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Optimization of Different Parameters for Biohydrogen Production by *Klebsiella oxytoca* ATCC 13182

[1]Veena Thakur, [1]S.K. Jadhav and [2]K.L. Tiwari
[1]School of Studies in Biotechnology, Pt. Ravishankar Shukla University, Raipur, Chhattisgarh, India
[2]Guru Ghasidas University, Bilaspur, Chhattisgarh, India

Corresponding Author: S.K. Jadhav, School of Studies in Biotechnology, Pt. Ravishankar Shukla University, Raipur, Chhattisgarh, India Fax: 0771-2262583

ABSTRACT

Increasing demand of energy due to industrialization has resulted into the shortfall of non renewable energy sources. Also the over consumption has raised many serious environmental issues, all these problem has resulted for the search of some alternative renewable energy sources which is eco-friendly and biohydrogen fulfils all these requirements. In the present study rice mill effluent was taken as a substrate and *Klebsiella oxytoca* ATCC 13182 strain was used for the fermentative production of biohydrogen. Hydrogen production was carried out at 40°C temperature and at 6 pH after optimization. Its quantity was measured by water displacement method. Immobilized bacteria were used for the process. Bacteria were immobilized in different concentrations of sodium alginate containing 0.3% activated charcoal. It was found that at concentration of 2 and 3% of sodium alginate 50.33±0.88 and 52.67±1.45 mL productions were observed, respectively with no significant difference. Therefore, effect of HRT (Hydraulic Retention Time) was studied at these 2 concentrations of sodium alginate. The rate of biohydrogen production was found better at concentration of 2%. Effect of metal ions, iron and cobalt was studied at different concentrations and it was found that maximum of 55.00±0.57 mL of hydrogen was produced at 80 mg L^{-1} concentration of $FeSO_4$ and cobalt nitrate at a concentration of 80 mg L^{-1} gave maximum production of 58.33±0.88 mL. Different physicochemical parameters of waste water was analyzed before and after fermentation process.

Key words: Renewable, biohydrogen immobilized, water displacement

INTRODUCTION

Due to globalization and industrialization, demand of petroleum and fossil fuel is increasing day by day which has lead to the shortage of petroleum reserve. Extensive use of fossil fuels and excessive emission of green house gases has also resulted into global warming and other serious environmental problems. As a result, development of an alternative eco friendly source of energy has become a serious issue in recent years. Among the various alternative sources developed, hydrogen is the most promising fuel with high energy content (122 kJ g^{-1}) (Kim *et al*., 2009). Hydrogen produces only water as a end product after combustion, hence it is considered as fuel of future (Das and Veziroglu, 2001). There are various methods for the production of hydrogen which includes photolysis of water, water gas shift reaction and fermentation (Levin *et al*., 2004). Biological hydrogen production from waste is the most challenging aspect of renewable energy production technology. Various waste substrates have been used for the production of biohydrogen by different workers. Wastes such as corn starch wastes, sweet potato, food industry wastes, waste sludge and waste water are the cheap sources of biohydrogen production (Kapdan and Kargi, 2006). The concept of biological hydrogen production with simultaneous wastewater treatment is an ideal method for pollutant removal and energy production (Vatsala *et al.*, 2008). There are many industries where water is utilized in huge quantities such as food, paper and textiles industries. At some places and for some industries, processes for recycling of water have been developed. Hence fermentative hydrogen production from waste water can be included as one of the method for biological treatment of wastewater.

Consortium of microorganisms work together for degradation of organic waste present in industrial effluents. Basically acid forming bacteria such as *Clostridium* sp., *Enterobacter* sp., *Lactobacillus* sp. and many more comes under the category of hydrogen producing bacteria (Tanisho and Ishiwata, 1995 ; Yokoi *et al.*, 1998 ; De Sa *et al.*, 2013; Nakashimada *et al.*, 2002) . Production of biohydrogen is being carried out in different types of fermentors, under different operating conditions. Mesophilic temperature is the best suited range for biohydrogen production. At pH in between 5-6 has been found most suitable as this range does not allow the growth of methanogenic bacteria which are the consumers of biohydrogen (Fang and Liu, 2002). State of the bacteria that is free or immobilised also effects the production as free state can result into over growth of bacteria in the waste water which will need further removal as an additional step for biological treatment of waste water. Hence, many of work has been done for continuous production of biohydrogen by different types of immobilized cells which can be reused again and again (Zhu *et al.,* 1999).

Therefore, the present study is focused on biological hydrogen production from rice mill effluent as a substrate. This study aimed to determine the favourable operational conditions for biological hydrogen production by using immobilized culture of a gram negative rod shaped bacteria *Klebsiella oxytoca* 13182. Also the different physicochemical parameters were analysed before and after the fermentation process to check the water quality.

MATERIALS AND METHODS

Collection, maintenance and assessment of physicochemical parameters of rice mill effluent: Rice mill effluent was collected in a clean plastic container from Khandelwal Rice mill, Tatibandh Raipur, Chhattisgarh. It was brought to the laboratory and different physicochemical parameters, pH, colour, odour, BOD, COD and turbidity was analyzed. Then it was kept at low temperature (4-5°C) for further study.

Bacterial strain and media: *Klebsiella oxytoca,* strain was taken from S.o.S in biotechnology for the fermentative biohydrogen production. The culture was grown in 100 mL nutrient broth for 24 h at 37°C.

Effect of temperature: Effect of different temperatures between 30-50°C was studied on biohydrogen production without maintaining the initial pH of the substrate.

Effect of pH: Effect of different pH was studied on biohydrogen production after optimization of temperature.

Immobilization: The 24 h old culture of bacteria was immobilized in different concentrations of calcium alginate ranging from 1-5%, also containing 0.2% of activated charcoal to increase the pore size.

Effect of hydraulic retention time: Hydraulic Retention Time (HRT) has a great effect on continuous biological hydrogen production. Three HRT were considered for this study, 24, 48 and 72 (HRT).

Effect of metal ions: Hydrogenase enzyme present in some bacteria are responsible for biohydrogen production. To enhance the activity of these enzymes different metal ions, Fe and Co were used. Ferrous sulphate and Cobalt nitrate were added to the waste water in different concentrations to see its effect.

Physicochemical analysis of waste water after fermentation: All the parameters of physicochemical analysis assessed before fermentation, were analysed after the fermentation also to check the water quality.

Statistical analysis: A complete randomized design was done in every experiment. Mean standard deviation and standard errors were calculated. The data were subjected to one way analysis of variance (ANOVA) to assess treatment differences and interaction using the SPSS version 6.0 significance between means was tested by DMRT's Test ($p \leq 0.05$).

RESULTS AND DISCUSSION

Physicochemical analysis of water before and after fermentation: The effluent sample was analysed for different physicochemical parameters before and after the biohydrogen production and it was found that pH of the raw effluent reduced from 8.2-6.5, when the initial pH of the water was not adjusted for the experiment. Turbidity from 110 NTU reduced to 89 NTU also with the reduction in TSS and TDS which were initially recorded as 113 and 450 mg L^{-1}, respectively were got reduced to 90 and 380 mg L^{-1}. Raw effluent was containing a very high amount of COD 580 mg L^{-1} which was reduced to 240 mg L^{-1}. BOD of the waste water also reduced from 30-17 mg L^{-1} after the fermentative biohydrogen production with help of immobilized bacterial culture (Table 1).

Effect of temperatures: Temperature plays a critical role in affecting the kinetics of cell growth and enzymatic activity of bacteria. Therefore effect of different temperature on biohydrogen production (Table 2) was examined between 30-40°C. At 30°C production was 32.67 mL and a maximum of 61.33 mL production was observed at 40°C , with further increase in temperature from

Table 1: Physicochemical analysis of effluent before and after fermentation

Parameters	Before fermentation (mg L^{-1})	After fermentation (mg L^{-1})
pH	8.20	6.50
Turbidity	110.00	89.00
TSS	113.00	90.00
TDS	450.00	380.00
BOD	30.00	17.00
COD	580.00	240.00

Table 2: Effect of different temperature on biohydrogen production

Temperature (°C)	Production (mL)
30	32.67±0.33
40	61.33±0.66
50	45.33±0.66
60	21.67±0.88

Each set up was repeated 3 times. All the values are Mean±SE, values differ significantly at 5% as analysed by Duncan multiple range test using SPSS

Table 3: Effect of different pH on biohydrogen production

pH	Production (mL)
4	22.00±0.57
5	45.33±0.33
6	67.33±0.88
7	20.33±0.33

Each set up was repeated 3 times. All the values are Mean±SE, values differ significantly at 5% as analysed by Duncan multiple range test using SPSS

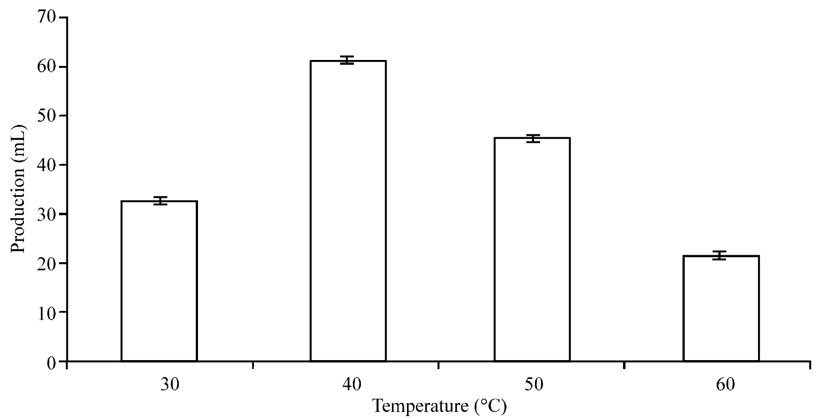

Fig. 1: Effect of different temperatures on biohydrogen production

50-60°C the production were 45.33 and 21.67 mL, respectively. Therefore, with increase in temperature growth of bacteria was strongly inhibited leading to a sharp decline in production. Therefore, among the temperature examined, 40°C seemed to be the best temperature for biohydrogen production (Fig. 1). Temperature and pH affects the activity of bacteria directly by effecting the production of biohydrogen. Veena *et al.* (2012) reported 35°C and pH 5 best for the production of biohydrogen. Suspended and immobilized cultures are preferred for this process.

Effect of pH: Change in pH, effects the growth and metabolic activity of bacteria as change in pH is responsible for affecting the enzymatic activity of hydrogenase enzyme. Effect of different pH range between 4-7 was studied on biohydrogen production (Table 3) and it was found that at pH 6 maximum production of 67 mL was recorded. Further increase in pH resulted in decrease in the production which was recorded as 20 mL (Fig. 2).

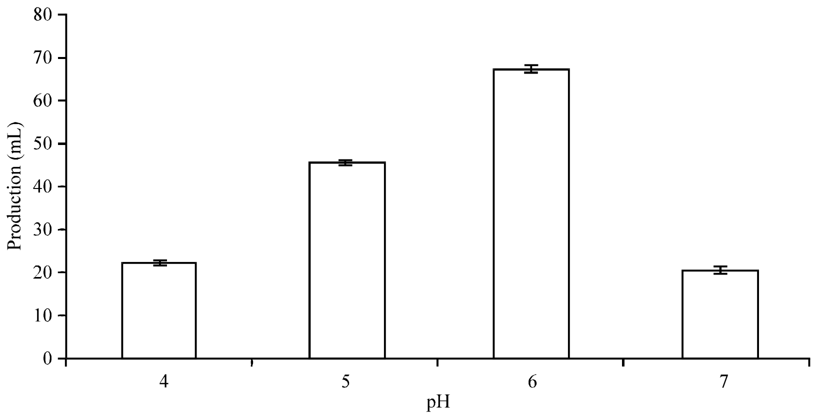

Fig. 2: Effect of different pH on biohydrogen production

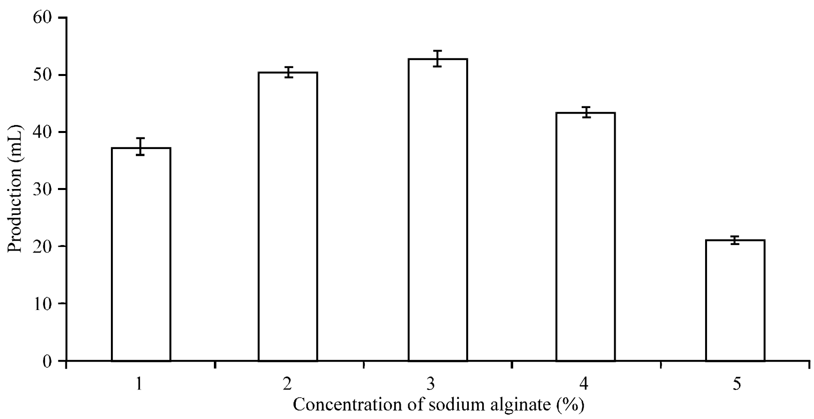

Fig. 3: Effect of different concentration of sodium alginate on biohydrogen production

Table 4: Effect of concentration of sodium alginate on biohydrogen production

Concentration of sodium alginate	Production (mL)
1	37.33±1.45[b]
2	50.33±0.88[d]
3	52.67±1.45[d]
4	43.33±0.88[c]
5	21.00±0.57[a]

Each set up was repeated 3 times. All the values are Mean±SE, means followed b different latters values differ significantly at 5% as analysed by Duncan multiple range test using SPSS

Effect of immobilized beads on biohydrogen production: Different concentrations of sodium alginate with 0.2% activated charcoal showed variegated effect, maximum mean production was found to 50.33±0.88 mL at 2% of sodium alginate and 52.67±1.45 mL at 3% concentration of sodium alginate with no significant difference (Table 4). By increasing the concentration of sodium alginate up to 5% gradual decrease in the production of biohydrogen was observed (Fig. 3). Calcium alginate is the most frequently used immobilization material due to easy preparation method but does not show mechanical stability. Use of certain supplementary material such as activated charcoal can increase the strength as well as porosity of the beads (Wu *et al.*, 2006a). Different

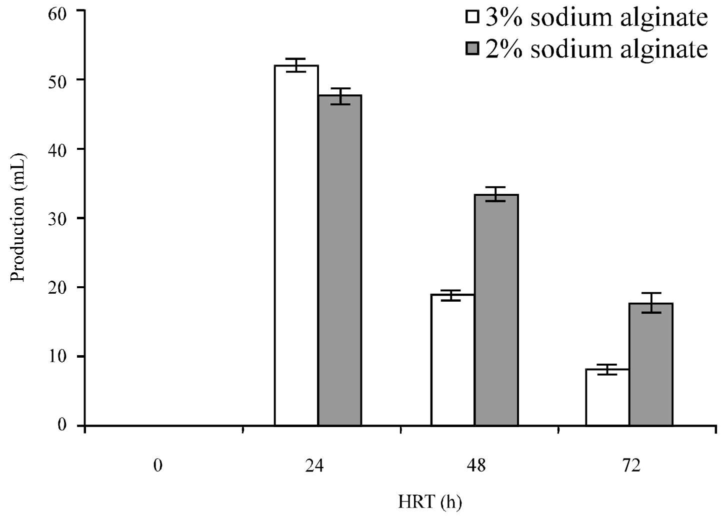

Fig. 4: Effect of different concentration of sodium alginate on biohydrogen production at different HRT

Table 5: Effect of HRT on biohydrogen production at different concentrations of sodium alginate

HRT (h)	Production 3% SA (mL)	Production 2% SA (mL)
0	0.0	0.0
24	51.67±0.88[c]	47.33±1.20[c]
48	18.67±0.67[b]	33.33±0.88[b]
72	08.00±0.57[a]	17.67±1.45[a]

Each set up was repeated 3 times. All the values are Mean±SE, means followed b different latters values differ significantly at 5% as analysed by Duncan multiple range test using SPSS. SA: Sodium alginate

concentrations of sodium alginate also effects the porosity and stability of sodium alginate which is directly proportional to the microbial activity of immobilized bacterial cell hence effecting the production of biohydrogen (Wu *et al.*, 2002). Best production of biohydrogen with 2% concentration of sodium alginate in combination with different types of carrier agents has been reported.

Effect of HRT on biohydrogen production: When effect of concentration of sodium alginate was studied, 2 and 3% gave maximum production with no significance difference hence another experiment was designed to see the mean production. Effect of HRT (Hydraulic Retention Time) was performed with 2 and 3% of sodium alginate to see the overall production (Fig. 4). It was found that although 3% SA was giving maximum production at 24 HRT but a reduction of more than 50% was observed at 48 and 72 HRT (Fig.2). In case of 2% though the initial production was less than 3% SA, reduction in production on 48 HRT was only 30% and the overall production on 72 HRT was 98.33 mL. With 3% SA after 72 HRT it was only 78.34 mL (Table 5). Therefore, 2% sodium alginate gave overall more production as compared with the other. Different HRT has different effect on biohydrogen production (Wu *et al.*, 2006b; Zhang *et al.*, 2006). Initially increase in biohydrogen with increases with HRT, with a gradual decrease in production when the HRT was further increased, due to depletion of carbohydrate source (Yusoff *et al.*, 2010).

Effect of metal ions: Effect of Fe^{2+} and Co was studied with a concentration range of 50-100 mg L^{-1} on biohydrogen production and it was found that with increasing concentration

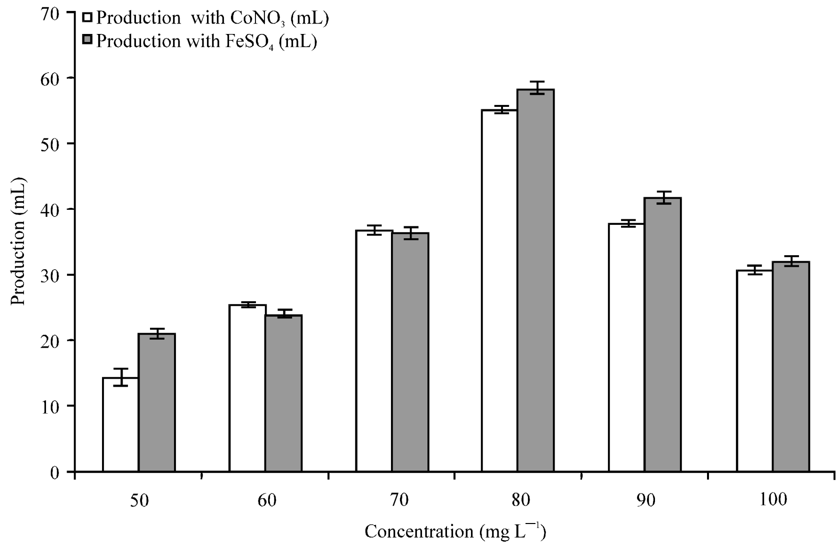

Fig. 5: Effect of Co^{2+} and Fe^{2+} on biohydrogen production

Table 6: Effect of metal ions on biohydrogen production

Concentration (mg L^{-1})	Production with $CoNO_3$ (mL)	Production with $FeSO_4$ (mL)
50	14.33±1.20[a]	21.00±0.57a
60	25.33±0.33[b]	24.00±0.57[b]
70	36.67±0.67[d]	36.33±0.88[d]
80	55.00±0.57e	58.33±0.88[f]
90	37.67±0.33[d]	41.67±0.88[e]
100	30.67±0.66[c]	32.00±0.57[c]

Each set up was repeated 3 times. All the values are Mean±SE, means followed b different latters values differ significantly at 5% as analysed by Duncan multiple range test using SPSS

of ions production also increased to maximum 55.00±0.57 and 58.33±0.88 mg L^{-1} at 80 mg L^{-1} concentration, respectively (Fig. 5). Further increase in concentration of ions resulted in decreased production of biohydrogen (Table 6). In anaerobic fermentation processes metal deficiency occurs, by addition of some trace metal ions production of biohydrogen can be enhanced. Liu *et al*. (2009) also found best production at 80 µmol L^{-1} of Fe^{2+} whereas Ying *et al*. (2010) has reported maximum production at 500 mg L^{-1} from molasses waste water as substrate. Fe^{2+} has been reported to be one of the best ions to enhance the metabolic activity of biohydrogen production efficiency of the fermentative bacteria.

CONCLUSION

This study was carried out to determine fovorable operational condition for biological hydrogen production from rice mill effluent as a substrate. Different physicochemical parameters were also analyzed before and after fermentation to check water quality. Results of study revealed that rate of biohgdrogen production was found better at concertration of 2% sodium alginate. It was concluded that at 2% sodium alginate, $FeSO_4$ (80 mg L^{-1}) and $CoNO_3$ (80 mg L^{-1}) gave better biohydrogen production rate.

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