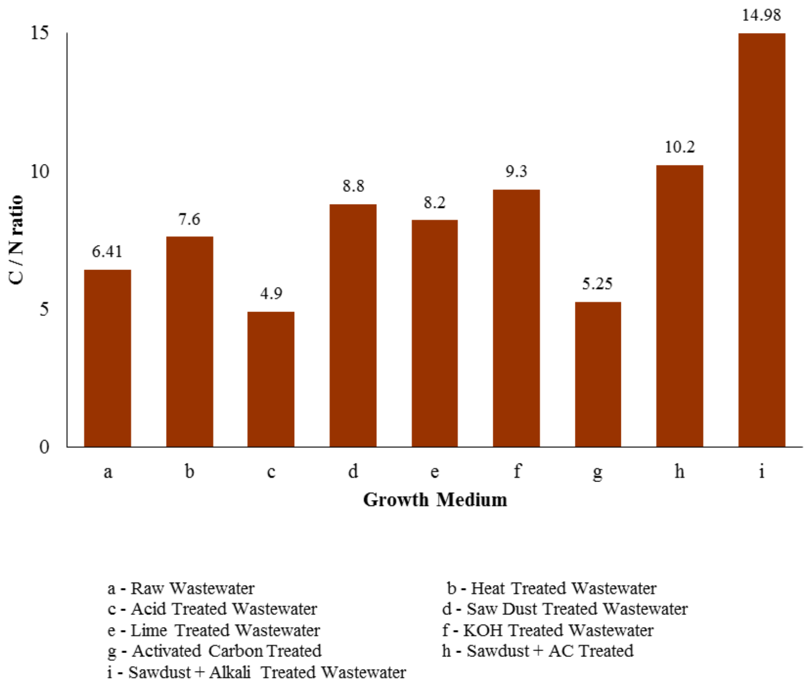


Table 2 Various pre-treatment methods and their effects in carbon and nitrogen values

Treatment method	*Quantity of additives*	*TC*	*TN*	*C/N*
Raw wastewater	–	1,255	195	6.41
Heat treatment	–	565.6	74.36	7.6
Acid treatment (%)	25%	442.4	90.29	4.9
	50%	410.7	89.29	4.6
	75%	334.3	115.3	2.9
Saw dust (g)	1 g	571.4	71.18	8.8
	2 g	709	87.51	8.5
	3 g	834.6	104.4	8.25
	4 g	1,012	134.5	7.78
	5 g	1,146	150.4	7.76
Lime	1 g	850.4	125	6.8
	2 g	868.32	120.6	7.2
	3 g	946.28	115.4	8.2
	4 g	875.94	112.3	7.8
	5 g	850.31	110.5	7.7

Table 2 Various pre-treatment methods and their effects in carbon and nitrogen values (continued)

Treatment method	*Quantity of additives*	*TC*	*TN*	*C/N*
KOH	1 g	845.3	124.3	6.8
	2 g	849.23	128.6	6.6
	3 g	876.1	121.6	7.2
	4 g	950.17	102.7	9.3
	5 g	930.48	113.4	8.2
Activated carbon	0.5 g	69.2	20.6	3.3
	1 g	52.22	18.01	2.8
	1.5 g	73.39	16.27	4.5
	2 g	78.4	14.92	5.25
Sawdust (g) + activated carbon (g)	5 + 0 (g)	1,146.21	150.4	7.6
	5 + 0.5 (g)	884.5	99.28	8.9
	5 + 1 (g)	697.71	68.22	10.2
	5 + 1.5 (g)	565.62	65.64	8.3
	5 + 2 (g)	525.33	53.52	9.8
Sawdust (1 g) + KOH (4 g)	1 + 4 (g)	1311	87.5	14.98

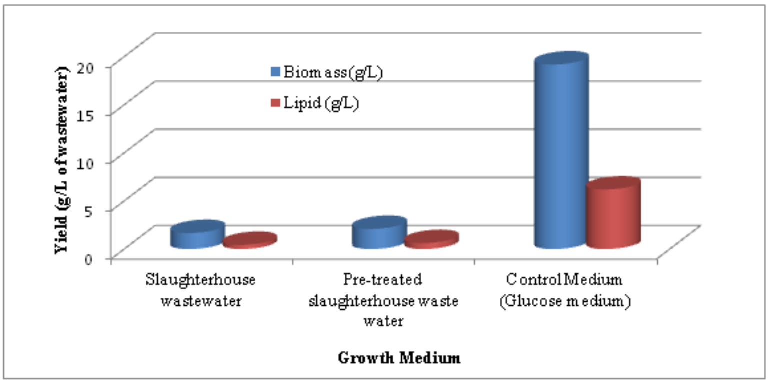
3.3 Biomass and lipid production

Three growth mediums, viz., raw slaughterhouse wastewater, pre-treated slaughterhouse wastewater and pure glucose medium were taken for the experimentation. After five days of incubation, the yeast biomass was harvested and the obtained yield for each medium is represented graphically in Figure 2. It was observed that the maximum biomass concentration of 2.4 g/L and lipid yield of 0.63g/L was obtained for pre-treated wastewater medium. For slaughterhouse raw wastewater, the yield was 0.43g/L and the results are given in Table 3. Chi et al. (2011) reported that the yeast *Y. lipolytica* grown on food waste, municipal wastewater and nutrient mixed municipal wastewater medium showed biomass growth of 10 g/L, 0.36g/L, and 15.3 g/L respectively. Park et al. (1990) suggested that C/N ratio of 5 in balanced nitrogen medium, oleaginous yeast *A. curvatum*, showed a lipid yield of 20% of its dry biomass. Also, for a C/N ratio of 18, the yield was 25%. Similar results were obtained in this present study with a dry biomass of 2.1 g/L of wastewater and a lipid yield of around 30% of its dry biomass. The biomass growth was slightly increased in raw wastewater whereas nearly two-fold increase in lipid content was observed in pre-treated wastewater. The increase in growth, as well as lipid yield, may be due to the habitat of the yeast strain *Y. lipolytica* since it was isolated from the fatty and oily environment. The lipid productivity of nearly 160 mg/L/day was achieved, which on scaling up provides a good platform for using the lipid as alternative feedstock for biodiesel.

Table 3 Biomass and lipid yield of wastewater medium

S. no.	*Wastewater medium*	*Biomass dry weight (g/L)*	*Lipid yield (g/L)*	*% of lipid in total biomass*
1	Slaughterhouse wastewater	1.673	0.439	26
2	Pre-treated slaughterhouse wastewater	2.1	0.629	30
3	Control medium (glucose medium)	19.2	6.2	32

Figure 2 Total lipid yield in different growth medium (see online version for colours)



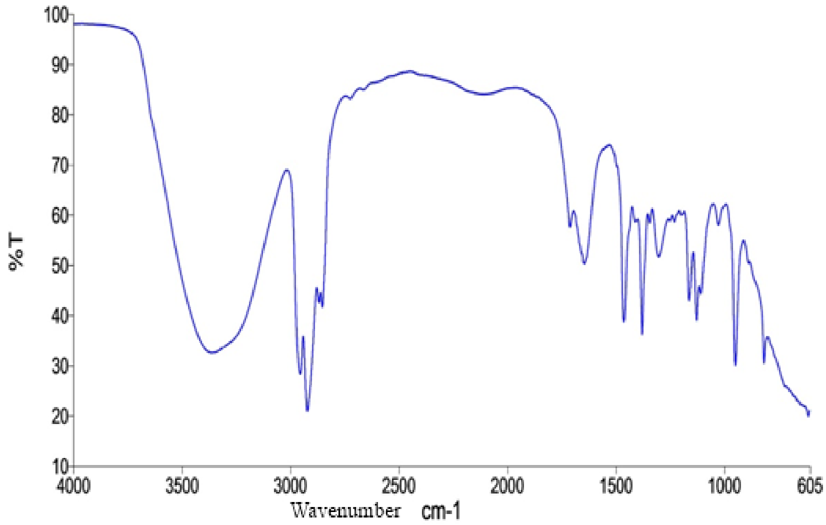
3.4 FTIR characterisation

The extracted lipid was characterised by FTIR spectroscopy (Figure 3). The wave number 3,360 cm^{-1} represented the –OH stretching of aliphatic – COOH. Between the range of 2,924 cm^{-1} and 3,000 cm^{-1} the C-H stretching of aliphatic fatty acids was observed. These bands were similar to the lipid extracted from green algae reported by Maity et al. (2014). A peak at 1712 cm^{-1} represents the possible bond assignment of C = O (ester groups) of lipids and fatty acids. From the data of vibrations (Table 4) and comparing with the previous literature, the lipid extracted in this study was well matched with the spectrum of linoleic acid methyl ester (Park et al., 1990; Thakur et al., 2013; Salimon et al., 2011).

Table 4 FTIR results

S. no.	*Wave number (cm^{-1})*	*Vibration*
1	3,367	– O-H molecule vibration
2	2,957	C = C
3	2,924	C-H stretching
4	2,854	C=H stretching
5	1,711	C=O, ester stretching
6	1,462	N-H bending
7	1,161	C-O stretching
8	605–1,100	–C-O-C stretching

Figure 3 FTIR spectrum of lipid extracted from *Yarrowia lipolytica* (see online version for colours)



4 Conclusions

The results of this study showed that slaughterhouse wastewater can serve as an effective substrate for microbial lipid production under favourable conditions. The effect of various pre-treatment showed that combined treatment methods can enhance the carbon content thereby increasing the lipid amount in *Y. lipolytica*. Maximum lipid content of 0.63 g/L with lipid productivity of 160 mg/L was achieved. Considering the lipid productivity of the yeast strain, the total lipid which can be made out from the wastewater will serve as good source of energy. Further studies need to be carried out for enhancing the yield of the lipid content and analysing it for the possible production of biodiesel.

References

Ageitos, J.M., Vallejo, J.A., Veiga-Crespo, P. and Villa, T.G. (2011) 'Oily yeasts as oleaginous cell factories', *Applied microbiology and Biotechnology*, Vol. 90, No. 4, pp.1219–1227.

American Public Health Association (APHA) (1992) *Standard Methods for the Examination of Water and Wastewater*, 18th ed., Washington, DC.

Carbon to Nitrogen Ratios of Various Waste Materials (2014) [online] http://norganics.com/wordpress/wp-content/uploads/2014/10/cnratio.pdf (accessed 21 September 2016).

Cheirsilp, B., Kitcha, S. and Torpee, S. (2012) 'Co-culture of an oleaginous yeast *Rhodotorula glutinis* and a microalga *Chlorella vulgaris* for biomass and lipid production using pure and crude glycerol as a sole carbon source', *Annals of microbiology*, Vol. 62, No. 3, pp.987–993.

Chi, Z., Zheng, Y., Jiang, A. and Chen, S. (2011) 'Lipid production by culturing oleaginous yeast and algae with food waste and municipal wastewater in an integrated process', *Applied Biochemistry and Biotechnology*, Vol. 165, No. 2, pp.442–453.

Folch, J., Lees, M. and Sloane-Stanley, G.H. (1957) 'A simple method for the isolation and purification of total lipids from animal tissues', *J. Boil. Chem.*, Vol. 226, No. 1, pp.497–509.

Ma, F. and Hanna, M.A. (1999) 'Biodiesel production: a review', *Bioresource Technology*, Vol. 70, No. 1, pp.1–15.

Maity, J.P., Hou, C.P., Majumder, D., Bundschuh, J., Kulp, T.R., Chen, C.Y., Chuang, L.T., Chen, C.N.N., Jean, J.S., Yang, T.C. and Chen, C.C. (2014) 'The production of biofuel and bioelectricity associated with wastewater treatment by green algae', *Energy*, Vol. 78, No. 1, pp.94–103.

Masse, O. and Masse, L. (2000) 'Treatment of slaughterhouse wastewater in anaerobic sequencing batch reactors', *Canadian Agricultural Engineering*, Vol. 42, No. 3, p.131–137.

Papanikolaou, S. and Aggelis, G. (2009) 'Biotechnological valorization of biodiesel derived glycerol waste through production of single cell oil and citric acid by *Yarrowia lipolytica*', *Lipid Technology*, Vol. 21, No. 4, pp.83–87.

Park, W.S., Murphy, P.A. and Glatz, B.A. (1990) 'Lipid metabolism and cell composition of the oleaginous yeast *Apiotrichum curvatum* grown at different carbon to nitrogen ratios', *Canadian Journal of Microbiology*, Vol. 36, No. 5, pp.318–326.

Salimon, J., Salih, N. and Abdullah, B.M. (2011) 'Improvement of physicochemical characteristics of monoepoxide linoleic acid ring opening for biolubricant base oil', *BioMed Research International*, No. 1, pp.1–8, Article Id:196565.

Taherzadeh, M.J. and Karimi, K. (2008) 'Pretreatment of lignocellulosic wastes to improve ethanol and biogas production: a review', *International Journal of Molecular Sciences*, Vol. 9, No. 9, pp.1621–1651.

Thakur, S., Shrivastava, B., Ingale, S., Kuhad, R.C. and Gupte, A. (2013) 'Degradation and selective ligninolysis of wheat straw and banana stem for an efficient bioethanol production using fungal and chemical pretreatment', *3 Biotech.*, Vol. 3, No. 5, pp.365–372.

Thiru, M., Sankh, S. and Rangaswamy, V. (2011) 'Process for biodiesel production from *Cryptococcus curvatus*', *Bioresource Technology*, Vol. 102, No. 22, pp.10436–10440.

Thirulogachandar, A., Priyadharshni, V.S., Anbarasan, T., Saraswathy, S. and Jayanthi, S. (2015) 'Production of microbial lipid using slaughterhouse wastewater as substrate', *International Journal of Applied Engineering Research*, Vol. 10, No. 67, pp.324–327.

Wismer-Pedersen, J. (1988) 'Use of haemoglobin in foods – a review', *Meat Science*, Vol. 24, No. 1, pp.31–45.