Recent Advances in using *Lipomyces starkeyi* for the Production of Single-Cell Oil

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**Abstract**

The clean energy demand and limited fossil fuel reserves require an alternate source that is sustainable and eco-friendly. This demand for clean energy steered the introduction of biofuels such as bioethanol and biodiesel. The third-generation biodiesel is promising as it surpasses the difficulties associated with food security and land usage. The third-generation biodiesel comprises biodiesel derived from oil produced by oleaginous microbes. The term oleaginous refers to microbes with the ability to accumulate lipids to about 20% of the biomass and is found in the form of triacylglycerols. Yeasts can be grown easily on a commercial scale and are amenable to modifications to increase single-cell oil (SCO) productivity. The oleaginous yeast *L. starkeyi* is a potential lipid producer that can accumulate up to 70% of SCO of its cell dry weight under optimum conditions. Compared to other oleaginous organisms, it can be grown on a wide range of feedstock and a good part of the lipid produced can be converted to biodiesel. This review presents the recent advances in single-cell oil production from *L. starkeyi* and strategies to increase lipid production are analyzed.

**Keywords:** Lipid, *Lipomyces Starkeyi*, Oleaginous Microbes, Single-cell Oil

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INTRODUCTION

In the pursuit to achieve net zero emissions of greenhouse gases by 2050, the energy sector needs to switch to clean energy options to meet their demand. Currently, fossil fuels meet about 80% of the world’s demand and other energy sources are nuclear power, renewable sources and biofuels. On the other hand, the limited oil reserves also drive the implementation of alternative energy sources.

Biofuel is derived from biomass and the most established biofuels are bioethanol and biodiesel. It is estimated that the global biofuel market size will exceed nearly 201.21 billion US$ by 2030. Bioethanol (C2) is usually blended with gasoline (C4-C9) while biodiesel is a substitute in diesel engines without any engine modification. Bioethanol is synthesized by the alcoholic fermentation of sugars that are derived from the hydrolysis of biomass. Biodiesel is an excellent choice because of its renewability, safety to use in any diesel engine, high efficiency and engine durability. Moreover, it is nontoxic, nonflammable and has a greater biodegradability. The first-generation biodiesel has been commercialized and its current production is about 50 billion litres on a global scale. Though it has good combustion quality in IC engines, it is a threat to food security as produced from vegetable oil and animal fat. Usually, vegetable oil such as peanut oil, soybean oil, sunflower oil, corn oil, rice bran oil, palm oil, coconut oil, olive oil, and rapeseed oil are used as feedstock. Nevertheless, currently, about 95% of the biodiesel demand is met by first-generation biodiesel. As an alternative to first-generation biodiesel, second-generation biodiesel that is derived from nonedible oil sources emerged. It is mainly derived from cheap, inedible and unconventional sources such as crops (e.g. jatropha, mahua), inedible oil (e.g. jojoba oil), inedible sources (e.g. wood, husk, tobacco seed).

Second-generation biodiesel is recognized to be an efficient and eco-friendly alternative to first-generation biodiesel. However, the crops’ cultivation requirements of fertile land and other resources led to its limited implementation. Biodiesel derived from oil produced by oleaginous microbes led to the discovery of third-generation biodiesel. It offers advantages as it is a renewable source, eco-friendly, has no threat to food and land usage, and has ease in manipulation to high cellular lipid accumulation. However, it poses challenges in the production of inadequate biomass on a commercial scale, high investments required for facility and setup at large scale.

In oleaginous microorganisms (OM), over 20% of the dry weight constitutes lipid under stress conditions of high carbon and low nitrogen nutritional sources. The lipid accumulation can be achieved up to 70% or more with appropriate stress conditions to the microbes. OM are constituted by microbial families viz. bacteria, fungi, yeast, and microalgae. The oil obtained from OMs has equivalent composition, thermal properties and low viscosity as that of oil obtained from plant and animal sources. Oleaginous yeasts are frequently a superior choice for lipid production at a commercial scale because of the higher growth rate, lipid accumulation and productivity. About 160 native yeasts have been reported as oleaginous and the most studied oleaginous yeasts are Cutaneotrichosporon oleaginosus, Rhodotorula toruloides, Yarrowia lipolytica, Rhodotorula glutinis, L. starkeyi, Trichosporon oleaginosus, and Candida tropicalis.

This paper aims to present the recent advances in single-cell oil production from L. starkeyi for its potential as biodiesel feedstock and the benefits of L. starkeyi in sustainable development. Moreover, the opportunities in the strategies to increase lipid production are discussed.

Oleaginous Organisms

The single-cell oil refers to microbial oil and its first commercial production dates back to 1985 by the filamentous fungus Mucor circinelloides. The microbial lipids include triacylglycerols (TAG) and glycolipids. The energy reserves, sterol esters and phospholipids constitute the former while membrane constituents the latter. The TAG part has been identified as the major portion of SCO and it is chemically identical to vegetable oils. However, the amount and type of lipid composition (Table 1) depends on the genotype of the microbe, its culture conditions and the substrate employed. The oleaginous microbes are capable of exploiting low-cost feedstocks such as agro-industrial residues for a
higher lipid synthesis. The lipid accumulated can be converted to biodiesel by transesterification reactions that are catalyzed by acid/alkali/enzymes for the conversion to fatty acid methyl esters.

In oleaginous yeast, the lipid accumulation happens by de novo (from carbon substrate in nitrogen limitation) or ex novo (from a lipid source or hydrophobic substrates) pathways. High TAG accumulation is observed among yeast and fungi compared to the bacteria as the latter is composed of polyhydroxyalkanoates as storage molecules. Among 100 genera with about 1500 species of yeasts, about 30 have been reported to accumulate lipids excessive 25% of their dry biomass.

Microalgae as a source of microbial oil have been investigated recently and it holds advantages in easiness in cultivation and product manufacturing. Above all, the lipid obtained from algae is equivalent to vegetable oils for the saturated and low-unsaturated long-chain fatty acids content. The commercial production of algal lipids is challenging as the outdoor cultivation systems for photoautotrophic algae gave lower than 20% (dry base) of lipid content. In addition, the high moisture content of algae cultivated in open ponds or photobioreactors requires dewatering and drying equipment during the downstream processing. These additional investments make the overall cost of algal biofuel much higher.

**Lipomyces starkeyi**

The genus Lipomyces belongs to the Lipomycetaceae family and about 16 species have been recognized in the genus so far. Among the species, *L. starkeyi*, unicellular eukaryotic yeast, is

### Table 1. Lipid production by oleaginous microbes

<table>
<thead>
<tr>
<th>Microbe</th>
<th>Substrate</th>
<th>Biomass (g L⁻¹)</th>
<th>Lipid content (% w/w)</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Bacteria</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rhodococcus opacus PD630</td>
<td>Sugarcane molasses</td>
<td>12.6 ± 0.3</td>
<td>18.8</td>
<td>80</td>
</tr>
<tr>
<td>Streptomyces strains</td>
<td>Cellobiose</td>
<td>2.6</td>
<td>47</td>
<td>81</td>
</tr>
<tr>
<td>Bacillus subtilis HB1310</td>
<td>Cotton stalk hydrolysate</td>
<td>5.7</td>
<td>39.8</td>
<td>82</td>
</tr>
<tr>
<td>Rhodococcus opacus</td>
<td>Biomass gasification wastewater supplementation with mineral salt media</td>
<td>-</td>
<td>62.8</td>
<td>83</td>
</tr>
<tr>
<td><strong>Yeast</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Rhodosporidium toruloides</em> CBS 14</td>
<td>40% hemicellulosic hydrolysate</td>
<td>19.4±0.0</td>
<td>54.6±1.5</td>
<td>84</td>
</tr>
<tr>
<td><em>L. starkeyi</em></td>
<td>Cassava starch</td>
<td></td>
<td>18.7</td>
<td>60</td>
</tr>
<tr>
<td>Cryptococcus curvatus</td>
<td>Cyanobacterial biomass</td>
<td>2.3</td>
<td>14.3</td>
<td>85</td>
</tr>
<tr>
<td>Yarrowia lipolytica</td>
<td>Crude glycerol prepared in Seawater</td>
<td>11</td>
<td>38</td>
<td>86</td>
</tr>
<tr>
<td><strong>Filamentous fungi</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mortierella (Umbelapsis) isabellina</td>
<td>glucose and xylose</td>
<td>28.8</td>
<td>61.0</td>
<td>87</td>
</tr>
<tr>
<td>Mortierella isabellina NRRL 1757</td>
<td>Glycerol</td>
<td>7.24±0.37</td>
<td>27.48</td>
<td>88</td>
</tr>
<tr>
<td>Mortierella wolfii AH12</td>
<td></td>
<td>3.81</td>
<td>41.2</td>
<td>89</td>
</tr>
<tr>
<td>Cunninghamella echinulata</td>
<td></td>
<td>10</td>
<td>51.3</td>
<td>90</td>
</tr>
<tr>
<td><strong>Microalgae</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dunaliella parva</td>
<td>Sweet sorghum bagasse</td>
<td>4.85</td>
<td>39.08</td>
<td>91</td>
</tr>
<tr>
<td>Chlorella vulgaris</td>
<td></td>
<td>3.44</td>
<td>40</td>
<td>92</td>
</tr>
<tr>
<td><em>Botryococcus braunii</em> EMS-mutant E1.0H15</td>
<td></td>
<td>3.30*</td>
<td>34.49*%</td>
<td>93</td>
</tr>
<tr>
<td>Chlorella minutissima CM7</td>
<td></td>
<td>2.27-3.48 **</td>
<td>32.01- 8.65**</td>
<td>94</td>
</tr>
</tbody>
</table>

* Single stage cultivation, ** double stage cultivation
the most widely studied because of its potential for lipid synthesis. *L. starkeyi* was isolated by R. L Starkey (strain number 74) from the soil in the USA. L. starkeyi grows in glucose mineral medium at pH=5 and biotin supplementation is required at pH 5.5 to 6.5. Biotin enhances cell growth and its synthesis is inhibited at pH more than 5.37 In the fermentation media, ions such as $\text{Mg}^{2+}, \text{Mn}^{2+}$, and $\text{Zn}^{2+}$ are required for cell growth and metabolism, $\text{Cu}^{2+}$ and $\text{Fe}^{2+}$ are required as cofactors and phosphate and sulphate are vital for structural components and cell physiology respectively.38 A biomass yield of 1.6 fold was achieved with an appropriate amount of $\text{Mn}^{2+}$ whereas a high lipid yield was obtained at a lower concentration of $\text{Zn}^{2+}$.39

### Table 2. Feedstocks used for SCO production

<table>
<thead>
<tr>
<th>Strain</th>
<th>Feedstock</th>
<th>Dry cell weight gL$^{-1}$</th>
<th>Lipid content %</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>L. starkeyi</em></td>
<td>Potato starch wastewater</td>
<td>2.59</td>
<td>8.88</td>
<td>95</td>
</tr>
<tr>
<td><em>L. starkeyi</em></td>
<td>Crude glycerol</td>
<td>9.1</td>
<td>46.2</td>
<td>96</td>
</tr>
<tr>
<td><em>L. starkeyi</em></td>
<td>WWS hydrolysate</td>
<td>8.2</td>
<td>42.7</td>
<td>96</td>
</tr>
<tr>
<td><em>L. starkeyi</em> DSM 70296</td>
<td>Hemi-cellulose hydrolysate</td>
<td>85.4</td>
<td>48.9</td>
<td>52</td>
</tr>
<tr>
<td><em>L. starkeyi</em> JAL 581</td>
<td>Cheese whey</td>
<td>4.26±0.07</td>
<td>3.27</td>
<td>97</td>
</tr>
<tr>
<td><em>L. starkeyi</em></td>
<td>Olive mill wastewater</td>
<td>2.7</td>
<td>17.8</td>
<td>98</td>
</tr>
<tr>
<td><em>L. starkeyi</em></td>
<td>Olive mill wastewater+30 gL$^{-1}$ of glucose</td>
<td>10.9</td>
<td>34.5</td>
<td>98</td>
</tr>
<tr>
<td><em>L. starkeyi</em></td>
<td>Glycerol</td>
<td>5.74 ± 0.32</td>
<td>50.49 ± 0.99</td>
<td>99</td>
</tr>
<tr>
<td><em>L. starkeyi</em></td>
<td>Rice bran hydrolysate</td>
<td></td>
<td>40 - 65</td>
<td>100</td>
</tr>
<tr>
<td><em>L. starkeyi</em> DSM 70296</td>
<td>Crude glycerol</td>
<td>9.0</td>
<td>32.7</td>
<td>101</td>
</tr>
<tr>
<td><em>L. starkeyi</em> DSM 70296</td>
<td>Crude hydrolysates from Flour-rich waste, by-product streams generated by bakery, confectionery and wheat milling plants</td>
<td>30.5</td>
<td>40.4</td>
<td>102</td>
</tr>
<tr>
<td><em>L. starkeyi</em> DSM 70296</td>
<td>Crude hydrolysates from Flour-rich waste, by-product streams generated by bakery, confectionery and wheat milling plants * Fed-batch mode</td>
<td>109.8</td>
<td>57.8</td>
<td>102</td>
</tr>
<tr>
<td><em>L. starkeyi</em> DSM 70296</td>
<td>Brazilian molasses</td>
<td>21.27</td>
<td>32</td>
<td>103</td>
</tr>
<tr>
<td><em>L. starkeyi</em></td>
<td>Olive mill</td>
<td>25</td>
<td>24–28</td>
<td>104</td>
</tr>
<tr>
<td><em>L. starkeyi</em></td>
<td>hemicellulosic hydrolysate (HH) from sugarcane bagasse</td>
<td>9.6*</td>
<td>26.1*</td>
<td>105</td>
</tr>
<tr>
<td><em>L. starkeyi</em> NBRC10381</td>
<td>Glucose</td>
<td>30.3</td>
<td>79.6</td>
<td>49</td>
</tr>
<tr>
<td><em>L. starkeyi</em> NBRC10381</td>
<td>Glucose + xylose</td>
<td>38.2</td>
<td>83.6</td>
<td></td>
</tr>
<tr>
<td><em>L. starkeyi</em> NBRC10381</td>
<td>Xylose</td>
<td>28.7</td>
<td>85.1</td>
<td></td>
</tr>
<tr>
<td><em>L. starkeyi</em> NBRC1038</td>
<td>oil palm trunks</td>
<td>27.7</td>
<td>55.2</td>
<td>106</td>
</tr>
<tr>
<td><em>L. starkeyi</em> NBRC1038</td>
<td>regular sap</td>
<td>30.1</td>
<td>64.4</td>
<td></td>
</tr>
<tr>
<td><em>L. starkeyi</em> NBRC1038</td>
<td>Sap+ mineral media</td>
<td>28.1</td>
<td>63.1</td>
<td></td>
</tr>
</tbody>
</table>

*Batch , ** Continuous

### Fermentation Strategies For Sco Production

**Fermentation feedstock**

Agro-industrial residues are rich in lignocellulosic biomass and are an abundant natural polymer to serve as a fermentation substrate. Pretreatment of the cellulosic biomass is required to make it accessible to the hydrolytic enzymes of the microbes. Pretreatment leads to the solubilization or separation of cellulose, hemicellulose and lignin to facilitate the digestion of lignocellulosic material.40 Approaches for the pretreatment include chemical, mechanical, and biological methods and their different combinations.41 The pretreatment methods influence the long-term storage of the biomass, the concentration of the pretreated biomass and creation of inhibitors in the medium.42 An
alternative method employing ionic liquid for the pretreatment of lignocellulosic biomass has proven successful. A study on employing seawater-based ionic liquid in the pretreatment of lignocellulosic biomass concluded that the use of seawater renders no negative effect on the pretreatment as well as the enzymatic hydrolysis of biomass obtained. It yielded 54–72% of reducing sugar and lipid yield of 4.5 g L\(^{-1}\) after cultivation of *Trichosporon fermentans* on wheat straw hydrolysate. The metal ions present in the feedstock also influences the lipid formation as observed by Zhang et al. in the utilization of municipal wastewater sludge as the feedstock in fermentation. The presence of Cd\(^{2+}\) in the fermentation reduced the lipid content from 51% to 41%. However, on the removal of metals from the sludge, lipid content was about half of the one with metals. This is due to the removal of metal ions such as Zn\(^{2+}\) that enhanced lipid accumulation.

*L. starkeyi* can assimilate a wide range of feedstock as the substrate for lipid production and various feedstock have been reported (Table 2). In the production cost of biodiesel, more than 70% is contributed by the raw materials used and the use of cheap feedstock can greatly reduce this cost. A large amount of raw glycerol produced during the process can also be recycled sustainably by using it as a substrate for fermentation.

**Enhancement of SCO production by *L. starkeyi***

**Fermentation strategies**

**Biphasic fed-batch fermentation strategy**

For *L. starkeyi* ATCC 56304, the biphasic system with a supply of glucose during the growth phase and xylose (at 120h) during the lipid accumulation phase resulted in 0.13 g L\(^{-1}\) h\(^{-1}\) oil productivity. It is higher than the fermentation with carbon source as glucose (0.06 g L\(^{-1}\) h\(^{-1}\)), xylose (0.12 g L\(^{-1}\) h\(^{-1}\)) and mixed sugars (glucose: xylose at 1:1)(0.09 g L\(^{-1}\) h\(^{-1}\)) in a single phase. However, both mixed (carbon) and biphasic cultures resulted in similar productivity (0.14 g L\(^{-1}\) h\(^{-1}\)) at a longer fermentation time. The mixed carbon sources (glucose and xylose) in a nitrogen-limited mineral medium resulted in the highest lipid content (84.9%) compared to the fermentation using a single carbon source using *L. starkeyi* NBRC10381.

**Mode of fermentation**

The carbon to nitrogen ratio (C: N ratio, mol mol\(^{-1}\)) in the media is vital in determining the cellular metabolism state of oleaginous yeast. Fed-batch fermentation is often followed to maintain the cells in the growth phase initially and subsequently in an oil accumulation phase. Fed-batch cultivation of *L. starkeyi* yielded an oil content of 27% while batch cultivation resulted in 23.7%. In a fed-batch study with two feedings using *L. starkeyi*, oil accumulated increased from 0.05 g g\(^{-1}\) to 0.11 g g\(^{-1}\) compared to the batch mode cultivation. A similar dependence as of low C: N ratio was observed for *L. starkeyi* for growth and lipid accumulation at a higher agitation rate. As in the case of nitrogen limitation in the culture media, oxygen limitation enhances lipid accumulation although it decreases growth in *L. starkeyi*.

The repeated batch cultivation of *L. starkeyi* DSM 70296 resulted in high cell (85.4 g L\(^{-1}\)) and lipid concentration (41.8 g L\(^{-1}\)) compared to the other cultivation modes such as batch, fed-batch, and continuous cultures with glucose and xylose as substrate. In the same study, the continuous cultivation (dilution rate = 0.03 h\(^{-1}\)) with hemi cellulose hydrolysate resulted in high biomass and lipid yield compared to media with glucose and xylose.

The substrate-feeding strategy affects cell growth and lipid production. As observed in study by Amza et al. that *L. starkeyi* D35 (Ls-D35 strain) achieved high-density culture on feeding with mixed glucose and xylose as substrates (0.15 w/w substrate) after 96 h while high lipid accumulation was observed in single xylose feeding (0.13 w/w substrate) after 120 h.

**Mixed culture of the microbes**

**Co-culturing oleaginous yeasts and microalgae**

The mutualistic interaction between oleaginous yeast and microalga has been greatly explored for the production of metabolites. The microalga synthesizes oxygen for the yeast, whereas yeast generates carbon dioxide for the microalga. Additionally, microalga converts the dissolved carbon dioxide in the medium to bicarbonate that on consumption, releases OH– ions and converts media to alkaline. On contrary, yeast growth makes the medium acidic.
The co-cultivation of *L. starkeyi* and *Chloroidium saccharophilum* resulted in lipid accumulation of 0.064, 0.064 and 0.081 g lipid·g biomass⁻¹ when grown on YEG (Yeast extract, glucose, (NH₄)₂SO₄, MgSO₄·7H₂O, KH₂PO₄), BBM + G (Bold Basal Medium+ glucose) and medium with *Arundo donax* hydrolysate respectively.⁵⁶

The symbiotic relationship between microalga *Chlamydomona reinhardtii* and *L. starkeyi* was demonstrated as algae growth was observed in media in which, the organic carbon in the feedstock was utilized in the absence of air showing the dependence on the CO₂/O₂ exchange between the two organisms.⁵⁶ Co-culturing of *L. starkeyi* and microalgae (wastewater native majorly *Scenedesmus* sp. and *Chlorella* sp.) with a 2:1 inoculum ratio was utilized for lipid production from urban wastewater. The easily assimilated organic substrates in the wastewater were absorbed during the first 3 days of fermentation and it limited the yeast growth. However, it resulted in 15% lipid accumulation at the end of cultivation time.⁵⁷

**Co-culturing oleaginous yeasts and bacterium**

The mutual relationship between yeast and bacterium results in the improvement of metabolic activities leading to enhanced biomass production and lipid accumulation. Karim et al.⁵⁸ co-cultured yeast (*L. starkeyi*) and bacterium (*Bacillus cereus*) for the simultaneous lipid production and palm oil mill effluent treatment. After the optimization of process parameters using statistical tools, lipid accumulation and COD removal efficiency was observed as 2.95gL⁻¹ and 86.54%, respectively. In a separate study, the synergistic relationship between yeast (*L. starkeyi*) and a bacterium (*Bacillus cereus*) resulted in high lipid accumulation. The co-culture on palm oil mill effluents resulted in 25.53% lipid yield and was greater than monoculture.⁵⁹

**Consolidated bioprocessing**

In the utilization of agro-industrial residues in lipid synthesis, a consolidated bioprocessing strategy proved to be technically and economically feasible. In this approach, *L. starkeyi* fermentation exhibited a starch hydrolysis mechanism as well as lipogenesis with a yield of 18.7% (w/w) of lipid with cassava starch as the substrate.⁶⁰ In the utilization of rice straw as the substrate, a consolidated bioprocessing consisting of *L. starkeyi* and *Aspergillus oryzae* resulted in lipid accumulation of 8.5 g/100 g oven-dry weight of rice straw. The rice straw was pretreated with lime at conditions of Ca(OH)₂ concentration of 12 gL⁻¹ and hydrolysis temperature of 110°C within 60 min.⁶¹

For the conversion of lignocellulose to lipid by *L. starkeyi*, the deficiency of β-glucosidase contributed to the utilization of cellulose and hence facilitated the simultaneous saccharification and enhanced lipid production.⁶² The two-step process utilizing the cellulosic paper mill waste as the substrate resulted in lipid accumulation of 37 wt%. In the first step, enzymatic hydrolysis (Celic® CTec2 25 FPU/g glucan, 48 h, biomass loading 20 gL⁻¹) yielded hydrolysates containing glucose and xylose. Subsequently, *L. starkeyi* was cultivated on the undetoxified hydrolysate in the second step.⁶³

**Lipid yield in the conversion of wastewater sludge is low as the limited availability of easily consumed nutrients in the sludge. The increase in soluble chemical oxygen demand after the pretreatment increased lipid accumulation. Lipid accumulation on the pretreated sludge on cultivation with *L. starkeyi* was 36.67% g/g, 18.42%, 21.08% and 26.31% for ultrasonication pretreatment, acid pretreatment, alkaline pretreatment and microwave pretreatment, respectively.⁶⁴**

**Two-stage cultivation**

In two-stage cultivation, cell growth and lipid production are spatially separated so that independent optimization of both stages can be achieved. It is a cost-effective approach with a relatively low C/N ratio during the first stage followed by a second stage with a relatively high C/N ratio for the fermentation of *L. starkeyi*. The nitrogen limitation during the second stage induces lipid accumulation. A two-stage fermentation using *L. starkeyi* AS 2.1560 with non-sterile glucose solution without additional nutrients resulted in 64.9% of lipid yield.⁶⁵ In another study, the fermentation media composed of 50% YPD + 50% Orange peel exhibited larger internal droplets in
the yeast cells and two-stage operations increased the lipid yield by 18.5-27.1%. The co-fermentation of glucose and xylose using *L. starkeyi* AS 2.1560 in a two-stage fermenter under unsterile conditions was studied by Liu. The fed-batch operation in the second stage with co-utilization of non-sterile lignocellulose-derived sugars accumulated high lipid (63.8%) after 46h of incubation. High cell density fermentation by fed-batch mode using *L. starkeyi* AS 2.1560 on unsterile xylose demonstrated 65.5% lipid content after 48h incubation. In the two-stage cultivation of *L. starkeyi* InaCC Y604 in a nitrogen-limited mineral medium, a mixed carbon source (glucose and xylose) resulted in the highest cell biomass compared to the single carbon source. However, the highest lipid accumulation (65.05% (w/w)) was observed when cellobiose was the carbon source.

**Immobilization**

*L. starkeyi* DSM 70296, immobilized on de-lignified porous cellulose with 30°C and pH 5.0 as the optimum conditions resulted in enhanced SCO production. In glucose media, the lipid accumulation was increased by 44% while 85% enhanced lipid accumulation was achieved in agro-industrial waste suspensions (orange juice and molasses) based media compared to free cell cultures.

**Integrated cascade bioprocesses**

Giant reed (*Arundo donax* L) as the substrate for SCO production has been studied by Fidio. The lignocellulosic feedstock from perennial grasses was treated by microwave-assisted hydrolysis and enzymatic hydrolysis. *L. starkeyi* DSM 70,296 cultivation on both the detoxified and partially-detoxified hydrolysates in the integrated cascade process achieved about 8 g SCO from 100 g biomass. Sugar cane bagasse on acid hydrolysis in the Parr reactor resulted in hemicellulose fraction (about 82% conversion) that on cultivation with *L. starkeyi* resulted in a 27.8% (w/w) lipid content.

**Genetic engineering approach**

Genetic engineering and metabolic approaches are widely used for improved lipid production by yeasts. The technique is to increase the metabolic flux rate by overexpression of enzymes associated with acyl-CoA synthesis and Kennedy pathways from glycerol-3-phosphate to TAGs. An alternative to enhance lipid accumulation is to inhibit the b-oxidation. Transformation methods based on metabolic engineering reported to enhance lipogenesis as well as in the synthesis of high-value metabolites. The electroporation procedure in the transformation of *L. starkeyi* was shown to be a better procedure compared to LiAc-mediated transformation and PEG-mediated spheroplast transformation methods in terms of transformation efficiency and time consumption, respectively. Agrobacterium-mediated transformation in *L. starkeyi* is an effective method for homologous recombination as well as expression of heterologous genes in *L. starkeyi.*

**Mutation**

Mutating the oleaginous yeast using techniques such as ethyl methanesulfonate (EMS) treatment and UV irradiation has been reported to increase lipid production. The selection of mutants after mutagenesis is achieved by methods such as cerulenin selection, Sudan Black B staining, and Percoll density gradient centrifugation. UV irradiation mutation of *L. starkeyi* E15 resulted in mutants with enhanced lipid accumulation compared to that of EMS-induced mutation. The lipid accumulation was observed as 32.0%, 44.2% and 68.1% for the wild-type, E15 strain and highest lipid-producing mutant (E15-15), respectively. In the UV irradiation mutation of *L. starkeyi* E15, three mutants, namely E15-11, E15-15, and E15-25, accumulated particularly higher TAG levels than their counterparts. The amounts of TAG per dry mass of the wild-type and E15 strains were 32.0% and 44.2%, respectively, on day 3, whereas those of E15-11, E15-15, and E15-25 on day 3 were 57.4%, 68.1%, and 60.5%, respectively. A higher TAG accumulation was observed on UV irradiation than that of EMS treatment mutation.

**Challenges and future research scope**

Presently, the high operating expense is a challenge in the industrialization of single-cell oil production by *L. starkeyi*. For an economically advantageous process, the lipid yield and productivity need to be further enhanced. A
metabolic engineering approach that targets potential genes can be an approach to enhance lipid production. Extensive research focusing on metabolic alterations should be undertaken. Using competent genetic tools, the possible gene targets in lipid synthesis can be studied. Recent techniques such as CRISPR/Cas9-based genome editing technology can be utilized in the improvement of lipid production.

CONCLUSION

Single-cell oil produced by oleaginous yeast is a non-plant type and renewable source that is used for biodiesel production. *L. starkeyi* is an excellent lipid producer and can assimilate a wide range of feedstock. The enhancement of lipid production is achieved by different strategies such as maintenance of a high C/N ratio, optimization of nutritional and process parameters, two-stage cultivation, mixed culture of microbes and genetic engineering. Metabolic engineering-based genetic modifications of the yeast *L. starkeyi* can result in even greater lipid production for the biodiesel industry.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

AUTHORS’ CONTRIBUTION

Both authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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DATA AVAILABILITY

All datasets generated or analyzed during this study are included in the manuscript.

ETHICS STATEMENT

Not applicable.

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