5. PHOTOBIOLOGICAL HYDROGEN PRODUCTION FROM CHLORELLA VULGARIS MSU-AGM 14 BY UTILIZING ACID HYDROLYSATE OF SEAWEED VALONIOPSIS PACHYNEMA

5.1. MATERIALS AND METHODS

5.1.1. Acidic Seaweed Extraction

Powdered seaweed sample (1g) were added into 1-5% of sulfuric acid solution (100 ml) and was kept at 60°C for 2 hours. The hot extract was filtered through double-layered cheesecloth and was authorized to cool at room temperature. The extract was treated with activated charcoal for detoxification process with a well-balanced elution pH (7.5). The extract was considered as 100% seaweed acid hydrolysate. Different concentrations of seaweed acid hydrolysate (10%, 20%, 30%, 40% and 50%) were prepared to carry out batch experiments with varied pH (4.0 - 9.0), temperature (20°C - 45°C) and substrate concentrations (10 - 50%) in triplicates.

5.1.2. Micro algal growth and H\textsubscript{2} production

The micro algae C. vulgaris MSU-AGM 14 (Accession No- KM189121) species was introduced into different concentrations of seaweed acid hydrolysate for determination of growth of algae at 30°C with 15 µmol photons m\textsuperscript{-2} s\textsuperscript{-1} of illumination under anaerobic condition. The algal growth curve was measured by using UV spectrophotometer (PC based double beam spectrophotometer Au - 2701– Systronics) at 550nm at regular intervals (24 hours). Simultaneously the gas samples were acquired from head phase of the culture medium for the estimation of hydrogen percentage by using Gas Chromatography (GC). Samples were estimated by injecting 500 µl of gas from the reaction vessels with the help of pressure lock gas syringe into a gas chromatograph (Chemito 7610 series) fitted with a poropak Q column to a thermal conductivity detector. During gas analysis the temperature of the column was set at...
60°C, injection port at 60°C and the detector at 90°C. The nitrogen gas was selected as carrier gas at a flow rate of 30ml/min. The amount of biohydrogen liberated by microalgae was evaluated from the peak height of the recorder (Software Iris 32 lite). The ultra pure hydrogen gas was also analyzed in the same protocol for reference peak calibration. For maintaining the positive pressure in the vessel, same amount of nitrogen gas was injected before withdrawing the test sample. The biohydrogen gas mass was calculated by Ideal gas law (STP-Standard Temperature and Pressure) using the following equation

\[ MW = \frac{m \times R \times T}{P \times V} \quad (1) \]

MW-Molecular Weight, m-Mass of the gas, R-Ideal gas constant, T-Temperature, P-Pressure, V- Volume of gas in liter.

5.1.3. Optimization of H\textsubscript{2} productivity

The central composite design was constructed at three different levels [optimum (code = 0), minimum (code = -1) and maximum (code = +1)] for all the independent factors such as substrate concentration, Carbon dioxide, pH and temperature (Table 8). The central point of the experiment was designed by using actual level of the variables. Using following equation the variables were exchanged into coded variables (2).

\[ X^i = \frac{X^i - X^i^*}{\Delta X^i} \quad i = 1,2,3 \ldots \quad (2) \]

\( X^i \) = Coded values; \( Xi \) = Uncoded values

\[ Y = \beta_0 + \beta_1 A + \beta_2 B + \beta_3 C + \beta_4 D + \beta_1 \beta_2 AB + \beta_1 \beta_3 AC + \beta_1 \beta_4 AD + \beta_2 \beta_3 BC + \beta_2 \beta_4 BD + \beta_3 \beta_4 CD + \beta_1 \beta_1 A^2 + \beta_2 \beta_2 B^2 + \beta_3 \beta_3 C^2 + \beta_4 \beta_4 D^2 \quad (3) \]
Independent variables | Parameters | Coded levels
--- | --- | ---
A | Substrate concentration (%) | -α, -1, 0, +1, +α
B | Temperature (°C) | 30, 32, 34, 36, 38
C | pH | 7.3, 7.5, 7.7, 7.9, 8.1
D | Carbon dioxide (%) | 3.6, 3.8, 4.0, 4.2, 4.4

Table 8: Level of the independent variables for Response Surface Methodology

The interactions between various factors were interpreted by 3D response graphs and each factor’s optimized values were analyzed. From the analytical data, the 3D curves and quadratic equations were developed in the following form of equation (3). Where Y is the predicted value, \( A, B, C, D \) are the coded independent variables, \( \beta_1\beta_2, \beta_1\beta_3, \beta_1\beta_4, \beta_2\beta_3, \beta_2\beta_4 \) and \( \beta_3\beta_4 \) are the interactive coefficients, \( \beta_1, \beta_2, \beta_3 \) and \( \beta_4 \) are the quadratic coefficients, \( \beta_0 \) is constant (Lakshmikandan et al., 2014; Radhika & Murugesan, 2012).

5.1.4. Selection of variables for response surface methodology

Based on central composite design, 30 experiments were generated to determine the experimental values of each factor such as substrate concentration (25%, 30%, 35%), pH (7.5, 7.8, 8.1) temperature (30°C, 32°C, 34°C) and carbon dioxide concentration (3.8%, 4.0%, 4.2%). All the experiments were carried out with 500 ml of culture medium along with 5% (vv\(^{-1}\)) seed inoculums at proper agitation with illumination for 8 days for the optimum hydrogen evaluation. The gas samples were collected and committed to Gas Chromatography for analyzing the percentage of biohydrogen production. The optimization of physical parameters like substrate concentration, temperature, pH and carbon dioxide levels were determined by Response Surface Methodology (RSM) with the help of software package Design Expert 7.0.0 (Stat-Ease, Inc., Minneapolis, USA).
5.2. RESULTS

5.2.1. Micro algal growth on various concentrated acidic extracts

Figure: 31 Microalgae *C. vulgaris* MSU-AGM 14 growth dynamics at (a) different diluted sulfuric acid hydrolysate of green seaweed *V. pachynema*

Figure: 32 Microalgae *C. vulgaris* MSU-AGM 14 growth dynamics at different concentrations of 3% acid hydrolysate.
The different acid hydrolysates of seaweed *V. pachynema* were selected for the assessment of cultural growth characteristics (Fig. 31). When compared with other concentrations of acid hydrolysate, 3% acid hydrolysate of *V. pachynema* showed greater growth under anaerobic condition (4%) at pH – 7.5 and 15 µmol photons m$^{-2}$s$^{-1}$ of illumination at room temperature. On 7$^{th}$ day of algal culture, growth reached maximum (OD~0.313) level with sustained growth upto 12$^{th}$ day. The selected hydrolysate (3% acid treated) was further analyzed to detect the specific concentration for optimal growth. The 3% acid hydrolysate was diluted upto 50% and was inoculated with same amount of micro algal culture at different dilutions (10-50%). Among which low concentration of acid hydrolysate (30%) recorded elevated growth (OD~0.81) of micro algae *C. vulgaris* MSU-AGM 14 (Fig. 32).

5.2.2. Photobiological Hydrogen Production

The elevation of algal cells density spontaneously reflects the biohydrogen production at peculiar condition. This study showed that the log or exponential phase cells actively generated molecular hydrogen at the rate of 0.0026g/h/l (Fig. 33). It shows that the production of hydrogenase enzyme also increased at exponential phase of culture and gradually decreased at stationery phase. The molecular hydrogen production reached the best possible level on 8$^{th}$ day (OD~0.605) wherein sustained hydrogen production lasted for 6 days.
Figure:33  Growth and photobiological hydrogen production of *C. vulgaris* MSU-AGM 14 at 30% concentrated acid hydrolysate in invitro culture bottles at 4% anaerobic condition with 15 µmol photons m$^{-2}$ s$^{-1}$ light illumination

5.2.3. Optimization of individual parameters

The optimization of photobiological hydrogen production was studied at specific concentration of extract along with selected variables under upheld environment.

The regression equation (4) of experimental data is to fit the cumulative biohydrogen production by using equation (3).

The interaction between four variables like pH, temperature, percentage of CO₂ and substrate concentration for the photobiological hydrogen production have been observed by 30 different experimental models (Table 9). The analysis of variance
(ANOVA) and regressions for biohydrogen production showed that the model has fitted well (Table 10). The coefficients of this experiment R2 were determined as 0.9511 with adjusted R² value of 0.9823 (Fig. 34). Hence the predicted and adjusted coefficients showed reasonable arrangement. In regression analysis the experimental results showed that all the variables have a positive linear effects (P<0.05). In addition, coded values such as AC, BC, BD. A², B², C² and D² were also obtained as significant model.

**Figure:34** Experimental and response surface methodology predicted photobiological hydrogen production.
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Table:10 ANOVA for response surface quadratic model of biohydrogen production obtained from the experiment.
Figure: 35 The 3D response surface plots showing the effect of various factors in biohydrogen production (a) Substrate concentration vs Temperature (b) Substrate concentration vs pH (c) Substrate concentration vs Carbon dioxide (d) Temperature vs pH (e) Temperature vs Carbon dioxide (f) pH vs Carbon dioxide.
5.3. DISCUSSION

The adequate requirement of nutrients for the growth of microalga is fulfilled by different concentrations of seaweed acid hydrolysate. Rohani-Ghadikolaei et al. (2012) reported that aqueous extract of selected seaweeds have potential biochemical compounds for culturing the micro alga *Isochrysis galbana*. Due to lack of hemicelluloses and lignin, seaweeds acts as substrate, as it has more essential components like carbohydrates and minerals (Matsumura et al., 2014; Wang et al., 2013; Wei et al., 2013). Alvarado et al. (2008) also described that the seaweed extracts are low cost medium for culturing the micro algae. In the present study low concentration of acid hydrolysate (30%) recorded elevated growth (OD~0.81) of micro algae *C. vulgaris* MSU-AGM 14. Likewise several reports have suggested that high substrate concentrations failed to encourage the micro algae growth (Alalayah et al., 2009; Mullai et al., 2013).

During acid hydrolysis process seaweeds forms 5-hydroxymethylfurfural which highly inhibits the hydrogen production (Park et al., 2011). The successful detoxification process will solve the 5-hydroxymethylfurfural inhibition in hydrogen production process for which activated carbon was employed by Park et al. (2011). Similarly the seaweed *V. pachynema* acid hydrolysates were treated with activated charcoal for detoxification process along with sulfur deprivation.

Several researchers suggested that pretreatment process of seaweed leads to elevation in hydrogen production employing bacterial strains (Jung et al., 2011; Liu & Wang, 2014; Park et al., 2011). Likewise, the seaweed *V. pachynema* acid hydrolysate showed elevated hydrogen production in acid treated extract when comparing to aqueous extract. In addition the CO₂ consumption of microalgae is reduced during hydrogen elevation.
The production of biohydrogen is sustained due to rich carbohydrate and nutritional property of seaweed \textit{V. pachynema} along with growth promoting hormones (Crouch & Van Staden, 1993). Various succeeded studies were performed to utilize the seaweed nutritional richness with the help of bacterial strains to produce various bioproducts like hydrogen (Jung et al., 2011; Liu & Wang, 2014; Xia et al., 2015a; Xia et al., 2015b), ethanol (Horn et al., 2000b; Jang et al., 2014; Kim et al., 2011) etc. The study reveals that the micro algae can utilize the seaweed extract for active growth and production of photobiological hydrogen at specific concentration.

The response surface 3D plots (Fig. 35) clearly described the interaction between two variables like substrate concentration and temperature on photobiological hydrogen production, which also represented the individual variables highest point in response surface plot. In this study the optimal values of substrate concentration, temperature, pH and carbon dioxide concentration were 30.73\%, 32°C, 7.66, and 3.96\% respectively. The optimal biohydrogen production was 0.0039g/h/l, which is higher than the batch fermentation. The outcome of pretreated seaweed hydrolysate clearly evidenced the elevation of photobiological hydrogen production in microalga \textit{C. vulgaris} MSU-AGM 14.

The present study evidenced to prove that seaweed is one of the sustainable solutions for biofuel production (Liu & Wang, 2014; Singh & Gu, 2010). It acts as a sustainable feedstock for microbial growth by enhancing their active metabolism. The richness of nutrients and beneficial biomolecules attract more attention and are considered as long term sustainable solution for biomass energy. The results obtained in this study clearly proved that \textit{V. pachynema} could be a feasible feedstock for microalgae \textit{C. vulgaris} MSU-AGM 14 hydrogen production.
5.4. CONCLUSION

The acid hydrolysate of *V. pachynema* enhances the growth and photobiological hydrogen production of microalgae *C. vulgaris* MSU-AGM 14 at specific concentration. With 3% sulfuric acid hydrolysate promising response on micro algae culturing were observed whereas 30% diluted acid hydrolysate was found to be an excellent source. The Response Surface Methodology clearly illustrated that low concentration (30.73%) acid hydrolysate had a significant impact on the production of biohydrogen. Simultaneously batch fermentation also proved that the increased concentration of substrate fails to encourage the culture growth. Under the optimum conditions, the variables expressed a similar response in both experimental and predicted values of photobiological hydrogen production. From the above prospective seaweed *V. pachynema* acid hydrolysate acts as an efficient source and low cost medium for the production of biohydrogen along with culturing of microalgae *C. vulgaris*. 