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Enhancing the nutrient removal and deposition of lipid by co-culturing Aspergillus tamarii and Chlamydomonas reinhardtii on potato processing waste water

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ABSTRACT

Removal nutrients by utilization from the culture medium and accumulations of lipid by oleaginous microorganisms are the promising route for the biodiesel feed stock production. Most of the oleaginous microbes cannot directly utilize starch, some of them modify the nutrient resources like cellulose and starch into monosaccharides initially, and then these fermentable sugars will be converted into fatty acids. In the present work, we investigated how the co-culturing of Aspergillus tamarii and Chlamydomonas reinhardtii involves in the nutrient removal, starch-digestion and lipid deposition when cultivated in potato processing waste water. We found that combined cultivation and consolidated bioprocessing the fungi and algae stimulate the nutrient removal, starch-digestion and biodiesel feed stock production effectively. The results exhibited the highest biomass (18.5g/L and 19.3g/L) with the lipid content of 43% and 57% on 10 and 25% diluted potato processing waste water after the 4th day of incubation. Maximum depletion of Ammonium nitrogen (34 mg/L) Orthophosphates (14 mg/L) and starch-digestion was 89% was obtained at 25% diluted potato processing waste water after the 4th day of incubation. Considering the yield, lipids derived from starch using Aspergillus tamarii and Chlamydomonas reinhardtii would be a promising alternative source for biodiesel feed stock production.

Keywords— *Co-cultivation, Aspergillus tamarii, Chlamydomonas reinhardtii, Lipid*

1. INTRODUCTION

Bio-found production of diesel has received increasing attention as a result of globally rising crude oil prices, increasing carbon dioxide emissions. Lipid contents, including triacylglycerol, produced by oleaginous microorganism have been confirmed to be among the most effective raw materials for biodiesel production [1, 2]. Cultivating oleaginous fungi and algae in the waste waters shows the utilization of nutrients and high potentiality to produce lipid from organic waste matter containing various types of polysaccharides, such as starch and cellulose [3, 4]. In general, food processing wastes contain large amounts of starch; to convert this starch from potato waste water into fatty acids, we have developed the consolidated bioprocessing method using *Aspergillus tamarii* and *Chlamydomonas reinhardtii*.

A range of starchy wastes, including potato processing and cassava pulp and other food processing wasters, are discarded without any use. Potato processing and cassava pulp processing waste water contain rich residual starch [5, 6]. This starch loaded wastewater contains high concentrations of chemical oxygen demand (COD) and biochemical oxygen demand (BOD), and they cause serious environmental threats [7]. It is reported that at least 0.60 m3 of wastewater is formed in the processing of one ton of cassava [8]. However, this wastewater can be used as a major resource for lipid production by cultivating microbes. In addition to algae, there are kinds of microorganisms that accumulate lipids, such as microalgae, bacteria, and yeast. Although microalgae accumulate lipids at high levels (60–70% of dry cell weight) [9] by using sunlight energy, carbon dioxide, and nitrogen, they cannot convert starch or other feedstocks to lipids. But when we cultivated algae with fungi or yeast they may convert starch or other feedstocks to lipids [10]. The filamentous fungus *Mortierella alpina* can assimilate starch and produce lipid [11] but challenges remain with respect to the accumulation speed and amount.

It is known that oleaginous yeasts, such as *Rhodosporidium toruloides*, *Cryptococcus curvatus*, *Lipomyces starkeyi*, and *Yarrowia lipolytica*, accumulate lipids in more than 20% of their dry cells [12, 13]. From a taxonomical viewpoint, it has been shown that several oleaginous fungi and yeasts can grow on medium containing soluble starch as a sole carbon source [14]. To the best of our knowledge, there has been no comprehensive screening for *Aspergillus tamarii* and freshwater *Chlamydomonas reinhardtii* that

directly produces lipids from soluble starch. Recently, Digby Wrede reported on the combined cultivation of algae and fungi may produce high level lipids [4]. Because the fungal and cynabacterial strains have broad assimilation spectra, we considered that these organisms would be suitable for lipid production from soluble starch. In this study, we showed how *Aspergillus tamarii* and *Chlamydomonas reinhardtii* are suitable characteristics for lipid production from potato processing waste water through consolidated bioprocessing.

2. MATERIALS AND METHODS

2.1 Materials

Potato processing wastewater was collected from a local vegetable processing industry in Chennai, India. Characteristics of the potato processing wastewater are given in Table 1.

2.2 Microorganisms

2.2.1 Fungal culture: Fungal culture was isolated from the tannery effluent soil and identified as *Aspergillus tamarii* MTCC 5152 and deposited in microbial technology Chandigarh India was used in this study. The organism was stored in the laboratory on potato dextrose agar medium. The cultures were grown for 7 days at 28°C and then maintained at 4°C. The slants were sub-cultured routinely every 4–5 weeks interval.

2.2.2 Micro-algal culture: *Chlamydomonas reinhardtii* was grown under heterotrophic conditions (10 g/l glucose), as suggested by the American Type Culture Collection (ATCC) was used in this study. Growth rates algae were analyzed by counting the cell numbers, measuring OD660 and OD750.

2.3 Preparation for seed fungal spores

For activation, the stored spores were grown at 25°C for five days on plates with Potato Dextrose Broth (PDB) containing 20 g/l glucose. Sterile water (10 ml) was added to harvest the spores and the spore solution was used as the inoculation for the co-culture after the numbers of spores in the solution were counted.

2.4 Pelletization of fungal- algal culture

To achieve pelletization spore solutions (2.0×10^6 spores/l) were cultivated at 28°C in liquid media containing 1g/L peptone, 0.6 g/L KH2PO4, 0.001 g/L, ZnSO4, 0.4 g/L, K2HPO4, 0.005 g/L, FeSO4, 0.1 g/L, MnSO4 and 0.1 g/l MgSO4. Carbon source we used was 10 g/l glucose with a shaking speed of 200 rpm for 72 h. Pellets were precipitated and the growth medium was removed. Algal cultures were precipitated, washed and re-suspended to achieve a final concentration of $6–7\times10^6$ cell/mL and added to fungal pellets. The fungal-algal mixtures were shaken at 200 rpm for 48 h under constant light at 28°C.

2.5 Dilution of potato processing wastewater

Sterilization of starch rich potato processing wastewater leads the solidification in the flasks causing the poor growth microorganism, to overcome this difficulty the potato wastewater was diluted with tap water and sterilized. Different dilution ratios were carried out: 10% (10 potato processing wastewater: 90 tap water; v/v, 25% (75 potato processing wastewater: 25 tap water; v/v), 50% (50 potato processing wastewater: 50 tap water; v/v). In all the trials raw potato wastewater (no dilution) was used for comparison.

2.6 Cultivation of fungus-algal cultures on potato processing waste water

The Aspergillus tamarii-algal pellets were harvested by filtration and 200 wet pellets were added to 250 ml of potato processing waste water (approximately, 1g/l DW). The mixtures were shaken at 200 rpm for 48 h.

2.7 Determination of cell dry weight

Determination of cell dry weight after the fermentation period, cultures were harvested by filtration (Whatman No.1) and the filtrate was rinsed thoroughly with sterile distilled water to remove unwanted constituents media present on the cell surface. The biomass was kept for drying in a hot air oven at 60°C till a constant weight was achieved. The cell dry weight was determined gravimetrically according to Devi et.al [16].

2.8 Starch, nutrient utilization analysis

Flasks were periodically removed from the incubator and subjected to starch and nutrient analysis. Samples taken from the flasks were centrifuged at 5000 rpm for 10 min. Total soluble starch was measured using the phenol sulfuric acid method [17]. Ammonium nitrogen (NH4+–N), orthophosphate phosphorus (Po43-P) concentrations were measured using Konelab-20 Analyzer.

2.9 Extraction and analysis of lipids content

The fungal–algal pellets cultivated in Erlenmeyer flasks containing various dilution of potato processing waste water at 28ºC, on a rotary shaker at 200 rpm were harvested after 1, 2, 3, 4,5, and 6 days of cultivation by centrifugation (15,000 rpm for 10 min), then washed twice with distilled water, the total residual biomass was used for measurement of lipids. A known amount of biomass was dried at 80°C until constant weight and lipids were extracted from the dried biomass using 2:1 (v:v) chloroform and methanol mixture [18].

3. RESULTS AND DISCUSSION

3.1 Pelletization of *A. tamarii* and *Chlamydomonas reinhardtii*

The cultures of filamentous fungi *A. tamarii* produced dense spherical pellets, approximately 2–5 mm in size when grown on fungal growth media containing glucose (10 g/l) under 200 rpm rotation. When A. tamarii pellets were mixed with high cell

density (6–7×106 cell/ml) cultures of *Chlamydomonas reinhardtii* culture, 90% flocculation was achieved after the first 24 h of co-cultivation.

3.2 Biomass production

Biomass productions by *Aspergillus tamarii* and *Chlamydomonas reinhardtii* jointly cultivated on differently diluted potato processing waste water were examined for the maximum yield (figure 1). The organism cultivated in the 25% diluted potato processing waste water for 4 days attained the highest biomass content (19.3g/L). Microalgae can tentatively be considered for the production of biomass and nutrient removal from waste water [19]. Avenda no-morales et. al [20] and Matsakas et. al [21, 22] reported that *Chlamydomonas reinhardtii*, *penicillium decumbens* and *Fusarium oxysporum* are able to accumulate a high level of lipids.

3.3 Starch consumption

Aspergillus tamarii and *Chlamydomonas reinhardtii* jointly cultivated and variously diluted potato processing waste water was analysed for the consumption of starch (figure 2). The organisms cultivated in the 25% diluted showed the maximum starch consumption (89%) on the fourth day of incubation. It was found that higher starch concentration does not promote lipid accumulation (figure 5). It can be surmised that high starch concentration led to a highly gelatinized solution, and then the diffusion of amylase was limited and its accessibility to glycosidic linkages was restricted [23]. It should be noted that the lipid contents of the organism increased rapidly between culture days 3 and 4. It is known that the lipid accumulation in oleaginous microbes occurs under stress conditions in the medium when cells begin the depletion of nutrients, such as nitrogen, the excess carbon in the culture medium is converted into cellular lipids. Nitrogen is one of the main components of media for microbial growth and metabolism. As previously reported, the role of nitrogen is to promote the growth of cells in order to prepare for the accumulation of lipids [24]. Unlike monosaccharides or oligosaccharides, starch is not assimilated directly by microorganism [25]. To grow on starchy substrates, microbes need to degrade starch to oligosaccharides enzymatically by using their own extracellular amylases. In the present work, the consolidated bioprocessing involves the growth phase (from days 2 to 4), and stationary phase (days 4 to 6). In the growth phase, the nutrient utilization and deposition of lipids are occurring simultaneously. Here fungal-algal pellets hydrolyze starch and assimilate the released glucose simultaneously. During the stationary phase, the nutrient utilization and deposition of lipids decrease gradually. During the growth phase assimilated starch was used for cell growth; the cell masses of *Aspergillus tamarii* and *Chlamydomonas reinhardtii* pellets attained the maximum level by the 4th day of incubation. It was proposed that higher starch assimilation does not necessarily follow the high deposition of lipid. On another hand, the deposition may be related to specific characteristics of the metabolic systems of these organisms.

3.4 Ammonium nitrogen (NH4+–N) and Orthophosphates (Po43-P) depletion

The depletion of Ammonium nitrogen and Orthophosphates by *Aspergillus tamarii* and *Chlamydomonas reinhardtii* pellets jointly cultivated and variously diluted potato processing waste water was shown in the figure 3 and figure 4. The organism cultivated in the 25% diluted showed the maximum nitrogen depletion (34 mg/L) and orthophosphates diminution (14mg/L) on the fourth day of incubation. Algal and fungal cells have been extensively used for efficient recovery of the main nutrients, N and P and microelements including heavy metals from different types of wastewaters (28, 29). Recent reports say that cultivation of microalga and bacterial consortia deplete the nitrogen from the waste water (30).

3.5 Lipid production using potato processing wastewater

Lipid productions by *Aspergillus tamarii* and *Chlamydomonas reinhardtii* jointly cultivated on differently diluted potato processing waste water were examined for the maximum yield (figure 5). The organism cultivated in the 25% diluted potato processing waste water for 4th day attained the highest lipid content (57%) on the fourth day of incubation. Extension of incubation tine does not produce any significant improvement in the deposition of lipids. The fatty acid profiles produced by the selected strains from starch were suitable for biodiesel production. Studies on oleaginous yeast revealed that lipid content achieved at most 61.53% from 3% glucose as a sole carbon source at day 4 26 [28]. However, unlike monosaccharides and oligosaccharides, starch is considered the feedstock with the greatest potential for biolipid production, as it is cheap and does not compete with food resources. Considering the feedstock and enzyme costs, lipid production from soluble starch by cultivating *Aspergillus tamarii* and *Chlamydomonas reinhardtii* together through consolidated bioprocessing would compensate for the loss and therefore has great potential for cost-effective and time-efficient biodiesel feedstock production.

4. CONCLUSION

In the present study, we found that the oleaginous fungus *Aspergillus tamarii* and *Chlamydomonas reinhardtii* jointly cultivated could directly assimilate soluble starch and utilize the nutrients available on the potato processing waste water, thereby accumulate a significant amount of lipids.

5. ACKNOWLEDGMENTS

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APPENDIX

Table 1: Characteristics of potato processing wastewater

Characteristics	Value (g/L)
Total solids	47
Total suspended solids	4.1
Volatile suspended solids	3.3
Total soluble starch	32.6
Total soluble Chemical Oxygen Demand (COD)	41
Total soluble nitrogen (TSN)	0.68
Total soluble phosphorus (TSP)	0.57
Ammonium nitrogen (NH_4^+–N)	170 (mg/L)
Orthophosphates (Po_4^{3-}-P)	126 (mg/L)
Sulfate	114 (mg/L)

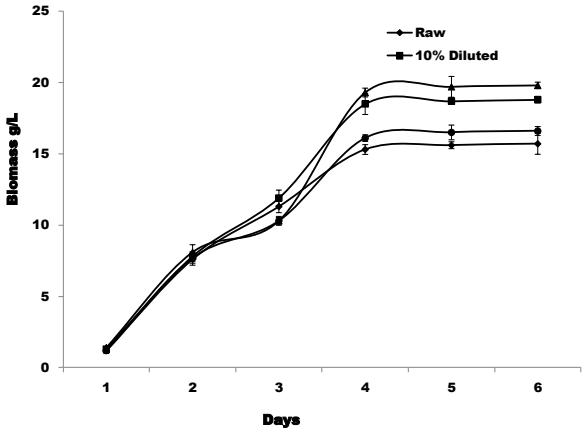

Fig. 1: Biomass produces by alkaliphilic Apergillus tamari and fresh water chlamydomonas reinhardtii using various concentration of potato processing wastewater

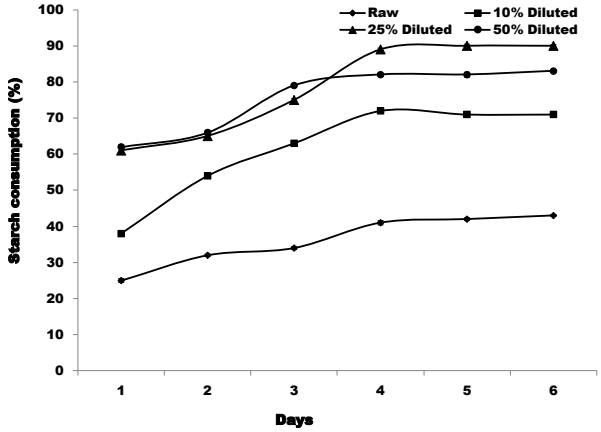

Fig. 2: Starch consumption from potato processing wastewater at various dilutions

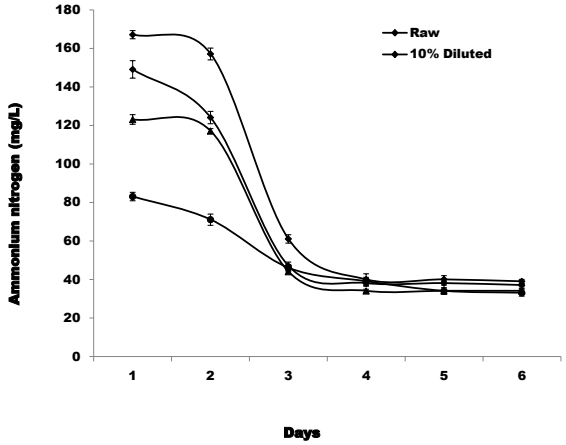

Fig. 3: Depletion of ammonium nitrogen

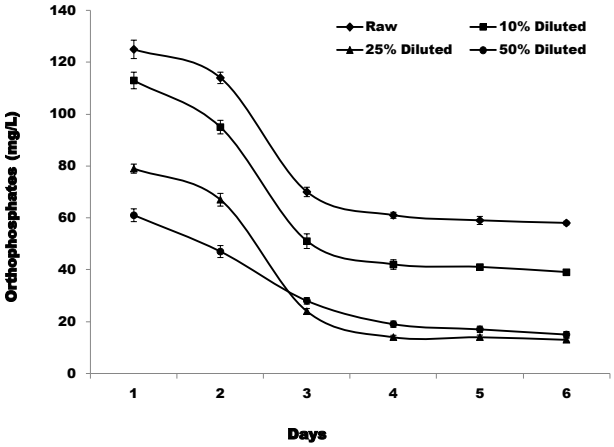

Fig. 4: Depletion of orthophosphates

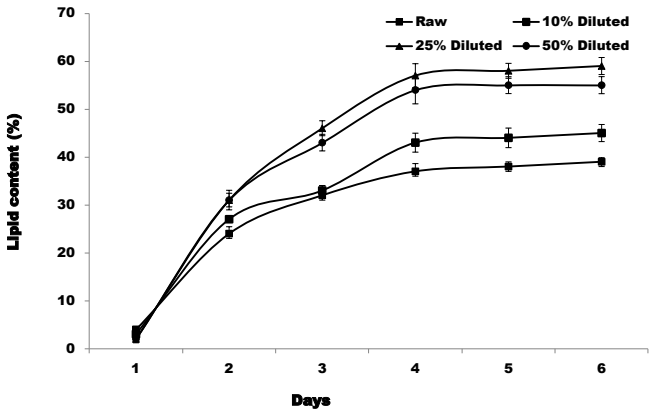

Fig. 5: Lipid production using potato processing wastewater at various dilutions

