Potential of Oleaginous Yeasts as Economic Feedstock for Biodiesel Production

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ARTICLE INFORMATION

ABSTRACT

Production of biofuels at commercial level has been perceived as sustainable solution to the problem of energy and economic crisis. Of all the liquid biofuels, an important one is biodiesel, the fatty acids methyl esters. Due to beneficial decomposition of bio-waste resources towards clear environments, oils from single cell microbes (yeasts) are gaining attention. Further cost reliance of these microbes to produce single cell oils (SCOs) could be reduced by using lignocellulosic waste (LCW) biomass produced in millions of metric tons annually all over the world covering 60% of annual crop produced. This review presents the yeasts SCO production from LCW, and its favorability towards commercialization in developing countries like Pakistan, which is eighth largest country among GDP sector for farm production.

Key Words: Oleaginous yeast, Single cell oil, Lignocellulosic wastes, LCW, Biodiesel, Yeast extract.

INTRODUCTION

Biofuels have taken the stern attention of researchers due to the increasing demands and depleting reserves of petroleum oils-based fuels. There is an immense need to recognize appropriate sustainable alternatives to conventional fossil fuels. Volatile as well as non-volatile, flammable hydrocarbons derived from plant or animal wastes or synthesized by autotrophic microbes such as microalgal oils able to be utilized as a rich source of energy represent biofuels. A variety of cellulolytic microorganisms capable of utilizing different lignocellolosic materials including agro-industrial wastes has been documented for the production of biofuels. Lignocellulosic biomass is broken down into cellulose or hemicellulose oligomers and monomers yielding ultimately sugars including pentoses and hexoses (Hahn *et al.*, 2007). Among all the biofuels, biodiesel, due to its clean burning, low viscosity, non- toxic and biodegradable nature low CO emissions and slightly higher NO_x with reduced production of SO_2 is an efficient biofuel. Therefore, biodiesel production from different feedstocks like animal fats and plants oils has gained much attention all over the world. Plants such as sunflower, rapeseed, soybean, olive, jatropha and palm oils have been employed so far to produce biodiesel. Animal processing unit wastes and tallow have also been used to generate biodiesel. But the oils such as vegetable and cooking oils are expensive and food competitive. To commercialize the process of biodiesel production, feedstock used must be abundant and more sustainable. In this scenario, the most abundant feedstocks are the lignocellulosic wastes (LCWs). These LCWs are produced in huge quantities all over the world throughout the year. LCWs are employed to fermentation using oleaginous yeasts to produce microbial lipids mainly consisting of triacylglycerides (TAG), which can be transesterified into biodiesel. The biodiesel derived from lignocellulosic biomass including certain crops' residues like wheat straw, sugarcane bagasse, corn stover, rice husk, municipal solid wastes and peels of fruits and vegetables are under colossal consideration.

Current progress and limitations in Biodiesel production

In the running scenario of the fossil fuels utilization and depletion, these fuels are no more questionable economically and ecologically. Production of sustainable and renewable biofuels has been under consideration since twentieth century. Transportation is the fastest growing energy consumption sector all over the world especially in US after electric energy sector. Alternative petroleum fuels are therefore under consideration of world energy producing companies to cope this crisis. Biodiesel is non-toxic, highly degradable and sustainable replacement of petroleum diesel accompanying existing petroleum engines with least modifications because of its chemical nature. Over the last decade, global

biofuel production increased rapidly; in 2008, about 15 billion liters of biodiesel were produced globally almost all of which was first-generation biofuel. In the European Union, biodiesel accounts for the major share of total biofuel production and is mainly derived from oil crops (canola and sunflower) as feedstock (Sustainable Production of SECOND – Generation Biofuels Potential and perspectives in major economies and developing countries, 2010). Some theoretical examples illustrate the vast amount of plant oil production necessary to replace conventional diesel. Converting the entire 2005 USA soybean crop to biodiesel would replace only 10% of conventional diesel consumed. Even the total world plant oil production of 2005 (approximately 120 million metric tons) would only satisfy approximately 80% of USA diesel demand (Weiss *et al.,* 2012; Velmourougane *et al.,* 2013). As discussed previously, devoting a greater proportion of plant oils for the production of biodiesel has already contributed to higher vegetable oil prices not only making biodiesel production more expensive but also having an impact on other sectors of the economy, such as food prices.

First generation biodiesel production includes the feed stocks like vegetable oils as well As oils extracted from corn, soybean, coconut, palm, sunflower etc. These feed stocks have proved to be renewable as well as environment friendly emitting lesser amounts of CO_2 resulting in remediation of Global warming. The amount of CO_2 emitted as a result of burning is directly balanced by the amount of CO_2 consumed during photosynthesis of plants thus resulting in comparatively cleaner environment (Osamu & Carl, 1989; Stevens & Verhi, 2004). These oils contains higher levels of fatty acids like oleic acid, linoleic acid and steric acid as compared to other fatty acids. More oleic acid containing oils are preferred for biodiesel production (Firestaone, 2006). These vegetable oils are of less worth as biodiesel feedsock for they are expensive and food competitive.

Non-edible oil seed plants have major significance for biodiesel production as they are not edible and could be grown in abundance over lands surrounding canal banks, road green belt and cultivator's leftover land during seasonal crop and naturally destructed forests area without the issue of food-fuel competition. Non-edible oil plants like Polanga (*Callophyllum inophyllum*), Mahua (*Madhuca indica*), Karanja (*Pongamia pinnata*), Rubber plant seed, Cotton seed, Jatropha, Jojoba, Neem, Linseed and Tobacco might be cultured in additional lands for cost-effective provision of second generation biodiesel feedstocks (Chhetri *et al.*, 2008; Mustafa, 2011; Atabini *et al.,*2013; Ong *et*

al., 2013; Ashraful *et al.*, 2014). But the oil produced from these seeds requires greater engine modifications as well as higher temperature zones to be applicable in transport sectors. In addition many categories of such oils are also non-favorable because of their higher viscosities.

For third generation biodiesel production, both prokaryotic and eukaryotic oleaginous microorganisms have been documented by researchers (Duong *et al.,* 2007; Li *et al.,*2008; Chatzifragkou *et al.*, 2010; Gorenberg *et al.,* 2013).These microbial oils/lipids are said to be Single Cell Oils (SCO). SCO can be made as best demanding and responsive because of their greater efficiencies, higher yields and productivity. The SCO are also independent of the issues regarding land, venue, season and climate (Ratledge & Evans, 1984; Li *et al.,* 2008; Moona *et al.*, 2008; Galafassi *et al.,* 2012; Tsigie *et al.*, 2012). Microalgae species of these genera like *Chlorella, Scenedesmus, Anabenae, Rhizocolonium* and many other have been reported to produce biodiesel based on the mechanism of CO_2 utilization giving out excess biomass able to be utilized as animal feed or fertilizers. The biodiesel from microalgae needs greater amounts of CO_2 and light as well as longer time period to yield the product.

Microalgae are on a great verge for biodiesel production having greater yields but the choice differs in the following aspects as they need hectors of area for their cultivation and maintenance as well as long cultural durations (Chisti, 2007). Further their oil composition differs mostly from that of vegetable oils being quite rich in mono as well as polyunsaturated fatty acids having four/ more double bonds (Belarbi *et al.*, 2000) making the oils more susceptible to oxidation when stored and acceptability for biodiesel production might face restrictions (Chisti, 2007). Although the process of microalgae biodiesel production has been commercialized in many countries both heterotrophically and photosynthetically (to reduce cost of carbon source) but the process needs serious attention for provision of culture able land as well as stability of microalgal oils as they are significantly better as compared to other microbial lipids because these oils are highly unsaturated and more prone to degradation at high temperature (www.Biofuel.org.uk). Bacterial cells, being much smaller than the yeasts, attain comparable intracellular lipid/oil contents for biodiesel conversion. While owing to their fast metabolic rate, rapid growth and their abilities of utilizing complete bio waste resources such as cellulose might make them appealing candidates for biodiesel production at least in select situation such as addressing the

lignocellulosic mass utilization. Table I shows different advantages and disadvantages of biodiesel produced from different feedstock.

Table I: Advantages and drawbacks of different biodiesel feedstock

Category	Feedstocks	Worldwide oil Production	Advantages	Drawbacks	References
First Generation Biodiesel	Palm oil, Rapseed oil, Coccunut oil, Sunflower oil, All vegetable oils	More than 95% of biodiesel production all over the world	i. Environment friendly ii. Social and economic security	i. Expensive ii. Food competitive iii. Used in blends with petroleum biodiesel iv. High viscosity v. Lower volatility	Demirbas, 2003; Gui *et al.*, 2008; Naik *et al.*, 2010; Sitepu *et al.*, 2014
Second Generation Biodiesel	Non-edible oil seed plants		i. Cheap ii. Non- food iii. No special nutritive requirements for growth	i. Cultureable land ii. High viscosity iii. Require engine modifications iv. Used in warmer areas v. Only annual or seasonal production	Fengrui *et al.*, 1999
Third Geneartion Biodiesel	Microalgae	Thousands of Gallons of oil/month	i. Strong oil producers ii. Strong CO_2 sequesters iii. Yields much biomass able to be utilized as animal feed or water purifier v. Similar fatty acids as that of vegetable oils v. 50-60% of oil contents per dry biomass vi. Higher growth periods (7-14 days)	i. Require more land area as well as water bodies for cultivation. ii. More susceptible to Bacterial or protozoan contamination in open ponds system. iii. Light required all the time for photosynthetic cultivation v. Most lipids are of lower fuel values as compared with diesel fuel v. Higher cultivation cost compared to plant oils	Solazyme, 2013; Meng *et al.*, 2009; Chisti 2007; Huang *et al.*, 2013
	Yeast		i. Quick growth to higher densities ii. Able to grow in variety of substrates iii. Able to control bacterial contaminations by low pH growth conditions in open culture systems v. Also could be	i. Heterotrophs ii. Needs Simple sugar to ferment, therefore require pretreatment of lignocellulosic biomass iii. Commertially not well known iv. Pathogenic nature of some of yeast species should be considered before	Sitepu *et al.*, 2014; Qi *et al.*, 2013

			grown on same conditions as hetertrophically grown algae v. Can accumulate upto 70-80% of lipids in four days vi. Resistant to fermentative inhibitors upto 0.5g/l of HMF, furfurals, and 2.5g/l acetic acid vii. No added vitamins or suppliments required viii. No hectors of areas required for growth	industrialization	
	Bacteria		i. Fast growth rate	i. Smaller size ii. Yields complicated lipoids instead of lipids	Huang *et al*., 2013

In this era overburdened land agricultural practices have directed the scientists to look for such microbial cultivations which can be managed in small area/volume owing to their rapid rates of multiplication coupled with their ability of using waste biomass as feedstock. Thus through the development of a process a value added product is obtained with concomitant consumption of waste(s). Therefore, in this review our main focus is on yeast biodiesel production using lignocellulosic wastes. In this regard certain species of oleaginous yeasts have much potential. The yeast can be cultivated on certain agri/food industrial wastes and byproducts. Depending upon the nature of complexity and recalcitrance of the waste substrates physicochemical and biological pretreatment is mandatory, however.

Oleaginous yeasts as renewable feedstock's for biodiesel production

The role of oleaginous precursors for the production of biodiesel depends upon their efficiency and environmental favorability. So the choice of oleaginous precursors should be according to the environmental concerns and coping competition of depleting fossil fuels. The biodiesel obtained from the respective precursors should also have the similar molecular structures as well as the energy density to that of petro diesel fuels. Yeast lipid production is a biphasic process: firstly the SCO produced are very much similar to vegetable oils and are highly compatible to be converted into the biodiesel and secondly, the cell biomass could be harvested and employed as a rich protein supplement to humans as well as aquaculture. Feasibility to this LCW based SCO production also leads to clear environments and surroundings improving health status of the local people.

Microbes like bacteria, yeasts and microalgae have up to 60% tendency of their dry cell mass to accumulate lipids/oil in them, while the yeast *Cryptococcus curvatus* has been reported to accumulate 82.7% lipid contents per 104.1 g/L of biomass utilizing glucose as sole source of carbon (Ykema *et al.,* 1988). The complex substrate utilization by yeast can be made possible by co-culturing cellulolytic bacteria and oleaginous yeast (Uchida *et al*., 2004; Zuroff *et al*., 2013).

While oleaginous yeasts have many advantages as being unicellular, having high growth rates with lipid accumulation in discrete lipid bodies in lesser time durations and are accomplishable within relatively short period of time. On the other hand usage of low-cost fermentable media including agricultural and industrial residues renders the process economically lucrative (Malisorn & Suntornsuk, 2007; Angerbauer *et al*., 2008; Yousuf *et al*., 2010; Koutinas *et al.,* 2014). Furthermore the accumulation of higher lipid contents depends mainly on the higher C/N ratio of the cultivation media (Angerbauer *et al*., 2008; Saenge *et al.,* 2011). Papanikolaou *et al.* 2003 reported 150 C/N ratios for giving out higher yields upto 68% of dry

cell mass of the yeast *Lipomyces starkeyi* utilizing pretreated sewage sludge. Moreover, *Yarrowia lipolytica* has been reported for 70% lipid accumulation utilizing agro-industrial wastes like stearin (waste industrial animal fats) and technical glycerol and glucose ultimately resulting in the increased production of citric acid and cocoa butter like lipids upto 14g/L and 3.4g/L, respectively. Papanikolaou and Aggelis (2011) found that the lipids accumulation in oleaginous yeasts increases in the nitrogen or to a lesser content on phosphorus or sulfate deficiency in media and is termed as *de novo* lipid accumulation which starts degrading soon after the depletion of carbon in the media, while ex-novo lipid accumulation is independent of the nitrogen content of the media. Lipid accumulation is also influenced by the factors like carbon source, nitrogen source, C/N molar ratios and physical growth parameters such as temperature and pH. Organic nitrogen (peptone and yeast extract) supplementation is more advantageous over

ammonium sulfate $[(NH_4)_2SO_4]$ and ammonium nitrate $[NH_4NO_3]$ in gaining higher cell mass as well as lipid yield from *Trichosporon fermentans*. Decrease in biomass using inorganic nitrogen source $[(NH_4)_2SO_4$ and $NH_4NO_3]$ might be due to the formation of inhibitor $(HNO_3$ and $H_2SO_4)$ acids after 72 hours growth of *Y. lipolytica* Po1g in sugarcane bagasse medium resulting the decrease in pH upto 6 and 5.8, respectively (Zhu *et al.*, 2008; Tsigie *et al.*, 2011). So a medium with surplus of carbon and other limiting supplements like nitrogen is required for higher lipid yields (Papanikolaou *et al.*, 2006). Arachidonic acid, a polyunsaturated fatty acid produced by yeasts and is of great importance as dietary supplement in infant formula (Ratledge & Wynn, 2002; Ratledge, 2004). Fatty acid composition of lipids from different sources varies greatly. Meng *et al.* (2009) have summarized ratio of different fatty acids constituents of lipids produced by microalgae, yeast, fungi and bacteria as shown in table II.

Table II: Fatty acid profiles of different microorganisms (Meng *et al.*, 2009)

Microorganisims	Lipid Composition (w/total lipid)					
	Palmitic acid C16:0	Palmitoleic acid C16:1	Stearic acid C18:0	Oleic acid C18:1	Linoleic acid C18:2	Linolenic acid C18:3
Microalgae	12-21	55-57	1-2	58-60	4-20	14-30
Yeast	11-37	1-6	1-10	28-66	3-24	1-3
Fungi	7-23	1-6	2-6	19-81	8-40	4-42
Bacterium	8-10	10-11	11-12	25-28	14-17	N.D

The typical biodiesel with maximum efficiency is defined as a mixture of long chain fatty acid methyl esters (typically C_{14}- C_{22}) being a non-toxic, biodegradable pollution reducer (Monoz *et al.*

2014). The relevant fatty acid profiles of different yeast strains have been shown in the following table III.

Table III: showing fatty acid profile of different oleaginous yeast species/ strains on glucose containing medium (adapted from Ratledge & Wynn, 2002; Liu & Zhao, 2007 & Beopoulos *et al.*, 2011)

Yeasts species/strain	Lipid accumulation (%D.W)	Major Fatty acid Residues (%w/w)					
		Palmitic acid C16:0	Palmitoleic acid C16:1	Stearic acid C18:0	Oleic acid C18:1	Linoleic acid C18:2	Linolenic acid C18:3
Cryptococcus curvatus	58	25	Trace	10	57	7	0
Cryptococcus albidus	65	12	1	3	73	12	0
Candida sp 107	42	44	5	8	31	9	1
Lipomyces starkeyi	63	34	6	5	51	3	0
Rhodotorula glutinis	72	37	1	3	47	8	0
Rhodotorulla graminis	36	30	2	12	36	15	4
Rhizopus arrhizus	57	18	0	6	22	10	12
Trichosporon pullulans	65	15	0	2	57	24	1
Yarrowia lipolytica	36	11	6	1	28	51	1
Rhodotorulla toruloides	58	24.3	1.1	7.7	54.6	2.1	N.d

Recently, Santamauro *et al*. (2014) have reported an oleaginous yeast *Metschnikowia pulcherrima* capable of utilizing waste lignocellulose derived sugar mixture (glucose, xylose, arabinose and cellobiose) to accumulate lipids upto 40% under non-sterilized conditions. Active growth rate was observed in stressed physical conditions like pH and temperature followed by nitrogen-starved medium. The heterotrophic organisms such as yeast may prove beneficial alternative to microalgae as biodiesel feedstock with an elevated production potential of lipids on per cell per 24 hours basis yielding upto 65% lipid/dry cell mass within 3-7 days from 10-100g/L of sugars (Li *et al*., 2008) comparing with microalgae giving only 0.15-0.25 g/L per day in open ponds (Sharma *et al*., 2011). Moreover, unlike microalgae yeast does not require light enabling 24 hours production of lipids on equal extent rendering further economical fermentation of both C_6 and C_5 sugars from lignocellulosic waste substrates (Sun & Chang, 2002; Larsen *et al*., 2008; Santamauro *et al*., 2014).

Lucrative substrates for yeast oil production
1-Lignocellulosic Wastes

Lignocellulose, a generalized term used to describe the three major components of plant material namely lignin, cellulose and hemicelluloses held together in complex matrices composed of several different polysaccharides, phenolic polymers and proteins. Large energy reserves are present in cellulose which is also the major portion of lignocellulosic biomass having existent potential for catalytic conversion into biofuels. Lignocellulosic biomass is the most abundant renewable organic source which could be exploited for biofuels production. It is produced around 200 billion tons annually comprising 60% of lignocellulosic wastes as crop residues, forest left over and industrial residues following food and vegetable processing (Silva *et al*. 2012; Gracia *et al*., 2014). Oleaginous yeasts produce a variety of SCOs having different fatty acid profiles depending upon the variety of nutrients/ carbon source. This property of the

oleaginous yeasts for biodiesel production is under consideration since last few decades. To improve economics of the product by using low-cost/ no-cost lignocellulosic wastes commonly called as crop/industrial residues are under consideration (Candia *et al*., 2014).

However, oleaginous yeasts are less active in cellulolytic activity and needs an efficient pretreatment of LCW for biodiesel production. Different pretreatment methods like acid/alkali treatments, steam and sulfur dioxide explosions, ammonia fiber explosion, ionic liquids and others are used to convert this lignocellulosic biomass into fermentable sugars. Many structural, compositional and physico-chemical parameters hinder conversion of the biomass into sugars. Further the pretreated lignocellulosic waste biomass is to be detoxified by over liming etc. for bypassing the inhibitory effects of certain LCW pretreatment/ hydrolysis derived molecules such as furfurals and 5-hydroxyl-methyl furfural (HMF) (Haung *et al*., 2011; Bochmann & Montgomery, 2013; Behera, *et al*., 2014).

Typical ratio of hexoses (glucose and mannose) to pentoses (xylose and arabinose) in LCW is reported to range from 1.5:1 to 3:1 (Balan *et al*., 2008; Balan *et al*., 2009; Lau & Dale, 2009). Pretreatment is also important so that the naturally occurring or genetically engineered or modified oleaginous yeasts could be able to hydrolyze sugars other than glucose such as xylose (second abundant sugar) to produce biodiesel. Xylose is well thought out for active consumption to produce biodiesel by oleaginous yeasts because it is less reported as fermentable for bioenergy production processes (Rahman *et al*., 2006). Microbial utilization of xylose will subsequently lead to the maximum utilization of LCW biomass. A thumbnail sketch of the whole process designed for converting LCW into biodiesel through the lipid accumulating metabolism of oleaginous yeasts is depicted in Fig.,1.
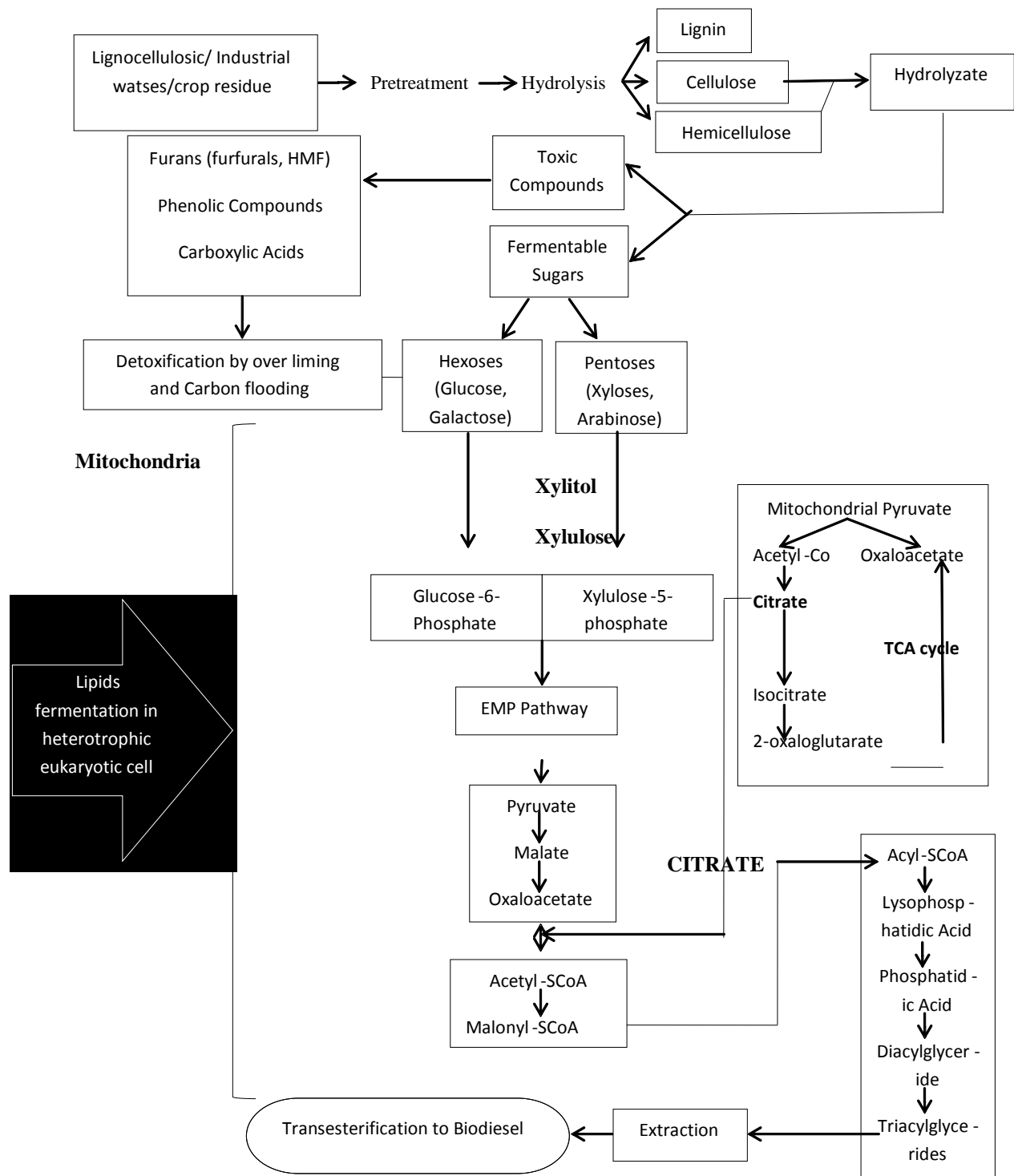
Fig.,1: A generalized overview and sequential organization of various steps involved in the process of upgrading agri/food industrial wastes to biodiesel routed through the eukaryotic microbial metabolism (Xue et al., 2013) EMP pathway; Embden-Meyerhof-Parnas pathway.

2- Industrial wastes

Increasing energy demands by world's population necessitate to recycling of every possible waste substrate. Industry is developing with the passage of time all over the world. Billions of tons of wastes like molasses, vegetable and fruits

processing wastes, glycerol, whey and waste waters including sewage sludge and from oil mills are rich sources of fermentable sugars. The feedstock from wastes could be utilized as potential substrates for SCO production worldwide (Haung *et al.,* 2013). The potential of SCO production using the low-cost substrates from wastes by variety of yeasts have been tested so far and the strains of *L. starkeyi, Rhodotorula glutinis, Y. lipolytica, C. curvatus, Apiotrichum curvatum* and *T. fermentans* have been found potentially active to produce SCO as biodiesel feedstocks (Akhtar *et al.*, 1998; Papanikolaou & Aggelis, 2003; Zhu *et al.*, 2008; Yousuf *et al.* 2010; Wu *et al.*, 2011). Lipid fermentation is not dependent upon the substrate selection solely. It also bases on microbial lipid production and suitability of the lipid produce for biodiesel production. Substrate should be cost- competitive and should not influence the quality of microbial oil.

Significance and abundance of wastes derived SCOs from yeasts

Microbial lipids, being an important feed stock for biodiesel production from oleaginous yeasts are of momentous significance around the world (Meng *et al.*, 2009). During last decade oleaginous yeasts *Rhodotorula glutinis, C. albidus, L. starkeyi,* and *Candida curvata* (currently called as *Cryptococcus curvatus*) capable of yielding higher quantities of SCOs utilizing acid/ alkali hydrolyzed lignocellulosic wastes as carbon sources like wheat straw, corn stover, rice husk, sugarcane bagasse, corncob, populus and Eucalyptus leaves, grass etc. have been documented. The SCOs are transesterified into biodiesel and other value added products. Some of the oleaginous yeast species are able to ferment xylose, a pentose sugar too, utilizing most of residual portion of lignocellulosic waste adding a positive clue to enhance the production of biodiesel (Haung *et al.*, 2013). Ability of xylose metabolism enhances the composition and nature of fatty acids chain lengths and degree of saturation. One of the most important selection criteria for oleaginous yeast strains is the chemical nature of fatty acids present in biolipids produced to ascertain their suitability for biodiesel production (Tanimura *et al.*, 2014).

Oleaginous yeasts could be preferable than fatty acid methyl esters (biodiesel) producing plants because they do not need any arable land and could be grown on low–cost degradable wastes using their carbon as energy source to accumulate lipids as well as non- food competitors resulting in cost effective biodiesel production (Bautista *et al.*, 2012).The nature of yeasts SCOs is similar to that of plant/vegetable oils as they are most commonly occurring saturated fatty acids. The major fatty acids

of SCOs comprises of palmitic acid (C16:0), stearic acid (C18:0), oleic acid (C18:1), linoleic acid (C18:2) and (C18:3) which is similar to vegetable oils and is species specific (Beopoulos *et al.*, 2009). Another way to favor SCOs from yeasts is the temperature and environmental favorability. Being rich in biodiversity yeasts are found in almost all habitats including hypertonic as well as hypotonic environments in aerobic as well as anaerobic conditions. Yeasts also favorably grow in a temperature ranging from 23-37°C within 48-72 hours in aeration. pH also ranges in a long range from 2-9 (Avallone *et al.*, 2001; Jackels & Jackels, 2005). Velmourougane (2013) reported that during the process of fermentation when 60% glucose is utilized the pH of the microbial culture is lowered because of the production of raw substances like acetic acid, lactic acid and ethanol which could further be used as supplement by yeast. Some of the criteria to choose yeast as SCO producer biodiesel feedstock have been reported by Pereira *et al.* (2014) and Sitepu *et al.* (2014). They have ability to grow in a wide range of lab media (LCW/ low-cost biomass as carbon source), fast growth log phase attained within 24-48 hours, faster lipid accumulation within 96-120 hours, maximum substrate utilization, 80 % maximum product yield of dry cell mass, higher metabolites' tolerance (12-15% ethanol, 2% lactic acid and 2% acetic acid), osmotic pressure tolerance because of rigid cell wall (upto 50% glucose or fructose), pH tolerance during fermentation (pH 2-8), heat tolerance (upto 43°C), lipids completely similar to plants/oil biodiesel, production all the year through- No seasonal boundary, environment friendly biodiesel, Xylose utilization from lignocellulosic waste, less viral vulnerability and lesser chances of bacterial contamination because of fermentation at low temperatures.

Effects of wastes derived carbon sources on yeast's lipids

Oleaginous yeast species are able to utilize diverse range of carbon sources for the production of maximum cell biomass and lipids. These sources can be glucose, xylose, glycerol, starch, hydrolyzed lignocellulosic biomass and others including industrial and municipal organic wastes. Independent of the carbon source lipid accumulation depends upon the limited supply of some nutrient other than carbon. *Trichosporan fermentans* produces lipids on a number of carbon sources (glucose, xylose, arabinose, mannose, galactose and cellobiose) derived from detoxified sulfuric acid treated hydrolyzate of rice straw (SARSH) and hydrolyzed pine/aspen lignocellulosic residues. The highest lipid contents were observed as 40.1% with

a cell biomass of 28.6g/L with a lipid yield of 11.5g/L utilizing detoxified SARSH and 13.9, 11.5 and 10.4g/L utilizing galactose, mannose and cellobiose, respectively (Parajo *et al.*, 1998; Ezeji *et al.*, 2007; Haung *et al.*, 2009). The fraction of fatty acids was dependent on carbon source attaining 65% lipids of *T. fermentans*. With sweet potato vines enzymatic hydrolyzate (SVH) *T. fermentans* was found to be an efficient lipid producer with a net yield of 9.6g/L lipid contents having 35.6% of lipid yield after 7 days of incubation utilizing 90% of reducing sugars. While with a supplementation of SVH with fructose, the yield was increased upto 27.6g/L (Zhan *et al.*, 2014). Wheat straw is an abundant lignocellulosic waste produced all over the world. Annual production of wheat straw is recorded to be 850 tons per annum based on straw/crop ratio of 1.3 (Talebnia *et al.*, 2010) which are exclusively increasing with the passage of time. Lipid producing potential of five oleaginous strains namely *Cryptococcus curvatus*, *Rhodosporidium toruloides*, *Rhodotorula glutinis*, *Yarrowia lipolytica* and *Lipomyces starkeyi* was tested using detoxified and non-detoxified acid hydrolyzed wheat straw having composition of 24.3 g/L of pentose and 4.9g/L of hexoses. Rest of the hydrolysate contained inhibitors like furfurals, acetic acid and hydroxyl methyl furfural (HMF). The resultant liquid hydrolysate contained 3g of glucose, 16g of xylose, 3.8g of arabinose, 1g of galactose and 3.3g of acetic acid per 100g of wheat straw. Out of these five oleaginous strains *C. curvatus* was found an efficient lipid producer having 33.5% and 27.1% lipid yield from 17.2g/L and 15.6g/L of dry cell mass on non-detoxified and detoxified liquid hydrolysate of wheat straw, respectively. All other strains also showed positive results on detoxified as well as non-detoxified liquid hydrolysate except *R. toruloides* for the non-detoxified liquid hydrolysate (Yu *et al.*, 2011). Chen *et al.* (2013) reported corncob acid hydrolysate as potential source of carbon for the growth of yeast *Trichosporon cutaneum*. The hydrolysate contained sugars namely glucose, xylose and cellobiose. This strain yielded 45.5% lipids with a lesser biomass of 22.9g/L. They also confirmed the effect of higher C/N ratio on the lipid accumulation resulting in reduced cell growth. Lipid production potential of sweet potato starch by *Lipomyces starkeyi* has been studied. These cells can consume dissolved starch in batch cultures fermentation processes containing 40% lipids with a dry cell mass of 0.41g of cells per g of starch (Wild *et al.*, 2010). *L. starkeyi* is also capable of giving higher lipid yields up to 6.4% in pretreated organic rich sewage sludge from 9.4g/l of cell biomass (Angerbauer *et al.*, 2008). *Lipomyces*

starkeyi is a distinctive strain for having ability of not utilizing its own lipids (Holdsworth *et al.*, 1988). Crude glycerol is the by-product of the industrial plants converting oils into biodiesel, constituting 10% of the oils fed as substrates. The increase in the production of biodiesel has tremendously increased the production of crude glycerol (Dasari *et al.*, 2005; Johnson & Taconi, 2007).This by-product cost high prices for its purification. Therefore, there is a need to utilize its crude form for producing SCOs. Oleaginous red yeast *Rhodotorula glutinis* has been reported to ferment this crude glycerol into valuable lipids for biodiesel production having 6.10g/L of lipid yield from 10.05g/L of biomass in a fed batch fermentation process (Saenge *et al.*, 2011). Another lignocellulosic waste of industrial sector is sugarcane bagasse. It is the major raw product of sugar industry. The presence of higher carbohydrate and lower lignin values makes it a suitable substrate for yeast fermentation. Sugarcane bagasse can yield 13.59 g/L xylose, 3.98 g/L glucose, and 2.78 g/L arabinose when treated with 2.5% of HCl. Detoxified sugarcane bagasse hydrolysate was found to be suitable substrate for yeast *Yarrowia lipolytica* yielding 6.68g/L lipids (Tsigie *et al.,* 2011). These authors also confirmed the higher lipid and biomass production utilizing peptone (organic nitrogen source) as compared to ammonium nitrate as a source of nitrogen. Molasses is also an industrial raw- product of sugarcane industry. It basically contains glucose, fructose and sucrose. This low-cost material has been found as a cheap medium formulation material for the fermentation of SCO from yeast. But its higher nitrogen contents prevent lipid accumulation in higher amounts (Zhu *et al.,* 2008 & Chatzifragkou *et al.,* 2010). It is concluded that conversion of agro-industrial by-products and wastes having quantitatively large amounts of carbon is a sustainable alternative for recycling and conserving resources. These raw materials could be recycled in the same industry they are being produced. The only need is to establish an extra plant for their fermentation. So the biodiesel fermentation by oleaginous yeast is a key step to be followed by the local governments to overcome the energy crisis at domestic levels ultimately making nations self-sustainable in industrial and transport sectors.

Yeast biodiesel production potential in Pakistan from wastes

In order to certify a sustainable energy prospect, Pakistan's prerequisite to broaden its stock blend cannot be overemphasized. Manipulation of native resources of energy should be the soul of future strategies to enhance the

energy security of the country through subsidizing reliance on energy imports. Renewable and sustainable energy might play a crucial role in the future of energy competence in Pakistan as the government had spent US $9 billion on energy import to fulfill current energy requirements during 2008-2009 (Asif, 2009; Rehman *et al.*, 2013).

Geographically, Pakistan is located in the Southwest of Asia, lying just above the tropic of cancer having an area of about 796,095 Km² comprising of 97.13% land, of which only 4% is covered by forests. Annually, the country receives a heavy rainfall upto 150mm on an average. Pakistan is blessed with four seasons ranging the average temperature as low as 5°C to maximally 55°C. The optimum temperatures vary from 23-37°C residing most of the part of the country for maximum part of the year, especially from March- June and September- November (on an exception of mid-December since last few years) (Weiss *et al.*, 2012). The climate conditions thus are quite favorable for the optimal growth and biolipid productivity of yeast (Chen *et al.*, 2013; Haung *et al.*, 2013; Zhan *et al.*, 2013). Moreover, on the world ranking, Pakistan stands at 14th position in having arable land equal to 20,714,000 hectors by the year 2011 and 25% of this land is under cultivation according to the FAO report, 2011. According to the list of countries by GDP sector composition Pakistan ranks eighth worldwide in farm output. The same report also ranks Pakistan for cotton, date palm, sugarcane, wheat and rice production at 4th, 5th, 5th, 7th and 14th positions, respectively (Food and Agriculture Organization, 2011).

Pakistan's economy is based on agricultural sector and is considered to be one of the world's top exporters of mango, orange, apricot, sugarcane, wheat and rice. Safe disposal of crop residue is a great problem to be overcome and to subsidize this problem careful attention is to be needed for the safe and valued utilization of the residues as a feedstock to produce microbial lipid and ultimately biodiesel (Khan *et al.*, 2010).

According to Pakistan Economic survey (2005-2006) the initial research for biodiesel production pilot scale projects are in progress. Mirza *et al.* (2008) reviewed all biodiesel power projects' in Pakistan and presented that the biomass produced annually should be utilized for centralized power generation by providing appropriate awareness among the farmers and crop cultivators to initiate the production at pilot scale so that more research could be done on the hurdles coming in the way to progress the production of biodiesel and more ways could be explored for a complete utilization of LCW. In accordance to biofuel production, Pakistan Agricultural Research Council (PARC), Islamabad has launched many projects regarding to second generation biodiesel production from oil-crops like jatropha, salicornia and castor oil requiring no agricultural land and less water. The technology is cost effective as compared to ethanol production. Soon in coming few years when these projects would be in progress then it would be more easy to produce biodiesel using LCW that are majorly wasted and only one third is used in burning purposes by the rural population (PSC (Science Technology: Plant Biotechnology- Sustainable Bioenergy for Pakistan, 2011; Kurian *et al.*, 2013; Anwar *et al.*, 2014).

Regarding to biofuel significance in Pakistan, the country has the potential to produce all types of energy including solar, wind, tidal, thermal, biogas, geothermal, biodiesel and biomass/biowaste energy, out of which biodiesel or biomass/biowastes energy production is under main progressive execution, because if Pakistan become self-sufficient in biodiesel production then the country could be among one of the developed nations and be able to withdraw US $ loans upto 60% by saving foreign exchange utilized for energy fulfillments (Sheikh, 2010). According to ministry of petroleum and natural resources by the Government of Pakistan, having highly favorable environments if Pakistan utilizes all uncultured land for biodiesel production then it would be able to produce 56 million tons of biodiesel per annum, however, the annual consumption including all sectors is just 8.5 million tons. Main consumer sector of petro diesel in Pakistan is transportation and power sector including all the industrial machinery with heavy engines. As modern economy is totally dependent upon the availability of cheap fuel and biodiesel is the only reciprocal petroleum fuel having same efficiency as fuel obtained from crude oil. Cheap fuel could be attained from cheap raw- materials otherwise there is no future of diesel engine after the depletion of fossil fuels (Khan & Dessouky, 2009).

Pakistan produces 69 million tons of only field based crop residues including wheat straw, rice husk, cotton sticks, maize stalks and sugarcane

tops. While industrial based crop residues and animal manure have been excluded from this calculation. These residues are not utilized beneficially and are wasted because of lack of their demand (FAO). This waste biomass is a low cost raw material for biofuel production and comprises of 60-70% of the total crop. So this abundant residual biomass can be utilized in the production of biodiesel to decrease the import of petro fuels and ensure an extra income of 200-300$ per acre annually per farmer. Hence the crop residues are considered viable options for Pakistan to cope energy crisis (DAWN News, 2009).

Being a clean and low-emission fuel biodiesel with its higher octane number is also advantageous for increasing engine efficiency and life. Another sustainability of SCO biodiesel (methyl esters) production is that the biolipids produced are reacted with ethanol to form esters and glycerol which in the presence of acid or alkali catalyst gives higher yields. Use of ethanol is favorable as per annum production of ethanol in Pakistan has been quantified as 400,000 tons from 2 million tons of molasses in 21 distillery units reported by ministry of industries, Production and initiatives, Pakistan. The ester produced is viscous enough to replace petro diesel leaving no need to modify the present engine manufacturing technology (Khan & Dessouky, 2009). Thus due to the abundant availability of waste derived feedstocks for cultivating oleaginous yeasts, climatic favors and availability of ethanol from local sources to process the biolipids into biodiesel and ease in use of the biofuel in the diesel engines without any modification are suffice to advocate strongly for initiating development project in this sector in Pakistan. It can be visualized as fortunate that big cities of Pakistan release millions of tons of industrial as well as household, cattle and poultry wastes each year. Due to less developed solid waste management facilities, heaps of such wastes can be observed around industrial and suburban areas. Such dumps and open heaps have caused serious health problems to the local people. Lack of awareness about the proper management and disposal of these wastes and their utilization as

potential energy source is one of the main barriers to promote renewable energy sector in Pakistan. Keeping in consideration all these aspects of energy subsidy in Pakistan there is an immense need to utilize all the gifted facilities in a proper way to put the country in the list of developed nations.

Economization and commercialization of yeast biodiesel production

To economize and commercialize a biotechnological process it is necessary to make it cost-effective with abundant supply of feed stocks required to run the process. The cost of biodiesel production almost doubles the cost of petroleum diesel. This is so because of the high price of feedstock and their transport to biodiesel production plants. Biodiesel has many advantages over petroleum diesel because of its environmental benefits with its biodegradable nature, reduced or none sulfur and aromatic contents and lesser toxic emissions (Demirbas, 2005 a.b). But still the cost of biodiesel production hinders the process to be commercialized. Since far, vegetable oils and non-edible oil seeds like jatropha, jojoba etc. have been used to cope the diesel crisis but the cost for trensesterification and purification alter the feedstock to be introduced commercially. Another main drawback is the availability of feedstock. All the above mentioned feed stocks are the seasonal crops and only available in the season. Further with the passage of time the food and feed requirements are increasing with the increase in population. This scenario will soon lead to the lack of agricultural and cultivatable land too. Therefore there is need to focus to economize the microbial oil production towards commercialization. Microalgal biodiesel production is on road towards commercialization but the doors shut when there comes the maintenance and land availability. Therefore, the focus should be transferred to the SCO production by yeast. Further SCO derived biodiesel from yeast could be viewed as an additional member of the co-product list so that the cell mass and the byproducts could be used as low-profit food or feed supplements as shown in the fig., 2.
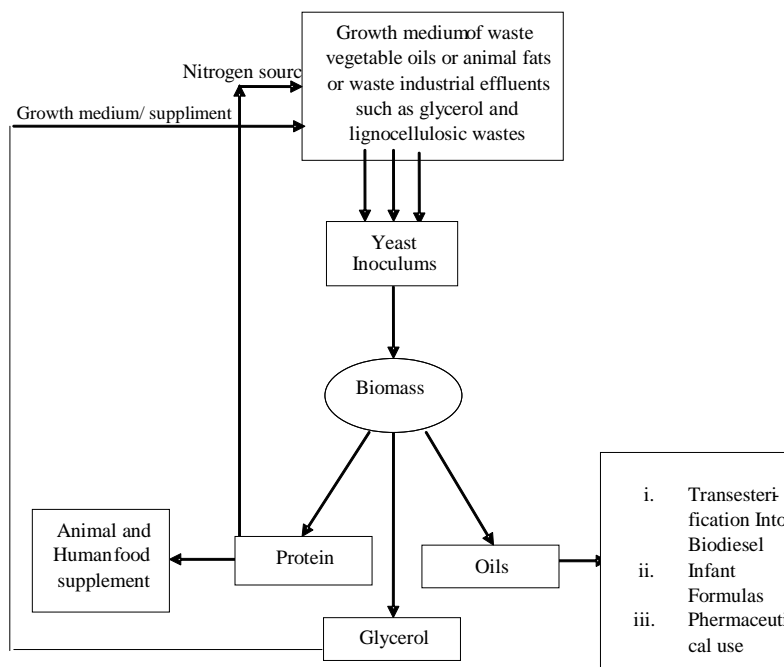
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                    ┌──────────────────────┐
                    │ Growth medium of waste│
                    │ vegetable oils or     │
    Nitrogen source │ animal fats or waste  │
                    │ industrial effluents  │
Growth medium/      │ such as glycerol and  │
suppliment          │ lignocellulosic wastes│
                    └──────────────────────┘
                              │
                        ┌──────────┐
                        │   Yeast  │
                        │ Inoculums│
                        └──────────┘
                              │
                          ( Biomass )
                         /    │    \
           ┌─────────┐  /     │      \  ┌──────────┐
           │ Protein │       Glycerol   │   Oils   │
           └─────────┘                  └──────────┘
   ┌──────────────┐                          ┌────────────────┐
   │ Animal and   │                          │ i. Transesteri-│
   │ Human food   │                          │    fication    │
   │ supplement   │                          │    Into        │
   └──────────────┘                          │    Biodiesel   │
                                             │ ii. Infant     │
                                             │    Formulas    │
                                             │ iii. Pharmaceu-│
                                             │    tical use   │
                                             └────────────────┘
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Fig., 2: Sustainable production of yeast Biomolecules.

The cost of yeast oil production has been estimated on an average of US$ 3,000 per ton recently excluding the cost of feedstock which is not economically viable till the increase in the prices of petroleum and vegetable oils further (Ratledge & Cohn, 2008). Another recent estimate by China claims the cost of SCO production from yeast and microalgae using lignocellulose biomass as US$ 1230 per ton including the cost of feedstock (Haung *et al.*, 2013). This process will lead to much more benefits towards the industrialization if considered the following facts:

i.   Non- pathogenic strain selection in case of yeast.
ii.   Economization of the cost of lignocellulosic biomass hydrolyzate with minimum inhibitors production.
iii. Development of a genetically modified strain to tolerate the inhibitors during sugars fermentation.
iv.   Valorization of the process end products other than the lipids such as biomass and glycerol making an improvement in economic viability of yeast biodiesel production process reducing the cost of additional supplements.

v.   Lowering the cellulose enzyme cost to produce hydrolysates.
vi.   Year-round production.
vii. Selection of Yeast strain able to grow without costly nutrients like Vitamins could also reduce the production cost of biodiesel. Recently, seven out of nine known species from basidiomycetous yeast of genus *Rhodosporidium* can grow on vitamin-free media and on a total account 38 out of 48 known oleaginous yeast strains can grow in vitamin-free media (Sampaio, 2011 & Sitepu *et al.*, 2014).

Construction of an economic process to improve yeast biodiesel production

The cost of biodiesel production is strongly influenced firstly, by the nutrients needed for the cultivation of yeast upto 50% and secondly, by the solvent needed for the extraction and transesterification of lipids (25%) into useable biodiesel. SCO production from yeast needs higher improvements to become economically viable process. These improvements include the cost reduction relative to the production, transport, pretreatment and hydrolysis of the feedstock. This production cost could be economized by creating a consortium based process of lipid production or by

using crude enzymes of bacteria to hydrolyze the lignocellulosic feedstock to become available for fermentation by yeasts. This natural remedy will reduce the cost of hydrolysis, pretreatment and detoxification of lignocellulosic biomass instead of using chemical based treatments. The recycling of waste lignocellulosic material and industrial effluents will also reduce the cost of waste disposal techniques. For further improvements the cost relevant to aeration and pH should also be controlled fig. 3. Illustrates the consortium based bioprocessing idea for yeast biodiesel production.
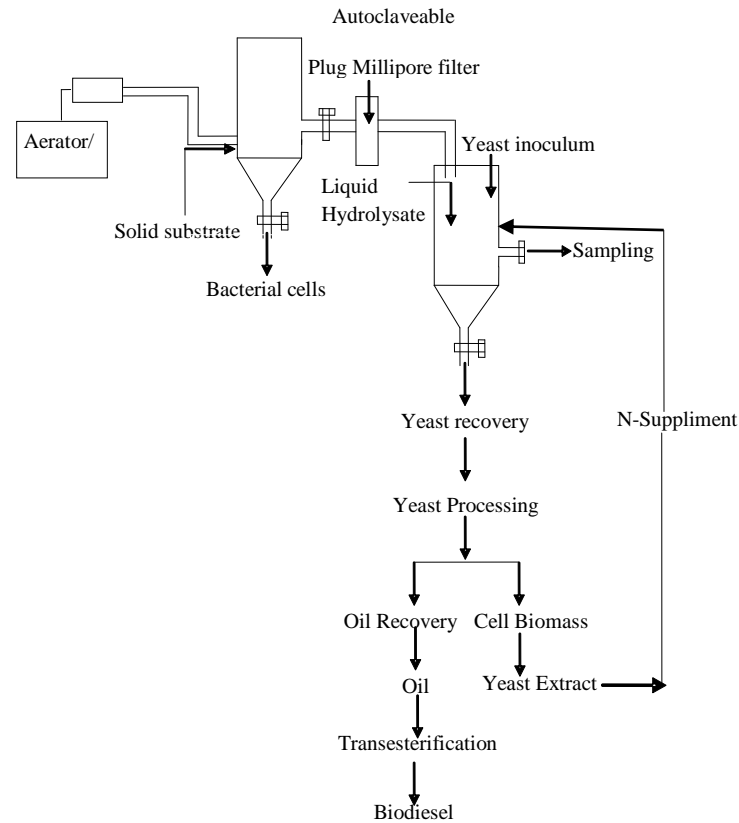

Fig., 3: Diagrammatic illustration of consortium based fermenter for oil recovery and yeast extract production

As a strong evidence of industrial interest in oleaginous yeast California- based Oil Company, Solazyme has been busy in applying several patents on use of yeast oil for food, chemical and fuel ingredients (Franklin *et al*., 2011; Trimbur *et al*., 2012). Other cost relevant improvements to the consolidated bio- processing of biodiesel production may include the improvement of the metabolic pathways in the selected yeast strains genetically. These improvements includes the incorporation of cellullase producing genes from bacteria, high oil and cell mass production, osmotolerence as well as inhibitors resistance, fast growth, simultaneous utilization of glucose as well as xylose to avoid time constrain, simultaneous fermentation and scarification and allowing desired array of fatty acid metabolism (Liu *et al*., 2012). Another way to economize the process of biodiesel production from yeast is to divert the carbon sources towards wastes such as municipal wastes, industrial wastes majorly glycerol from brewery industry, waste vegetable oils and other carbonaceous wastes (Fakas *et al*., 2009a, b). This is so because the 75% cost of biodiesel production depends on the feedstock. Once the feedstock is economized then this process is no more far from commercialization in next few years. *Lipomyces starkeyi* was checked for its biodiesel production in starchy wastes from sweet potato processing industry as sole source of carbon by Patil (2010). The economic analysis resulted in the production of microbial lipids produced at a factory gate price of $2.30 per gallon. This could support the biodiesel price at $3.00 per gallon with a

continuous subsidy of $1.00 per gallon which is quite reasonable to become commercialized.

CONCLUSION

From the above discussion it is concluded that there is a massive need to become self-sufficient in biofuel production. This is so because the development and economy of every nation is a direct reflection of its energy sources and their useful exploitation. To attain the target, the feedstock should be abundant and cost-effective. Lignocellulosic and industrial wastes could be used in biodiesel production processes as they are produced in abundance and problem of their disposal will be solved by recycling them. Further SCO from yeasts are same as vegetable oils so they are proved to be appropriate pre-biodiesel oils. The fermentation processes could be made lucrative by overcoming the cost consuming steps like pretreatment of waste substrates. This could be done so by consortia between valuable yeast and cellulolytic bacteria or by using bacterially saccharified sugar syrups of wastes as biologically treated substrates to produce yeast origin biolipids. To take the biodiesel production upto maturation in Pakistan the need is just to provide the general awareness to the local industrialists for sustainable production of biodiesel at domestic levels.

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