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Biohydrogen fuel from cow dung-enriched cellulose rich microcrystalline betel nut shells

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ABSTRACT

Sustainable renewable biohydrogen fuel is produced by microbial conversion of acid hydrolysed cellulose rich microcrystalline betel nut (*Areca catechu*) shells enriched with cow dung. Gram positive, rod shaped, motile, obligatory anaerobic, spore forming, catalase test negative, indole test positive, methyl red test positive, Voges-Proskauer test negative, citrate test negative, cellulase producing, sulphate reducing organism that utilises glucose, fructose, lactose, and sucrose with acid and gas were isolated from aging cow dung collected from South Kanara district, and the strain identified as *Clostridium acetobutylicum* was used for subsequent study. Cellulose rich microcrystalline betel nut shells were hydrolysed by 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 4.0 and 5.0% of either sulphuric acid or hydrochloric acid. Hydrochloric acid at 5% for 2 hours was efficient in hydrolyzing cellulose compared to other as it produced highest percentage of glucose of 0.23g/ml. Acid hydrolysed microcrystalline betel nut shell and pulverized dry aging cow dung at the ratio of 1:1, 1:2 and 2:1 were sterilized by Tyndallisation method, and inoculated with 100mL of culture broth of *Clostridium acetobutylicum* per 1000 mL of the mixture. Hydrochloric acid hydrolysed microcrystalline betel nut shell at 5% and pulverized dry aging cow dung at the ratio of 1:2 inoculated with culture of *Clostridium acetobutylicum* produced hydrogen gas of 260 mL/g, hence has a potential to produce biohydrogen.

KEYWORDS

Cow dung;
Biohydrogen;
Betel nut;
Microcrystalline cellulose;
Acid hydrolysis.

INTRODUCTION

Biohydrogen as a fuel is a fascinating way to provide a sustainable renewable energy source, as only byproduct of reacting hydrogen with oxygen is water and no carbon dioxide or other greenhouse gases are produced. Biohydrogen is a potential biofuel produced commonly by bacteria through number of biological process using waste organic materials. Since hydrogen gas is much lighter than air, it rises fast to quickly eject from the atmosphere, and hence not found in the earth. However, this is compounded with other elements such

as water, methane, coal and petroleum. Growing biomass is the good source of hydrogen and this energy carrier has the highest energy content of any common fuel by weight that is about three times more than gasoline, but the lowest energy content by volume that is about four times less than gasoline. Even though hydrogen is not presently been used widely as an energy carrier, it has potential as an energy carrier in the future.

Hydrogen can be produced from variety of resources such as water, fossil fuels, or biomass and is a byproduct of other chemical processes. Hydrogen is generated in the industry from fossil fuels by steam reforming and the electrolysis of water produces hydrogen at a price that is always affected by the cost of the available electricity. Hence, utilisation of abundantly available cheap cellulose wastes for bacterial production of hydrogen gas has a greater potential in overcoming this difficulties and also helps us to reduce the environmental burden. Microorganisms such as *Cyanobacteria spp.*, *Clostridium spp*., *Rhodobacter sphaeroides* and *Enterobacter cloacae* are known to produce biohydrogen. Of which biohydrogen produced from organic waste materials by microorganisms such as *Clostridium spp*. to reduce oxidized substrates is a promising alternative as a sustainable energy sources[1]. Hydrogen production using cellulose has received lots of attention of recently because of its abundance in earth and potentials of hexose and pentose sugars of the cellulosic material for the production of fuel. Such cellulosic materials abundantly available in nature can be degraded by microbial consortia consisting of cellulolytic and non-cellulolytic bacteria depending on the substrate. Hydrogen production from celluloses is a complex task, as we need to have a combination of high-active cellulose-hydrolyzing bacteria and hydrogen-producing bacteria for synergistic hydrogen production, and in nature all highly efficient hydrogen-producing organisms are either none or low-cellulose-degrading fermentative bacteria[2]. This complexity can be overcome by hydrolyzing cellulose by acids such as sulfuric acid and hydrochloric acid to facilitate the subsequent production of hydrogen by hydrogen-producing bacteria[3].

Lay[4] investigated the potential of producing hydrogen from microcrystalline cellulose under mesophilic digestion condition using heat-shocked digester (HSD) sludge, and he concluded that *Clostridium spp.* was the predominating hydrogen-producing bacteria in the HSD sludge and the hydrogen percentage in the headspace of the digesters was greater than 50%. He further stated that methanogenesis was observed and hydrogen significantly inhibited the hydrogen-producing activity of sludge when initial microcrystalline cellulose concentrations exceeded 25g/L. Lo and others[5] reported that mixed culture of cellulosic-hydrolysis sludge and *Clostridium pasteurianum* produced 1.09 mmol of hydrogen per gram of cellulose from 10g of l-1 carboxymethyl cellulose. Ren and others[6] produced hydrogen from cellulose at rate of 272 ml/g cellulose (2.09 mol/mol-hexose) at substrate concentration 10 g/l by the cow dung compost enriched continuously in defined medium containing cellulose, and he concluded that cow dung compost enriched cultures were ideal microflora for hydrogen production from cellulose.

Biomethane gas production is very popular and success story in the villages of Western Ghat. However, hydrogen has been considered as one of the most potential energy for the future, as in one hand it produces only water upon combustion, and on the other hand it is advantageous with respect to the energy security and environmental protection. Biohydrogen production from renewable aging beetle nut shell wastes available abundantly in the villages of Western Ghat, and is been an exciting area of bio-energy production because of its environmental friendly and energy saving process. The bio-conversion of aging beetle nut shell wastes into hydrogen is challenging to us because of its complex chemical structures and hard biodegradation. Majority of study on hydrogen production, are confined to using pure carbohydrates and carbohydrate-rich wastewater or straw that is commonly used as feed. No work has been done on pretreated aging beetle nut shell wastes enriched with cow dung manure as a source of hydrogen. The pretreatment of aging beetle nut shell wastes plays a vital role in the effective conversion of cellulose or cellobiose into cellulose hydrogen by mixed culture[3]. The anaerobic atmosphere is beneficial to the enzymatic hydrolysis of beetle nut and the hydrogen production using microflora isolated from aging cow dung. Hence this work has great potential as it generates employment, produces environmental friendly fuel, efficient, and additional income for the village folks. In this study, we

have hydrolysed microcrystalline cellulose of Beetle nut shells by acid treatment and product of this is used as a base that in turn enriched with the cow dung to product hydrogen using hydrogen producing bacteria isolated from the cow dung available in the Dakshina Kannada District.

MATERIALS AND METHODS

Materials

Aging cow dung manure was collected from the villages near the Western Ghat of Dakshina Kannada District. Dried beetle nut shell samples were collected from the same villages after cutting opening the dried beetle nuts to separate the inner nuts. Standard buffer, chemicals and ingredients were of analytical grade and were manufactured by Merck Ltd (Mumbai, India). Glassware used for the present study was manufactured by Borosil (Mumbai, India).

Collection of samples and isolation of microorganisms

Aging cow dung and aging beetle nut shells collected from these villages were transported in sterile containers in aseptic condition to the laboratory with the lag period not exceeding four hours. 1 g of aging dry cow dung samples were suspended in a sterile 10 mL of culture medium and incubated at 70°C in water bath for 10 minutes to inactivate vegetative cells for counter selecting against non-spore formers. The tubes were subsequently incubated for 24 hours at 37°C in an anaerobic jar. Samples having gas production and increased turbidity were re-incubated for 96 hours at 37°C and checked daily for growth and gas production in sugar medium with Durham's tube, and biochemical tests were performed to identify the isolates[7].

Pretreatment of cellulose rich betel nut sample

The pretreatment of aging beetle nut shell feedstock enriched with cow dung manure will be essential for adequate conversion of the cellulose into biohydrogen. Dried aging beetle nut samples were disintegrated into coarse powder using warring blender (Philips, Bombay). Cellulose rich beetle nut sells were hydrolyzed into cellulose hydrogen by treatment with 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 4.0 and 5.0% by sulphuric acid or hydrochloric acid at ambient temperature. Acid hydrolysis was carried out by adding 10g of microcrystalline betel nut into 100 mL of sulphuric acid or hydrochloric acid maintained at the predetermined level of 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 4.0 or 5.0% in tightly capped containers at ambient temperature for 2 hours. Hydrolysis is stopped by neutralizing the acids by slightly reversing the pH with the addition of predetermined quantity of concentrated potassium hydroxide solution, and the quantity was noted down for subsequent estimations. Hydrolyzed beetle nut samples were sterilized by Tyndallisation method, where solution was free steamed for one hour on first day and for thirty minutes on the next two successive days.

Production of biohydrogen using cellulose rich betel nut samples

Biohydrogen was produced in anaerobic condition in batch culture using sterilized microcrystalline beetle nut shells feedstock enriched with cow dung manure at 1:1, 1:2 or 2:1 proportion. Sterilized microcrystalline beetle nut shells feedstock enriched with cow dung manure at different combinations were sterilized before further processing by Tyndallisation method, where solution was free steamed for one hour on first day and for thirty minutes on the next two successive days. Gas produced is collected in the collector and measured. Production of biohydrogen was estimated using a simple test, as biohydrogen has no color or smell, has no effect on moist litmus paper or moist universal indicator paper, and burns with typical 'pop' sound.

Proximate analysis

Samples were collected and analysed in triplicates. Microbiological methods were performed as per APHA method[7]. Carbohydrate such as glucose is estimated by Dinitrosalicylic acid method[9]. Gram staining, motility test, indole test, catalase test, methyl red, Voges-Proskauer test, citrate test, cellulase test, hydrogen sulphide production test, carbohydrate utilisation test, heat resistance test and glycerol utilisation test were performed on bacterial isolates[9]. Glassware and prepared media were sterilized using moist heat at 121°C for 15 minutes (ELECO, Cochin). Petri dishes, flasks and pipettes were sterilized at 180°C using dry heat for 1 hour in a hot air oven (Rotex, Kerala). Nutrient agar

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and broth used as culture media were sterilized at 121°C at a pressure of 15 lb in moist heat for 15 minutes. Sample preparations, plating were performed in laminar air flow filter hood (Kemi Labs). Standard laboratory practices were followed to avoid contamination risk.

Statistical analysis

One- and two-way ANOVA was performed using Statographics 2.1 (STSC Inc., Rock vile, and MD). The difference in means was analyzed using a Turkey HSD test ($p<0.05$).

RESULTS AND DISCUSSION

Isolation and characterisation of microorganisms

Aging dry cow dung collected from the villages were transported to the laboratory and incubated at 70°C in water bath for 10 minutes to destroy vegetative cells for counter selecting against non-spore formers. These samples were inoculated on nutrient agar and cultures were incubated at 37°C for 24 hours in an anaerobic jar. Three different types of strains having gas production in sugar medium with Durham's tubes and increasing turbidity were isolated and re-incubated at 37°C for 96, and checked daily for growth and gas production in sugar medium. These cultures were purified by single colony isolation and incubated on agar plates under strict anaerobic condition at 37°C for 12-15 days. Such isolates were characterised to identify the gas producing strains, are presented in the TABLE 1. Clostridial forms were re-streaked for purification and re-tested for gas production and spore formation.

The colonies of the isolated pure strain were mostly circular to slightly irregular, whole, raised, and dull in appearance, gram positive rods, spore forming, motile in semi-solid agar, obligatory anaerobic, non-hydrogen sulfide reducing, and showed growth optimum of 60°C, and according to this criteria all the three strains were behaved like member of Clostridia. Along with this, strain-I was only indole positive and identified as *Clostridium acetobutylicum*, Strain-II only utilised glycerol as carbon source and identified as *Clostridium butylicum*, and Strain III was neither showed positive indole test results nor utilised glycerol as carbon source

TABLE 1 : Biochemical tests for differentiating strains isolated from aging cow dung

Tests		Strain-I	Strain-II	Strain-III
Gram staining		Gram positive bacilli	Gram positive bacilli	Gram positive bacilli
Spore forming		+	+	+
Motility		+	+	+
Catalase		-	-	-
Indole		+	-	-
Hydrogen sulfide reduction		-	-	-
Methyl red		+	+	+
Voges proskauer		-	-	-
Citrate		-	-	-
Cellulase		+	-	+
Heat stability		Stable at 60°C for 30min	Stable at 60°C for 30min	Stable at 60°C for 30min
Sugar utilisation	Glucose	Acid and Gas	Acid and Gas	Acid and Gas
	Sucrose	Acid and Gas	Acid and Gas	Acid and Gas
	Lactose	Acid and Gas	Acid and Gas	Acid and Gas
	Fructose	Acid and Gas	Acid and Gas	Acid and Gas
Glycerol utilisation		-	+	-

and was identified as utilised glycerol as carbon source and was identified as *Clostridium beijerinckii*. However, all the strains produced acid and gas in the sugar test within 24 hours. The strains that grew and produced gas in sugar medium within 24 hours were considered as better source for the production of hydrogen than those that showed same results latter than 48 hours. Keeping within the scope of the present work, only the strain *Clostridium acetobutylicum* is considered for the subsequent work.

Acid hydrolysis of microcrystalline betel nut shells

Cellulose rich beetle nut sells were hydrolyzed by treating with 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 4.0 and 5.0% sulphuric acid or hydrochloric acid at ambient temperature in a tightly capped containers for the period of 2 hours. At the end of this process, mixture is neutralized by adding predetermined quantity of concentrated potassium hydroxide solution, and was sterilized after neutralization by Tyndallisation method, where solution was free steamed for one hour on first day and for thirty minutes on the next two successive days. Percentage of the glucose produced by this process is estimated by Dinitrosalicylic acid method[8]; keeping in mind the dilution occurred during the neutralization process. Concentration of glucose in either sulphuric acid or hydro-

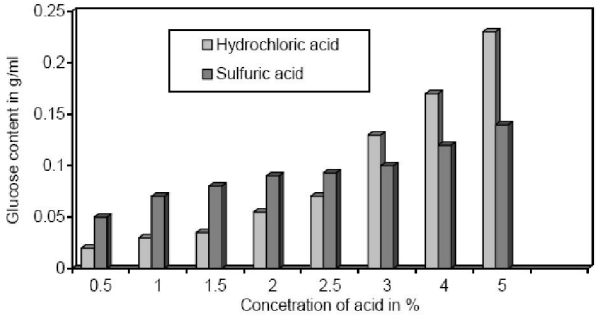

Figure 1 : Percentage of gluocose in acid hydrolysed microcrystalline betel nut shells

chloric acid hydrolysed samples is represented in the figure 1. Percentage of glucose available in acid hydrolysed mixture increased ($p<0.05$) with increase in the concentration of either sulphuric acid or hydrochloric acid. Two hours digestion of microcrystalline betel nut shell at 0.5% of sulfuric acid produces 0.02 g/ml of glucose or at 0.5% of hydrochloric acid produces 0.05g/ml of glucose, and on the other hand two hours digestion of microcrystalline betel nut shell at 5% of sulfuric acid produces 0.23 g/ml of glucose or at 5% of hydrochloric acid produces 0.14 g/ml of glucose. Here it is interesting to note that up to 2.5% sulfuric acid is effective ($p<0.05$) in hydrolyzing cellulose to glucose compared to hydrochloric acid, but beyond 2.5 % hydrochloric acid is effective ($p<0.05$) in hydrolyzing cellulose to glucose compared to sulfuric acid. At 5 % hydrochloric acid production of glucose was 1.64 folds greater compared to 5 % sulfuric acid, however at 0.5 % sulfuric acid production of glucose was 1.66 folds greater compared to 0.5 % hydrochloric acid. It is very important to note here that unlike bacterial cellulose degradation, acid hydrolysis of the cellulose into glucose can be manipulated by varying the concentration of the acids and type of acid. However, much work has to be done to establish concrete results in this regard. Glucose produced here is the starting material for the production of hydrogen. Microcrystalline betel nut shells hydrolysed by 5% hydrochloric acid for two hours will be used for subsequent processing.

Formulation of biomass and production of biohydrogen

Conversion of glucose into product such as hydrogen by *Clostridium acetobutylicum* is very interesting, because the raw material used for doing so abundant and renewable, the whole world will be depending on this for food, fuel and chemical supplies in future. Pulverized and hydrolysed betel nut shells and cow dung at the ratio of 1:1, 1:2 and 2:1 were air tightly packed in the 1000mL Erlenmeyer flask, plugged by cotton and were sterilized by Tyndallisation method, where solution was free steamed for one hour on first day and for thirty minutes on the next two successive days. Each flask was inoculated with the 100mL of culture broth of *Clostridium acetobutylicum*, and connected to biohydrogen production unit and incubated at ambient temperature on successive days. Hydrochloric acid hydrolysed at 5% microcrystalline betel nut shells and cow dung at the ratio of 1:1 1:2 and 2:1 inoculated with culture of *Clostridium acetobuty- licum* produced hydrogen gas of 220 mL/g, 260mL/g and 216 mL/g, respectively. In the present study productivity is not that high. No concrete ($p<0.05$) conclusions can be made out of the former result, and to arrive at conclusive remarks we need to make lots of work.

CONCLUSION

Biohydrogen is the energy of the future as it is a clean energy source with high energy content. Even though microorganism can convert cellulose into glucose, acid hydrolysis is promising method as it can be manipulated by varying the concentration of acid and type of the acid at room temperature to improve productivity. In one hand, 5 % hydrochloric acid was effective in producing glucose compared to 5 % sulfuric acid, and on the other hand at 0.5 % sulfuric acid was effective in the production of glucose compared to 0.5 % hydrochloric acid. Beetle nut shells are abundantly available in South Kanara District of Karnataka State as a waste product that can be enriched with cow dung to produce biohydrogen. However, much work has to be done to arrive at a conclusion to improve the state of art biohydrogen production as a sustainable energy source.

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REFERENCES

[1] I.K.Kapdan, F.Kargi; 'Biohydrogen Production From Waste Materials', R.Noris, D.W.Ribbon, C.Booth; Methods in Microbiology, Academic Press Inc., NY, **38**, 569-582 **(2006)**.

[2] S.Kota, S.Harutaa, Z.J.Cuia, M.Ishiia, Y.Igarashia; FEMS Microbiology Ecology, **51(1)**, 133-142 **(2006)**.

[3] F.Carvelheiro, L.C.Daurte, F.Girio; Journal of Scientific and Industrial Research, **67**, 849-864 **(2008)**.

[4] J.J.Lay; Biotechnol.Bioeng., **74(4)**, 280-287 **(2000)**.

[5] Y.C.Lo, M.D.Bai, W.M.Chen, J.S.Chang; Bioresource Technology, **99(17)**, 8299-8303 **(2008)**.

[6] N.Q.Ren, J.F.Xu, L.F.Gao, L.X.Jie Qiu, D.X.Su; International Journal of Hydrogen Energy, **35(7)**, 2742-2746 **(2010)**.

[7] APHA, 'Recommended Procedure for the Examination of Seawater and Shellfish', 2nd Ed., American Public Health Association, Washington, **(1982)**.

[8] G.L.Miller; Anal.Chem., **31**, 426 **(1959)**.

[9] R.Noris, D.W.Ribbon, C.Booth; Methods in microbiology, Academic Press Inc., NY, **4**, **(1971)**.