Lipid and gamma-linolenic acid production by two oleaginous fungi from potato processing wastewater

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Abstract
This study examined the utilization of potato processing wastewater as the feedstock by two oleaginous fungi, *Aspergillus flavus* I16-3 and *Mucor rouxii*, for lipid and gamma-linolenic acid production. The high strength wastewater contained soluble carbohydrates of 36.2 g/l, soluble chemical oxygen demand (COD) of 40 g/l, total soluble nitrogen of 0.62 g/l, and soluble total phosphorus of 0.24 g/l. Its total carbohydrates and total soluble nitrogen ratio was 72.4. Without addition of any external nutrients, 3.3 g/l and 4.1 g/l of lipids were produced by *Aspergillus flavus* I16-3 and *Mucor rouxii*, respectively, with corresponding maximum GLA yields of 132 mg/l and 180 mg/l. *Aspergillus flavus* I16-3 and *Mucor rouxii* reduced COD from the wastewater by 60% and 90%, total soluble nitrogen by 100% and 98%, and total soluble phosphorus by 92% and 81%, respectively. Thus the oleaginous fungi can produce lipids and GLA from the potato processing wastewater as well as efficiently remove COD and nutrients.

Keywords: *Aspergillus flavus* I16-3, Gamma-linolenic acid, Lipid, *Mucor rouxii*, Potato processing wastewater

Introduction
Potato is the primary carbohydrate source at main meals in Ireland. The crop was grown over an area of 20,000 - 30,000 hectare in 2008 (Department of Agriculture, Fisheries and Food, Ireland, 2008). Potato processing industries producing processed foods like chips, peeled potatoes, etc. generate a large amount of potato processing wastewater. Starch is the main organic compound in potato processing wastewater and is responsible for high chemical oxygen demand (COD), biochemical oxygen demand (BOD₅) and suspended solids (SS) (Koby et al., 2006). The wastewater can cause environmental pollution if without proper treatment. Different methods, such as aerobic and anaerobic biological biotechnologies, lagoons, land applications and electrocoagulation, have been used or developed to treat the potato processing wastewater (Koby et al., 2006; Gelinas et al., 2007). However, these processes are only focused on the removal of pollutants and do not recycle the wastewater as a resource.

Potato processing wastewater has been studied for producing value-added products, such as biogas, lactic acid, and other related products (Abu et al., 2000; Huang et al., 2005; Gelinas et al., 2007). Its starch content has a high potential for microbial lipids and gamma linolenic acid (GLA) production. Microbial lipids are rich in polyunsaturated fatty acids and have been used as food supplements and nutraceuticals for decades (Horrobin 1992). Members of lower fungi belonging to the order Mucorales are known for accumulating GLA (Gema et al., 2002). It is known that the physiology of oleaginous fungi requires high carbon and limited nitrogen sources (namely high C:N ratios) for lipid production (Ratlledge 2004).
However, high costs of carbon sources make the technology less economically competitive (Meng et al., 2009); thus search for low cost raw materials is very important (Papanikolaou et al., 2010). This study aims at using the potato processing wastewater for lipids and GLA production by two oleaginous fungi - Aspergillus flavus I16-3 and Mucor rouxii, in addition to treatment of this wastewater.

**Methods**

**Microorganisms**

Aspergillus flavus I16-3, an oleaginous, amylolytic, and neutrophilic fungus, was isolated from Irish soils by the authors. Mucor rouxii was obtained from German Collection of Microorganisms and Cell Cultures (DSMZ, Germany). Both the cultures were maintained on potato dextrose agar slants at 4 °C.

**Inoculums preparation**

1.0 g of fungal mycelium grown on potato dextrose plates was inoculated in a shaker incubator at 28±2 °C at 180 rpm in yeast extract malt extract agar (YM agar) broth containing (in g/l): glucose, 10; peptone, 5; yeast extract, 3; malt extract, 3. After 48 h the culture was harvested and homogenized using sterile glass beads (0.5 mm in diameter). 0.8 ml of the homogenized mycelium was used as the inoculum.

**Potato processing wastewater**

The potato processing wastewater was collected from the Glynn fruit and vegetable processing plant, Galway, Ireland, and was stored in the lab at 4 °C. The wastewater contained the peeled potato skin, soil particles, and potato solids.

The characteristics of the potato processing wastewater are described in Table 1. The wastewater had high carbohydrates and low nitrogen contents with the total carbohydrates: total soluble nitrogen ratio of 72, which would favour lipid accumulation by the oleaginous fungi.

<table>
<thead>
<tr>
<th>Item</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total solids (TS)</td>
<td>6.2 g/l</td>
</tr>
<tr>
<td>Volatile solids (VS)</td>
<td>2.2 g/l</td>
</tr>
<tr>
<td>Total carbohydrates</td>
<td>36.2 g/l</td>
</tr>
<tr>
<td>Starch</td>
<td>32.6 g/l</td>
</tr>
<tr>
<td>Total soluble nitrogen</td>
<td>0.5 g/l</td>
</tr>
<tr>
<td>Total soluble phosphorus</td>
<td>0.24 g/l</td>
</tr>
<tr>
<td>Soluble COD</td>
<td>40 g/l</td>
</tr>
<tr>
<td>pH</td>
<td>4.5-5.5</td>
</tr>
</tbody>
</table>

pH of the potato processing wastewater was 4.5-5.5, which was slightly lower than the optimum value of 5.5-7.0 for lipid accumulation (Wu et al., 2011).

**Cultivation conditions**

Cultivation of A. flavus I16-3 and M. rouxii on potato processing wastewater for lipid production was studied under two conditions - the raw wastewater and the wastewater added with extra nutrients (Synthetic wastewater). 50 ml of properly mixed potato processing wastewater (raw and synthetic) was added in 250 ml conical flasks and autoclaved at 121 °C for 20 min. The pH of the wastewater was adjusted to 6.0±0.5 using 1 N NaOH before sterilization and then confirmed after sterilization. A synthetic wastewater was made by adding extra nutrients (10 ml) to the raw wastewater (40 ml); the nutrients added to the raw wastewater were (in g/l): Yeast extract, 0.5; CaCl₂, 0.1; KH₂PO₄, 2.5; FeSO₄, 0.02; NH₄Cl, 0.01; MgSO₄, 0.5; MnSO₄, 0.003; CuSO₄, 0.002. pH of the synthetic wastewater was adjusted to 6.0±0.5. 0.8 ml of the
homogenized mycelium was added to these flasks and incubated at 28±2 °C in an incubator shaker at 180 rpm for 10 days.

Analytical methods
Flasks were periodically removed from the incubator and subjected to analysis. Samples were centrifuged at 5000 rpm for 10 min. The supernatant was used for analysing pH, the amylase activity, and the concentrations of reducing sugars, COD, total carbohydrate, total soluble nitrogen (TSN) and total soluble phosphorus (TSP). Lipid was extracted from dried biomass according to (Bligh et al., 1959) method using 2:1 (v:v) chloroform and methanol mixture as the extractant and the soxhlet apparatus (Sarstedt, Germany). Fatty acid methyl ester extraction was performed via the direct transesterification method (Lewis et al., 2000). Concentrations of GLA were measured using gas chromatography (Fakas et al., 2009). COD was measured according to standard APHA methods (American Public Health Association. et al., 1960). Total carbohydrates were measured using the phenol sulphuric acid method (Dubois et al., 1956). Reducing sugars and amylase were determined using the dinitrosalicylic acid method (Miller, 1972). TSN and TSP were measured by the nitrogen and phosphorus kits (Hach Lange, Ireland) according to the supplier’s protocol.

Results
Lipid production and carbohydrate utilization by A. flavus I16-3 and M. rouxii
Aspergillus flavus I16-3 started to accumulate lipids two days after incubation and the lipid yield reached the maximum of 3.3 g/l in raw wastewater condition and 4.1 g/l in the synthetic wastewater with extra nutrients in Day 5 (Fig. 1). M. rouxii also presented a high lipid accumulation capacity under both conditions and started to accumulate lipids three days after incubation. In Day 5 the lipid yield reached the peak values of 4.1 g/l under the raw wastewater condition and 5.1 g/l under the synthetic wastewater condition (Fig. 2). These results show that potato processing wastewater can be an efficient alternative raw material for microbial lipid production. Occurrence of reserve lipid turnover was not observed in Aspergillus flavus I16-3. Whereas, M. rouxii utilized its assimilated lipid after day 5 when the soluble carbohydrate level was zero. Both the fungi utilized a significant amount of carbohydrates (Fig. 1 and 2). The soluble carbohydrate concentrations decreased from about 36 g/l to about 7 g/l on day 6 under both conditions for Aspergillus flavus I16-3. For M. rouxii, carbohydrates were nearly utilized on day 3, indicating that M. rouxii can uptake more carbohydrates than Aspergillus flavus I16-3. The reason that no reserve lipid turnover by Aspergillus flavus I16-3 may be due to the surplus carbohydrates in the medium after day 7.
GLA production by *Aspergillus flavus* I16-3 and *Mucor rouxii*

A comparison of GLA production in the raw and synthetic wastewater by the two oleaginous fungi was given in Table 2. GLA production rose with the fermentation time. The maximum GLA yield (60 mg/l) by *Aspergillus flavus* I16-3 was observed during the stationary phase. The synthetic wastewater led to increased GLA yields to 72 mg/l.

GLA percentages were 2% and 1.7% (Table 2.) in lipids of *Aspergillus flavus* I16-3 in the raw wastewater and the synthetic wastewater, respectively. The GLA percentages were higher in *Mucor rouxii* (4.8% and 5.7%, respectively) under the raw wastewater and the synthetic wastewater conditions. The maximum yield of GLA per gram of starch consumption was 7.7 and 9.8 mg/g for *Aspergillus flavus* I16-3, and 35.8 and 307.7 mg/g for *Mucor rouxii* from the raw wastewater and the synthetic wastewater, respectively.

Reduction in COD, total soluble nitrogen and total soluble phosphorus

*Aspergillus flavus* I16-3 reduced the soluble COD concentration in the raw wastewater from 40 g/l to 14 g/l as a result of 7 days fermentation. Addition of extra nutrients into the raw wastewater did not enhance COD removal with the final soluble COD of 16 g/l). *M. rouxii* was able to reduce COD to 12 g/l in the raw wastewater and its efficiency was increased when treating the synthetic wastewater, with the final soluble COD concentration of 4 g/l (Fig. 3).
Fig. 3. Total soluble COD reduction by *Aspergillus flavus* I16-3 (T1, T2) and *Mucor rouxii* (T3, T4). (T1 and T3: raw wastewater; T2 and T4: synthetic wastewater)

*Aspergillus flavus* I16-3 reduced the TSN by 98% from the raw wastewater, and removed it completely when treating the synthetic wastewater. The nitrogen removal efficiency of *Mucor rouxii* was 95% from the raw wastewater and 98% from the synthetic wastewater (Fig. 4).

Fig. 4. Removal of total soluble nitrogen by *Aspergillus flavus* I16-3 (T1, T2) and *Mucor rouxii* (T3, T4) (T1, T2, T3 and T4 are defined as in Fig. 3)

Changes in pH values during the whole incubation period were monitored. For *Aspergillus flavus* I16-3, pH slightly increased in the first two days and then decreased gradually to the final values of 5.3 and 4 at the end of the experiment in the raw wastewater and the synthetic wastewater, respectively (Fig. 6).

*Aspergillus flavus* I16-3 reduced the TSN by 98% from the raw wastewater, and removed it completely when treating the synthetic wastewater. The nitrogen removal efficiency of *Mucor rouxii* was 95% from the raw wastewater and 98% from the synthetic wastewater (Fig. 4). Regarding removal of TSP, both the fungi seemed to be less efficient in phosphorus removal than nitrogen. The phosphorus removal efficiency in raw wastewater of *Aspergillus flavus* I16-3 was 84% and 92% from the raw wastewater and the synthetic wastewater. *Mucor rouxii* reduced 85% phosphorus from raw wastewater and 81% from the synthetic wastewater (Fig. 5).

Fig. 5. Removal of total soluble phosphorus by *Aspergillus flavus* I16-3 (T1, T2) and *Mucor rouxii* (T3, T4) (T1, T2, T3 and T4 are defined as in Fig. 3)

Changes in pH values during the whole incubation period were monitored. For *Aspergillus flavus* I16-3, pH slightly increased in the first two days and then decreased gradually to the final values of 5.3 and 4 at the end of the experiment in the raw wastewater and the synthetic wastewater, respectively (Fig. 6). Reduction in pH during fermentation are due to secretion of organic acids such as citric acid (a fatty acid synthesis intermediate) and volatile fatty acids. On the contrary, for *Mucor rouxii*, pH gradually increased to about 8 until day 10 of fermentation. Similar results were reported when oleaginous fungi were grown on orange peel, that the pH of the medium was stable for few days and then increased until the end of fermentation (Gema *et al.*, 2002). However, the reason for pH increase has not been known.
Discussion and Conclusions

The potato processing wastewater contains high concentrations of nutrients and has not been recycled to produce products of added value in Ireland (Wijngaard et al., 2011). Utilizing the nutrients for lipids and GLA production will provide an alternative approach to recycle the wastewater. The high total carbohydrates: N ratio of 72, in addition with high concentrations of other essential nutrients, enabled these two fungi to grow and produce lipids. Without addition of any external nutrients a significant amount of lipids was produced (3.3 g/l) and (4.1 g/l) by Aspergillus flavus I16-3 and Mucor rouxii, respectively. This shows that raw potato wastewater alone would be an efficient feedstock for low cost microbial lipid production. In recent years, microbial lipids are considered to be potential candidates for biodiesel production (Meng et al., 2009). Reducing the lipid production cost has been given lots of attention by many researchers (Zong-bao 2005; Huang et al., 2009; Xue et al., 2010). This study gives a positive solution of using potato processing wastewater to reduce the microbial lipid production cost since the raw material is available free of cost. Further increase in the lipid yields (4.1 and 5.1 g/l) was achieved when adding nutrients to the raw wastewater. This can be explained as follows: added nutrients to the wastewater might support the metabolism of lipid synthesis or favoured the growth of fungi; this is confirmed with the increased uptake of total carbohydrates by both the oleaginous fungi in the wastewater with extra nutrients.

Ability of these fungi to produce GLA was also observed, with the yields of 60 mg/ L and 70 mg/l by Aspergillus flavus I16-3 from the raw wastewater and the synthetic wastewater, respectively. This yield was comparable to other mucaraceous fungi. Strains of Mortierella spp. produced 2.6-4.9 mg/l/h of GLA when grown on low cost substrates (Hansson et al., 1988). However, the GLA in lipids for Aspergillus flavus I16-3 was only 2% with the raw wastewater as the medium and was reduced to 1.7 % with the synthetic wastewater. The lipid yield of Aspergillus flavus I16-3 was higher under the synthetic wastewater condition, so the GLA content in lipids would be decreased (Gema et al., 2002). In addition the content of GLA in lipids was not increased significantly by prolonging the fermentation time after day 7 because there was no lipid turnover occurred in Aspergillus flavus I16-3 (Fakas et al., 2007). On the other hand Mucor rouxii produced much higher yields of GLA, with the yield of 100 and 120 mg/l from the raw wastewater and the synthetic wastewater, respectively, confirming that lower fungi belonging to the order of Mucorales are efficient GLA producers. In addition the amount produced by Mucor rouxii is comparable to other strains in the literature. Mucor rouxii CBS416.77 cultivated on cheap nitrogen and carbon sources produced 2.9-4.6 mg/l/h of GLA (Lindberg et al., 1991) and Mucor rouxianus CBS120-08 cultivated on glucose produced 80 mg GLA/L of medium (Kavadia et al.,...
The present study shows that high GLA yields could be obtained when *Mucor rouxii* was cultivated on potato processing wastewater. Again the GLA percentage in lipids was low (4.8% and 5.7%) compare to other cultures because of the higher lipid yields. However, in *Mucor rouxii*, the GLA concentration was increased as the fermentation time increased. This was due to its ability to use the accumulated lipid after exhaustion of carbon source for synthesis of fat free biomass in the medium, thereby increasing the GLA percentages in lipids. This phenomenon has also been observed in mucaraceous class fungi (Kavadia *et al.*, 2001; Gema *et al.*, 2002).

Furthermore, the two fungi significantly reduced COD and nutrients from the potato processing wastewater, which would be beneficial for environmental protection. Nitrogen removal efficiencies were high by both the fungi, 98% and 100% achieved by *Aspergillus flavus* I16-3 and 95% and 98% achieved by *Mucor rouxii* from the raw wastewater and the synthetic wastewater, respectively. In the primary anabolic process of lipid accumulation, ammonium ions from the medium is utilized by oleaginous fungi to make the fungi to grow slower to channel the excess carbon source into lipids. A significant amount of phosphorus was also removed by both fungi. *Aspergillus flavus* I16-3 reduced 83% and 92% and *Mucor rouxii* reduced 85% and 81% of total soluble phosphorus from the raw wastewater and the synthetic wastewater, respectively. Since the wastewater contained essential nutrients especially nitrogen and phosphorus, both oleaginous fungi didn’t require external addition of nutrients; this could further reduce the cost of lipid production.

Thus it is concluded that potato processing wastewater can be used as an alternative feedstock for microbial lipid production and GLA accumulation by oleaginous fungi. In addition, COD and nutrients can be removed, providing an environmentally sound method for potato processing wastewater management.

**References**


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**Disclosures**

The authors have nothing to disclose.