Isolation and Characterization of Biohydrogen Producing Bacteria from Rice Bran with Optimization of Different Parameters

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Abstract

Biohydrogen is a renewable source of energy and is an alternative to the traditional fuels because of its less harmful impact on the environment. Anaerobic mixed fermentative biohydrogen production was carried out using rice bran as the substrate, a by-product of the rice milling industry. A total of six bacterial sp. were isolated and screened, out of which bacterial sp. B1 and B6 were selected for further biochemical characterization and for parameters optimization. Pretreatment of the substrate was carried to make production more rapid and efficient. The effects of Hydraulic Retention Time (HRT), temperature and pH were studied. The maximum biohydrogen production was 112 ml at 48 hours of Hydraulic Retention Time, 92 ml production at 35 ºC and 180 ml at initial pH 8. The co-culture of inoculum B1 and B6 was efficient in biohydrogen production.

Keywords: Biohydrogen, Displacement method, Pre-treatment, Rice bran

1. Introduction

Hydrogen, a potential source of energy offers advantages of being renewable, high energy-density (122 kJ/g) and cleanliness as it gives water upon combustion thus causing no harm to the atmosphere and preventing the green house gases which are major contributors toward global warming (Hawkes et al., 2002 and Wang and Wan, 2009). Anaerobic fermentative biohydrogen production holds an importance because of its simple operation, cost effectiveness as it uses a wide variety of organic wastes that serve as renewable source of energy (Xing and Zhang, 2005). Fermentative production can be carried out at ambient temperature and pressure (Nishio and Nakshimada, 2004; Das and Verizoglu, 2001). The high naturally occurring microbial diversity allows the process to make use of variety of substrates and mixed culture thus allows the process to operate more easily and rapidly by dealing with a variety of substrates (Kleerebezem, 2007). The use of organic wastes has two important functions: it cleans up the environment by giving water upon combustion and produces energy that will be sustainable for future use (Fang and Zhang, 2004). Rice bran is a by-product of rice mill in which the brown rice gets converted to white rice. Bran particularly contains 50 % carbohydrate, 20 % protein, 15 % oil and is a rich source of starch. Bran is particularly important in dietary fibre (beta-glucan, pectin and gum), oryzanol and vitamin E potent anti-oxidant in oil and rich in B-complex vitamin (Taha et al., 2007).

The aim of the study was to optimize the conditions for maximum production of biohydrogen. Availability of the substrate, low cost, abundance and biodegradability are important for sustainable production. Thus, organic wastes as substrates will serve as a renewable form of energy source with sustainability in the future.

2. Materials and Methods

2.1 Collection of sample

Rice bran sample was collected from rice mill Kumhari, Durg, Chhattisgarh, India. The sample was stored at room temperature in air tight container.

2.2 Isolation and screening of fermentative bacteria

Bacteria were isolated from the rice bran. A 5g of rice bran was added to 100 ml of distilled water. Bacteria were isolated by serial dilution method and dilution of sample was prepared from 10^{-1} to 10^{-9}. The series 10^{-3}, 10^{-4} and 10^{-5} was used for isolation by the spread plate method (Thakur et al., 2012). Plates were incubated for 24 hrs and mixed culture of the bacteria was obtained on the plates of dilution at 10^{-3}, 10^{-4} and 10^{-5}. Pure culture was prepared for each of the bacterial colony by streak plate method and marked as B1, B2, B3, B4, B5 and B6. Fermentation medium was prepared for testing bacteria having the gas producing ability. Phenol red was used as an indicator (pH 6.8-7.0) which turns the medium from yellow to red in color. Durham tubes were placed in an inverted position in the tubes having the fermentation medium for detection of gas production. Bacteria inoculated in the tubes were then placed for incubation for 24-48 hrs (Prescott and Harley, 2002).

2.3 Biochemical characterization of selected bacteria

Different biochemical tests of the bacteria were performed for their characterization. Amylase test, Cellulase test, Casein

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hydrolysis, urease, IMViC test (indole test, methyl red test, Voges-Proskaeur test, citric acid utilization test) and catalase tests were performed for the characterization of isolated bacteria (Prescott and Harley, 2002).

2.4 Pre-treatment of the sample

B1 was found to be amylase producing bacteria which was confirmed by using starch agar medium and iodine as an indicator (Prescott and Harley, 2002). As rice bran contains complex sugar, initially the substrate was inoculated with 5 ml bacterial broth of B1 for hydrolysis of these complex sugars and B6 for utilization of these sugars for fermentative biohydrogen production. The process was carried out at 37 °C which is the optimum growth temperature for bacteria.

2.5 Experimental set up for the process

The pre-treated inoculated sample was placed in a flask on the magnetic stirrer. This flask was connected to another flask containing 10 % KOH for the absorption of carbon dioxide produced as a byproduct of the fermentation process. This flask was again connected to a measuring cylinder for collection of KOH in the process of liquid displacement to measure the production of biohydrogen (Zanchetta et al., 2007). The experiment was submerged fermentation carried out in a 250 ml conical flask.

2.6 Effect of hydraulic retention time (HRT), temperature and pH on biohydrogen production

The physical parameters have a marked influence on the production process. Hydraulic Retention Time (HRT) is the time retained by the medium in the bioreactor. The influence of retention time on the production was studied by varying the time period from 24 hrs to 148 hrs. Both temperature and pH have a profound effect on the metabolic activity of microorganism's and their conversion process. Each microorganism has its activity at a specific temperature and pH. The effect of temperature on biohydrogen production was studied by varying the temperature from 35 °C- 50 °C. The effect of pH was also studied on the biohydrogen production by raging the initial pH of the medium from 4, 5, 6, 7, 8, 9.

2.7 Effect of immobilized and suspended form of bacteria on biohydrogen production

Bacterial cultures were taken in suspended as well as in immobilized state to see the effect of state of bacteria in biohydrogen production. 5 ml of 24 hrs old bacterial cultures broth were mixed with 10 ml 2 % sodium alginate. This solution was taken in syringe and dropped in 0.1 M $CaCl_2$ to form sphere like structure having diameter of 0.3-0.4 mm (Wu et al., 2006).

3. Results and Discussion

3.1 Screening of amylase and fermentative bacteria

Six bacterial sp. were isolated from the rice bran sample named as B1, B2, B3, B4, B5 and B6 and each of them were screened for the production of gas. Bacterial sp. B5 and B6 were found to be gas producing and bacterial sp. B1, B2, B3 and B4 showed negative results. All isolated bacterial sp, were tested for their enzymatic activity. As rice bran is a complex source of carbohydrates, the amylase producing bacteria degrades the complex sugars to simpler forms (Taha et al., 2007). Bacterial sp. B1, B2, B4, B5 and B6 were found to be amylase positive with B1 showing the highest activity. It was studied by Lui et al. (2008) that the hydrogen-producing ability of hydrogen-producing bacteria was enhanced when mixed cultured bacteria were used. Thus, in the further study, B1 (amylase producing) and B6 (fermentative) co-culture was used.

3.2 Characterization of hydrogen producing microorganism

3.2.1 Microscopic characterization of selected bacterial sp.

In total six bacterial sp. were isolated from the sample. Colony characterization of isolated bacterial sp. was done. As shown in Table 1 the bacterial sp. was characterized based on morphological characters. Bacterial sp. B1 was found to be rod in shape, gram negative, non-acid fast, non endosporic and motile as shown in Table 2. Zhang et al. (2007) identified the spore forming rod shaped during their studies on enhanced biohydrogen production from corn stalk wastes with acidification pretreatment by mixed anaerobic cultures. Vatsala et al. (2008) found rod shaped, gram negative, motile bacteria during the studies of pilot-scale biohydrogen production from distillery effluent using defined bacterial co-culture. These results were similar to bacterial sp. in this study. Bacterial sp. B6 was rod shaped gram negative, non acid fast, endosporic and motile as shown in Table 2. Fang et al. (2001) revealed that there were two predominant bacterial sp. The fusiform *Bacilli* and spore forming rod shaped bacteria and illustrated that the presence of endospore, is characteristics of hydrogen producing bacteria. Fang et al. (2006) reported isolation of rod shaped bacteria during their studies on acidophilic biohydrogen production from rice slurry and by further molecular characterization of bacteria confirmed it as *Clostridium* species. Thus, the characteristics were similar to bacterial sp. in this work.

Table 1. Colony characterization of bacteria isolated from rice bran

S. No	Bacterial sps.	Form	Elevation	Margin	Opacity	Colour	No. of colonies
1	B1	Irregular	Flat	Filamentous	Opaque	White	13
2	B2	Irregular	Flat	Rhizoidal	Opaque	Cream	12
3	B3	Small irregular	Flat	Lobate	Opaque	Cream	6
4	B4	Irregular	Flat	Entire	Opaque	Cream	8
5	B5	Punctiform	Flat	Entire	Opaque	Yellow	Numerous
6	B6	Small circular	Flat	Lobate	Opaque	Cream	Numerous

Table 2. Microscopic characterization of bacteria isolated from rice bran

S. No	Characters	B1	B2
1	Simple staining	Rod	Rod
2	Grams staining	Gram negative	Gram negative
3	Acid fast staining	Negative	Negative
4	Endospore staining	Negative	Positive
5	Motility test	Positive	Positive

3.2.2 Biochemical characterization of the selected bacterial sp.

Biochemical characterization of isolated bacterial sp. was done. Bacterial sp. B1 showed positive carbohydrate fermentation test (glucose, fructose, sucrose, mannitol, maltose, sorbitol) positive amylase test, positive cellulase test, positive casein, positive urease test, positive indole test, positive methyl red test and negative results for Voges-proskauer test, citrate utilization test and catalase test (Thakur et al., 2012).

Table 3. Biochemical characterization of fermentative bacteria isolated from rice bran

S. No.	Biochemical tests	Bacteria B1	Bacteria B6
1	Carbohydrate fermentation test	+ ve	+ ve
2	Amylase test	+ ve	+ ve
3	Cellulase test	+ ve	+ ve
4	Casein hydrolysis test	+ ve	- ve
5	Urease test	+ ve	- ve
6	Indole test	+ ve	+ ve
7	Methyl red test	+ ve	+ ve
8	Voges- Proskaeur test	- ve	- ve
9	Citrate utilization test	- ve	+ ve
10	Catalase test	- ve	+ve

Bacterial sp. B6 showed positive carbohydrate fermentation test (glucose, fructose, sucrose, mannitol, maltose, sorbitol), positive amylase test, positive cellulase test, negative casein test, negative urease test, positive indole test, positive methyl red test, negative Voges-proskauer test, positive citrate utilization test, positive catalase test as shown Table 3. Vatsala et al. (2008) also found similar results during the studies of pilot-scale biohydrogen production from distillery effluent using defined bacterial co-culture.

3.3 Enzymatic pre-treatment

The co-culture of bacterial sp. B1 and B6 was inoculated in autoclaved sample. This biologically pretreated substrate was subjected for biohydrogen production process. No production resulted on the first and second day of the fermented sample process. Initial rise in the production resulted on third day and further no rise in production occurred. Hence, three days of pretreatment is required for production.

3.4 Effect of hydraulic retention time (HRT)

HRT plays an important role in biohydrogen production as the time of retention in the fermentor increased after the optimum, accumulation of more and more metabolites and exhaustibility of the nutrients takes place which results in less or no production. Initially the production increases from 24 HRT which was 10 ml to 112 to 44 to 14 and to 8 ml at 48, 72, 96 and 120 respectively. But when further HRT was increased it was found that production did not increase. Therefore at 48 HRT the maximum of 112 ml production was observed as shown in Table 4.

Table 4. Effect of different HRT on biohydrogen production.

S. No.	HRT (hrs)	Production (ml)
1	24	10.00
2	48	112.00
3	72	44.00
4	96	14.00
5	120	8.00
6	148	0.00

3.5 Effect of temperature and pH

Temperature is one of the most important factor affecting production either by enhancing or inhibiting the process. Sinha and Pandey (2011) and Thakur et al. (2014) studied the affect of temperature on the growth and their pathway and concluded that, mesophilic temperatures best enhance the production. Herbert et al. (2006) conducted fermentation reactions at (25–40 ^oC) mesophilic, (40–65 ^oC) thermophilic, extreme thermophilic (65–80 ^oC), or hyperthermophilic (>80 ^oC) temperatures and concluded that dark fermentation favours best at 25-40 ^oC. In the present work, maximum production was found at 35 ^oC with 92 ml production and rise in further temperatures decreased the production process as shown in Table 5. So, mesophilic temperature was the optimum for biohydrogen production. pH is one of the vital factor influencing the hydrogenase activity of hydrogen producing and fermentative H_2 producing bacteria. The study of pH resulted in 180 ml of biohydrogen at pH 8 and further increase in pH decreased the production as shown in Table 6.

Table 5. Effect of different temperature on biohydrogen production

S. No.	Temperature (^oC)	Production (ml)
1	35	92
2	40	86
3	45	52
4	50	36

Table 6. Effect of different pH on biohydrogen production.

S.No	pH	Production (ml)
1	4	0
2	5	0
3	6	30
4	7	45
5	8	130
6	9	90

3.6 Effect of immobilized and suspended form of bacteria on biohydrogen production

Effect of suspended and immobilized bacterial culture was studied on biohydrogen production and it was observed that initially at 24 HRT suspended form gave production of 10 ml which increased to 112ml at 48 HRT and the started decreasing with further increase in HRT to 72 and 96 it resulted in decreased production i.e. 54 ml and 14 ml respectively. When effect of immobilized bacterial culture was studied it was found that initially at 24HRT the production was 7 ml, at 48, 72 and 96 HRT the production were 83, 20 ad 20 ml respectively as shown in Table 7.

Table 7. Effect of immobilized and suspended form of bacteria on biohydrogen production at different HRT.

S.No	HRT (hrs)	Suspended form	Immobilized form
1	24	10	7
2	48	112	83
3	72	54	20
4	96	14	20

3.7 Confirmatory test of hydrogen

Presence of hydrogen was confirmed by flame test, hydrogen when burns produce popping noise (Paul et al. 2014).

4. Conclusions

The utilization of rice bran for biohydrogen production is a cost-effective and environmentally friendly approach. Rice bran serves as a potential source rich in carbohydrates which is utilized the microorganisms. A total of six bacterial sp. were isolated and screened for biohydrogen production, from which the bacterial sp. B1 and B6 were selected for their high amylase and fermentative ability. Further these bacteria were biochemically characterized. Enzymatic pretreatment of three days was found to enhance the production process. The maximum production was 112ml of biohydrogen on 48 hours. The maximum biohydrogen production with optimized temperature was 92 ml at 35 ^oC, and 130 ml at pH 8 and 48 hrs incubation the production was 112 ml was found. Effect of immobilized and suspended form of bacteria on biohydrogen production at different HRT was compared and maximum of 83 ml at 48 HRT was found whereas suspended bacteria gave maximum production of 112 ml at 48 HRT. Waste biomass can serve as a best source for production of a non-polluting fuel. Further work is in progress for optimization of other effecting parameters for enhanced biohydrogen production from rice bran.

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