Acclimatization Strategy of *Chlamydomonas* sp. BTA 4152 for Growing in Natural Rubber Latex Processing Wastewater

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Abstract: Algal biomass a potential renewable feedstock for 3rd generation biofuel only if the production costs are substantially reduced avoiding added stress on water resources. The waste water generated from various sources has substantial amounts of nutrients load are useful for algae growing. Selection of proper waste water and its ready access in large volume round the year remain a big concern. While wastewater as such cannot be used for algal culture it needed dilution to right concentration and robust algal strains either screened or acclimatized. This research finding reports the three steps of acclimatization process of *Chlamydomonas* sp. BTA 4152 for growing in rubber wastewater (RW). In the first step 13.3 x 10⁶ cells/ml of cells were grown in 20, 40, 60, 80, 100% RW recorded highest growth (83.1 x 10⁴ cells/ml) with net increase of 5.2 folds in 20% RW after 2 weeks. The same cells cultured in 20,40,60,80 and 100% RW showed highest growth (1024 x 10⁶ cells/ml) with net increase of 2.22 fold in 40% RW in 2 weeks. Finally the acclimatized cells in 40% RW cultured in 40, 60, 80 and 100% RW highest growth (1450 x10⁶ cells/ml) with net increase of 39.28 folds and 504mg/l dry biomass in 5 weeks.

Keywords: *Chlamydomonas* sp. BTA 4152, rubber wastewater, acclimatization, biomass

1. Introduction

Third-generation biofuels refers to non-arable crops as precursor with easier extraction process such as algae. Algae biomass is considered as viable energy feedstock is devoid of the major drawbacks associated with first and second generation biofuels [1, 2]. Nutrients and carbon are the essential and expensive input for microalgal growth therefore wastewater is explored as a cheap alternative to expensive synthetic growth media for large-scale microalgal cultivation [3]. *Chlamydomonas reinhardtii* is grown in municipal wastewater as a feedstock for biofuel production and also remove about 80% of nitrate and 15% of phosphate [4]. Algal can be grown on marginal land using wastewater, can be harvested repeatedly and many species contain high lipid content which is essential for biodiesel production [3, 5].

India’s rubber plantation sector is dominated by small holdings that accounts for 92% of the production and 89% of the area. Since 1986, Rubber Board a statutory body constituted by Government of India promote formation of voluntary associations of small growers called the Rubber Producers’ Societies (RPS) for facilitating extension communication, and to adopt suitable measures to increase the production and productivity of small and marginal holdings. Presently there are over 2500 RPSs across India, representing more than a million farmers, cultivating over seven lakh hectares of which the average holding is 0.54 ha. The most of the RPSs are registered in Kerala and nearly 250 of them are registered in the North East region. Each RPS has a Rubber Processing Centre (RPC) for group processing of latex collected from surrounding farmers where Ribbed Smoked Sheet (RSS) are produced. The RPC consume large volume of water and chemical for processing of rubber. The effluent of RPC contains wash water, small amounts of uncoagulated latex and serum with small quantities of protein, carbohydrates, lipids, carotenoids and salts [6], contain strong colour, a large amount of suspended solids, a highly fluctuating pH, COD, BOD, etc. [7,8]. Because of these characteristics, treatment of rubber wastewater is an essential requirement before its disposal to natural water system [9, 10, 11]. During 2008-09, out of 6.61 lakh ha area covered under rubber plantation in India latex are tapped in 4.63 lakh ha area producing 8.645 lakh MT of dry natural rubber @ an average yield of 1.867 MT per hectare land. Rubber processing industry usually generates large quantities of wastewater containing high concentration of organic matter, suspended solids and nitrogen [12]. Processing of a kilogram of dry rubber requires 15-20L of water thus India generated about 12.96 -17.29 billion liters of wastewater from processing of 8.645 lakh MT dry natural rubbers during 2008-09. Rubber plantation and subsequent processing for natural rubber in the northeast India has been considered as rehabilitation package due economic viability at the farmer’s perspective and is slated for expansion for 4.5 lakh ha area from existing 0.88 lakh ha. Likewise the group processing of latex for RSS rubber in RPC is set to increase many fold in years to come leading to severe stress of water resources. Besides stress of water resources the waste water discharge of these RPC contains high load of nutrient nitrogen and phosphorous besides other may cause serious threat to environment endangering millions of livelihood associated. The high level of phosphate and ammonia in rubber effluent makes it a good medium for algal growth and can result in eutrophication of surface waters if discharged without proper treatment [11]. The published report on rubber wastewater focuses on its bioremediation and none of the research has directed for use of rubber waste water as resource. Therefore present experiment has been attempted to optimize the
acclimatization process for utilization of the rubber wastewater in algae cultivation.

2. Materials and Methods

A. Materials

Rubber wastewater (RW) used in the study was collected from Jalabasa Rubber Producers Society, Jalabasa, North Tripura district, Tripura, India. *Chlamydomonas* sp. BTA 4152 strain used in this work was isolated from natural stream at Dhupdhara, Goalpara district, Assam, India (Latitude 25°57'11.6: Longitude 91°04'31.2) and deposited to Fresh water algal Repository, Institute of Bioresources and Sustainable Development, Government of India.

B. Inoculum Preparation and Experiments

Seed culture was prepared by inoculating *Chlamydomonas* sp. BTA 4152 into BG11 media and it was grown till it reached early logarithmic phase (6–7 days). The seed culture thus prepared was then inoculated in 150 ml Erlenmeyer flask containing 50 ml culture medium with inoculum size of 10% (v/v). Algal strain was grown at 28±2 °C and at pH 6.8–7 under 14:10 light–dark cycle with light intensity of 2000 lx. During incubation, the cultures were periodically gently mixed to ensure homogeneous mixing, release of O2, and to avoid settling and sticking of algae on to the flask wall. The stationary phase algal cells were inoculated for acclimatization experiment hereafter (table1).

| Acclimatization stage | Inoculum source | Rubber wastewater concentration in % v/v in tap water |
|-----------------------|-----------------|--------------------------------------------------|
| 1                     | Fresh           | 0 20 40 60 80 100                                 |
| 2                     | Acclimatized in 20% RW | 20 40 60 80 100                                  |
| 3                     | Acclimatized in 40% RW | 40 60 80 100                                    |

C. Acclimatisation stage I. Cultivation of *Chlamydomonas* sp. BTA 4152 in Rubber wastewater without any supplementation:

RW used as raw without any interference except dilution with tap water. RW was diluted with tap water to produce dilution percentages of 20, 40, 60, 80, and 100. In another set of experiment RW was enriched with BG11 medium and pH was adjusted for only for control (BG11 media). The experiment was set in 500ml Erlenmeyer conical flasks and replicated three times. The stock solution of *Chlamydomonas* sp. BTA 4152 was maintained at 28 °C±1 with photoperiod of 12hrs light/dark period provided by fluorescent lamps at the light intensity of 2000lx. Experiment was initiated with inoculation of 10% v/v inoculum density 13-18 x 10^4 cells/ml of *Chlamydomonas* sp. BTA 4152 stock culture. The experiment run for 14 days at temperature 28°C±2, and photoperiod of 14/10 light/dark with light intensity 2000 by fluorescent tubes (Havels) orbital shaker (Thermo Fisher make).

D. Acclimatisation stage II. Cultivation of *Chlamydomonas* sp. BTA 4152 in Supplemented Rubber wastewater:

In the second experiment, *Chlamydomonas* sp. BTA 4152 acclimatized in 20% RW was used as inoculums and subcultured in supplemented RW at 20, 40, 60, 80 and 100% in tap water. All the culture conditions were kept same as in experiment I.

E. Acclimatisation stage III. Cultivation of *Chlamydomonas* sp. BTA 4152 in Supplemented Rubber wastewater:

In the third experiment, *Chlamydomonas* sp. BTA 4152 acclimatized in 40% RW was as inoculum and subcultured in supplemented RW at 40, 60, 80 and 100% in tap water. All the culture conditions were kept same as in experiment I none of culture medium for acclimatization was adjusted for pH.

F. Growth Evaluation:

Growth was measured in terms of dry biomass and cell count. At the different interval of experiment cells were counted using a Haemocytometer (Neubauer). Net growth fold= C1-C0/C0

Where C0 is the initial cell count, C1 is the subsequent cell count after incubation.

For dry weight method, the algal cultures were pelletized by centrifugation at 7500 rpm (Eppendorf, 5810R) for 15 minutes. Cells were washed with glass-distilled water, again centrifuged and dried in an oven (Thermo Fisher) at 105 °C for 24 hours or until constant weight. Simultaneously pH of the culture media was recorded using pH meter (SenTix® 81, Merck). The net growth fold was calculated based on following equation.

3. Result

Rubber wastewater discharge from processing facility was opaque in colour, malodour due to ammonia and H2S and recorded pH 3.5. This discharge is usually treated in floating dome anaerobic digester for biogas production, however due to non replenishment of proper C/N ratio the anaerobic digester remain non operation for the 50 processing facilities visited.

1) First Acclimatization

Growth performance for *Chlamydomonas* sp. BTA 4152 species in various concentration of Rubber wastewater (20, 40, 60, 80 and 100% v/v) without adjusting pH for 2 weeks of incubation is illustrated in table 2,3,4 and Fig 1.
The highest growth 83.1 x 10^4 cells/ml and net 5.248 fold increase was recorded in 20% RW where pH rose from 6 to 9. The enrichment of BG11 media with various concentration of RW (20, 40, 60, 80 and 100%) recorded no advantages over tap water (table 5, 6, 7). A similar finding was reported for Chlorella vulgaris growth of textile wastewater supplementation of with nutrients of BBM [13]. This could be attributed to super salinity situation in rubber wastewater due to enrichment of BG11.

Table 2: Growth performance of Chlamydomonas sp. BTA 4152 in tap water supplemented with rubber wastewater

| Tap water+RW % | Cell count (x 10^4/ml) after days |
|----------------|-----------------------------------|
| 0%             | 0 1 4 7 10 14                     |
| 20%            | 13 19 27.4 40.3 51.3 54           |
| 40%            | 18.5 19.1 18.8 19.3 20.1 38.4     |
| 60%            | 16.2 20 19.8 21.4 20.8 22.3       |
| 80%            | 17.7 20.3 20.9 21.5 20.6 20.5     |
| 100%           | 14.6 20.5 20.4 24.4 20.8 21.9      |

Table 3: Net growth fold of Chlamydomonas sp. BTA 4152 in tap water supplemented with rubber wastewater

| Tap water+RW % | Starting cell count | Fold increase in Cell count (x 10^4/ml) after days |
|----------------|---------------------|-----------------------------------------------|
| 0%             | 0.46 1.11 2.10 2.95 3.15 |                               |
| 20%            | 13.3 0.96 1.63 2.52 3.74 5.25 |                               |
| 40%            | 18.5 0.03 0.02 0.04 0.09 1.08 |                               |
| 60%            | 16.2 0.24 0.22 0.32 0.29 0.38 |                               |
| 80%            | 17.7 0.15 0.18 0.22 0.17 0.16 |                               |
| 100%           | 14.6 0.40 0.40 0.67 0.43 0.50 |                               |

Table 4: Changes of pH in Chlamydomonas sp. BTA 4152 grown in tap water supplemented with rubber wastewater

| Tap water+RW % | pH values after days |
|----------------|---------------------|
| 0%             | 0 1 4 7 10 14       |
| 20%            | 6 6.2 6.6 7 7.8 8   |
| 40%            | 5 6.2 6.3 7 7.8 7   |
| 60%            | 4.5 6 6.2 6 7 7    |
| 80%            | 4 6 6.5 6.5 7 6.5  |
| 100%           | 3.5 5.5 6.4 6.5 7.1 7.5 |

1) Second Acclimatization
The two weeks old Chlamydomonas sp. BTA 4152 culture in 20% waste water when inoculated in 20,40,60,80,100 % RW the highest growth recorded (1024 x 10^4 cells /ml) with calculated net increase of 2.22 fold in 2 weeks culture in 40% RW followed by 60% RW with 932 x 10^4 cells/ml and 1.8 fold increase (table 8,9). The pH rose from 5 to 8 in all the concentration of waste water (table 10).

Table 5: Growth performance of Chlamydomonas sp. BTA 4152 in BG11 supplemented with rubber wastewater

| BG11+RW % | Cell count (x 10^4/ml) after days |
|-----------|-----------------------------------|
| 0%        | 17 21.9 38.2 41.2 47 70           |
| 20%       | 16.94 18.8 24 35 40.6 40           |
| 40%       | 24.4 19.4 19.1 22.2 24 18.3        |
| 60%       | 23.5 20 20.4 21 24.6 20.6         |
| 80%       | 19.1 20.17 23.3 24.5 29 22.7      |
| 100%      | 21 20.2 24 23 27.3 22             |

Table 6: Net growth fold of Chlamydomonas sp. BTA 4152 in BG11 supplemented with rubber wastewater

| BG11+RW % | Starting cell count | Fold increase in Cell count (x 10^4/ml) after days |
|-----------|---------------------|-----------------------------------------------|
| 0%        | 17 0.29 0.89 1.42 1.77 3.12 |                               |
| 20%       | 16.94 0.11 0.42 1.07 1.40 1.36 |                               |
| 40%       | 24.4 0.21 0.22 0.90 0.02 0.25 |                               |
| 60%       | 23.5 0.15 0.13 0.11 0.05 0.12 |                               |
| 80%       | 19.1 0.06 0.22 0.28 0.52 0.19 |                               |
| 100%      | 21 0.04 0.14 0.10 0.30 0.05       |                               |

Table 7: Changes in pH for Chlamydomonas sp. BTA 4152 grown in tap water supplemented with rubber wastewater

| BG11+RW % | pH values after days |
|-----------|---------------------|
| 0%        | 0 1 4 7 10 14       |
| 20%       | 6 6.3 7.6 7 7.3 7   |
| 40%       | 5.5 6.6 6.5 7 7.1 6 |
| 60%       | 5 6.9 7 6.5 6.7 6.5 |
| 80%       | 4.5 6.8 7.4 7 7.2 6.5 |
| 100%      | 4 6.8 7.4 7.5 7.5 6.5 |

Figure 1: Acclimatization stage I of Chlamydomonas sp. BTA 4152 in Rubber wastewater (optimum growth in 20%RW)
Table 8: Growth performance of *Chlamydomonas* sp. BTA 4152 acclimatized in 20% RW cultured in tap water supplemented with rubber wastewater

| Tap water + RW % | Cell count (x 10^4/ml) after days |
|------------------|----------------------------------|
|                  | 0      | 2                 | 4      | 7      | 9      | 11     | 14     |
| 20%              | 304    | 288               | 144    | 464    | 464    | 396    | 356    |
| 40%              | 312    | 368               | 192    | 320    | 320    | 520    | 1024   |
| 60%              | 324    | 336               | 320    | 400    | 400    | 408    | 932    |
| 80%              | 304    | 256               | 256    | 320    | 320    | 476    | 408    |
| 100%             | 324    | 356               | 176    | 400    | 400    | 492    | 384    |

Table 9: Net growth fold of *Chlamydomonas* sp. BTA 4152 acclimatized in 20% RW cultured in tap water supplemented with rubber wastewater

| RW % | Starting cell count | Fold increase in Cell count (x 10^4/ml) after days |
|------|---------------------|-----------------------------------------------|
|      | 0       | 2                 | 4      | 7      | 9      | 11     | 14     |
| 20%  | 304     | -0.05             | -0.53  | 0.53   | 0.30   | 0.17   |
| 40%  | 312     | 0.18              | -0.39  | 0.03   | 0.03   | 0.67   | 2.28   |
| 60%  | 324     | 0.04              | -0.01  | 0.24   | 0.24   | 0.26   | 1.88   |
| 80%  | 304     | -0.16             | -0.16  | 0.05   | 0.05   | 0.57   | 0.34   |
| 100% | 324     | 0.10              | -0.46  | 0.24   | 0.24   | 0.52   | 0.19   |

Table 10: Changes in pH of *Chlamydomonas* sp. BTA 4152 acclimatized in 20% RW cultured in tap water supplemented with rubber wastewater

| Tap water + RW % | pH values after days |
|------------------|---------------------|
|                  | 0      | 2      | 4      | 7      | 9      | 11     | 14     |
| 20%              | 5.6    | 6.2    | 7.5    | 8.4    | 8.78   | 9.0    | 9.2    |
| 40%              | 5.6    | 5.8    | 8      | 8.29   | 8.61   | 8.8    | 8.84   |
| 60%              | 5.5    | 5.7    | 7      | 8.1    | 8.54   | 8.5    | 8.67   |
| 80%              | 5      | 5.65   | 7.5    | 7.9    | 8.49   | 8.4    | 8.57   |
| 100%             | 5      | 5.61   | 4.5    | 7.4    | 8.12   | 8.2    | 8.36   |

2) Third Acclimatization

The *Chlamydomonas* sp. BTA 4152 acclimatized in 40% waste water subcultured in 40, 60, 80,100% concentration incubated for 5 weeks and growth performance illustrated in Fig. 2,3,4,5. The 40% concentration of RW showed the highest cell counts 124 x 10^4 cells/ml, 240 x 10^4 cells/ml, 260 x 10^4 cells/ml, 530 x 10^4 cells/ml and 1450 x 10^4 cells/ml with net increase of 2.44, 5.67, 6.22, 13.72 and 39.28 folds after 1st, 2nd, 3rd, 4th and 5th weeks of incubation respectively. In the same period the pH rose from 5 to 8.73, 8.95, 9.19, 9.24 and 10.2 after 1st, 2nd, 3rd, 4th and 5th weeks of incubation respectively.

The highest biomass productivity (504 mg dry weight/l) of *Chlamydomonas* sp. BTA 4152 was recorded in 40% waste water (Fig.6) and productivity decreased with increasing RW concentration even after acclimatization.

![Figure 2: Acclimatization satge III of Chlamydomonas sp. BTA 4152 in Rubber wastewater (optimum growth in 40%RW)](image)

![Figure 3: Growth performance of Chlamydomonas sp. BTA 4152 acclimatized in 40% RW cultured in tap water supplemented with rubber wastewater](image)

![Figure 4: Net growth fold of Chlamydomonas sp. BTA 4152 acclimatized in 40% RW cultured in tap water supplemented with rubber wastewater](image)

![Figure 5: Changes in pH of Chlamydomonas sp. BTA 4152 acclimatized in 40% RW cultured in tap water supplemented with rubber wastewater](image)

![Figure 6: Biomass productivity of Chlamydomonas sp. BTA 4152 acclimatized in 40% RW cultured in tap water supplemented with rubber wastewater](image)
The raise in pH values is attributed to rapid photosynthesis by algae, which consume CO2 as a result, the carbonate and bicarbonate ions dissociate, as follows:

\[ 2\text{HCO}_3^- \rightarrow 3\text{CO}_2 + \text{H}_2\text{O} + \text{CO}_2 \]

\[ \text{CO}_3^{2-} + \text{H}_2\text{O} \leftrightarrow 2\text{OH}^- + \text{CO}_2 \]

The resulting CO2 is fixed by the algae, and the hydroxyl ions accumulate thereby raising the pH to values above 10.

Conventionally NaOH is used to neutralize acidic effluents and to reduce the cost of NaOH, iron oxidation and limestone neutralization processes are adopted because limestones are less expensive than lime [14]. The present findings indicate pH correction of rubber waste water in algal biomass production that would reduce cost and sludge significantly in environment friendly way besides generating raw materials for biofuel, fertilizer etc. Lee (2001) [15] reported tolerance of microalgae and utilization of nutrients from several domestic and industrial wastewaters. Both nitrogen and phosphorus present in wastewater discharge are the major source of eutrophication by algal bloom [16]. Screening and scrutiny of efficient algal species are necessary for assessing the biofuel prospects of algae. The native or indigenous algal species thriving in the wastewater conditions with high N and P [17, 18] concentration would be more beneficial than the commercially available strains which have a narrow range of tolerance. The locally isolated algae easily adapt to the wastewater conditions and are found to grow at higher biomass densities [17]. The *Chlamydomonas sp.* BTA 4152 used in the experiment isolated from fresh water also equally tolerate the wastewater toxicity and grow efficiently after the 3 stage acclimatization.

3) **Strategy flow chart (9 weeks)**

1. Inoculated *Chlamydomonas sp.* BTA 4152 in waste water at 0,20,40,60,80,100 % strength in tap water without pH correction and incubated for 2 weeks
2. Selected the best concentration of waste water (20%) with regard to cell count and pH change in the medium
3. Inoculated the *Chlamydomonas sp.* BTA 4152 strains from selected waste water concentration (20%) to its higher strength of waste water (20,40,60,80,100%) and incubated for another 2 weeks
4. Again selected the best concentration of waste water (40%) with regard to cell count and raise in pH in the medium
5. Reinoculated the *Chlamydomonas sp.* BTA 4152 strains from 40% waste water concentration to its higher strength of wastewater (40,60,80,100%) and incubated for another 5 weeks

6. The appropriately acclimatized *Chlamydomonas sp.* BTA 4152 strains ready for utilizing the wastewater as growing medium for production of biomass @500mg/l after 5 weeks.

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