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BIOCONVERSION OF SYNGAS TO BIOETHANOL USING ACETOGEN UNDER SUBMERGED FERMENTATION

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Abstract–Crude oil extinction and booming energy consumption coupled with climate crisis to pave the way of academia & industry for development of a sustainable biofuel. Hence, economic & eco-friendly bioethanol comes into limelight which is a superior substitute of gasoline used in transportation sector as it doesn't contribute noxious gases to global warming. This research deals with synthesis of bioethanol through syngas fermentation using acetogens. Therefore, in toto 07 acetogens were isolated from compost sample in ATCC medium no. 1754 under anaerobic condition. Among them, the acetogen S1 a has the ability to produce bioethanol as confirmed from pyridinium chlorochromate assay. The screened acetogen produced 1.44% of bioethanol through syngas (60% CO+ 20% CO_2+ 15% H_2+ 5% CH_4) fermentation carried out at 37 ^oC for 72 hours in aspecially designed culture bottle. The bioethanol production and its concentration was confirmed by GC analysis. The bioethanol producing acetogen was identified as *Clostridium* sp. S1a by API 20A system. Further, bioethanol production under optimized condition and molecular characterization of the acetogen is highly essential in this regard.

INTRODUCTION

Over the last eras, rapid industrialization coupled with population explosion amplified global energy consumption. It has been accomplished by crude oils which causes environmental pollution that eventually led to global warming. As, these crude oils are in the verge of extinction and also its production is predicted to be declined from current 30 to 5 billion by 2050 (Puttaswamy *et al*., 2016). Thus, it is the need of the hour to search for a suitable alternative transportation fuel from the renewable sources like, lignocellulosic biomass (LCB) (Wyman, 2012). Recent advancement in agricultural and industrial sector proposed economic and eco-friendly technology for bioethanol production from LCB. As bioethanol depicts various excellent properties such as, high heat of vaporization, flame speed and restricted flammability, thus it needs to replace gasoline in transportation fuel. Further, bioethanol is the only transportation fuel that doesn't subsidize to greenhouse gas effect as the CO_2 released during its burning reused by plant through photosynthesis process (Brooks, 2008; Gnansounou and Dauriat, 2005).

Earlier bioethanol production was achieved by thermo-chemical(Fischer-Tropsch) and bio-chemical process. In biochemical process, LCB is converted to simple sugar by enzymatic or acid hydrolysis followed by fermentation to produce bioethanol. However, excessive pre-treatment, high operational cost, toughness and fussiness of the above two process shifted attention of researchers towards syngas fermentation (Griffin and Schultz, 2012). On account of that, intensive research is going on since last decade for bioconversion of LCB such as, rice straw, corn stover, wood chips, saw dust and sugarcane bagasse to bioethanol through syngas fermentation under second generation technology (2G). In syngas fermentation, syngas act as a flexible source of energy for acetogens to produce various

value-added products. (Mohammadi *et al*., 2012). Acetogens are the anaerobic or facultative, chemolithotrophic bacteria that are able to utilize unicarbon components of syngas through Wood-Ljungdahl pathway to produce several value-added organic products like bioethanol, acetate and butanol (Sim *et al*., 2008; Elshahed, 2010).

Reports are available in favour of bacteria such as, *Clostridium carboxidivorans*, *Clostridium ljungdahlii*, *Clostridium ragsdalei*, *Butyribacterium methylotrophicum*, *Clostridium autoethanogenum* for bioethanol production through syngas fermentation (Phillips *et al*., 1993; Shen *et al*., 2014a; Maddipati *et al*., 2011; Bengelsdorf and Durre, 2012). Bioethanol production through syngas fermentation have a flourishing future as it will reduce utilization of crude oils and environmental pollution by proper waste disposal. Despite of several advantages, still the ratio of bioethanol to acetate (1:20) production through syngas fermentation, which is developing a point of interest among the researchers for higher bioethanol production (Mohammadi *et al*., 2012). Moreover, lack of high bioethanol yielding strains and advancements in the bioprocess technology holding back the thriving commercialization of syngas fermentation. Therefore, objective of the study is to screen and monitor bioethanol production from LCB through syngas fermentation using acetogens.

MATERIALS AND METHODS

Sample collection and isolation of acetogens

In this study, compost samples were collected aseptically from Baramunda, Bhubaneswar, Odisha for bacteriological analysis. Isolation of acetogens was conducted by enrichment culture technique where, 1g of compost sample was inoculated to 99 ml of ATCC medium no. 1754 containing NH_4Cl 1g, KCl0.1g, $MgSO_4$. $7H_2O$0.2g, NaCl0.8g, $KH_2PO_4$0.1g, $CaCl_2$. $2H_2O$20mg, yeast extract1g, trace elements 10 ml, wolf's vitamin 10 ml, $Na_2CO_3$2g, fructose5g, reducing agents 10 ml, distilled water 980 ml. Composition of trace elements includes nitrilotriacetic acid 2g, $MnSO_4$. H_2O 1g, $(NH_4)_2$ $Fe(SO_4)$-$_2$. $6H_2O$0.8g, $CoCl_2$. $6H_2O$0.2g, $ZnSO_4$. $7H_2O$0.2mg, $CuCl_2$. $2H_2O$20mg, $NiCl_2$. $6H_2O$20 mg, Na_2MoO_4. $2H_2O$20mg, $Na_2O_3$20mg, $Na_2WO_4$20 mg.

Reducing agent present in the medium contains NaOH0.9g, L-cysteine hydrochloride4g, Na_2S. $9H_2O$4g and Wolf's vitamin contains biotin 2 mg, folic acid 2 mg, pyridoxine hydrochloride 10 mg, thiamine hydrochloride5mg, riboflavin 5mg, nicotinic acid5mg, calcium D-(+)-pantothenate 5mg, vitamin B120.1mg, p-aminobenzoic acid 5mg, thioctic acid 5 mg with pH 5.9and incubated in anaerobic workstation (Whitley DG250) at 37 °C for 72 hours. This process was repeated 04 times consecutively and discrete colonies were sub-cultured to make pure culture. Then, pure cultures were preserved in Fluid thioglycolate (FT) agar at 4°C for further study (Sun *et al*., 2018; Gagen *et al*., 2014).

Screening of bioethanol producing acetogen

All the acetogens were screened for bioethanol production by pyridinium chlorochromate (PCC) assay. Acetogens were inoculated individually in specially designed anaerobic culture bottle containing 100 ml of FT medium, N_2 gas was purged at flow rate 0.5LPM for 6 sec to remove oxygen followed by syngas ($CO:CO_2:H_2$/55:20:25) at a flow rate of 0.5LPM for 6 sec as carbon source and incubated at 37 °C for 72 hours. Then, culture was centrifuged in a cooling centrifuge (Eppendorf 5430R) at 3200 g for 20 minutes and the collected supernatant was boiled in rotary vacuum evaporator (Heidolph) at 78 °C (boiling point of ethanol 78.37 °C) for collection of distillates. Subsequently, 1 ml of distillates was mixed with 1 ml of PCC (1M) and incubated at RT for 1hour. PCC reacts with bioethanol and colour changes from brilliant orange to dark brown was monitored by spectrophotometer (Perkin Elmer UV/VIS Lamba 365) at 570 nm. Similarly, PCC assay of various concentration (1, 2, 3, 4, 5, 6, 7%) of standard ethanol was conducted to derive the slope of the line for etermination of bioethanol concentration using the formula;

$$y = (-318.71x^2 + 46.169x + 0.0229)$$
$$R^2 = 0.9981$$

Generic level identification of acetogen

The generic level identification of screened bioethanol producing acetogen was conducted by Gram's staining followed by biochemical characterization using API 20A biochemical test kit (Bio Merieux India) along with oxidase, catalase and sporulation test. The acetogen was cultivated in FT agar medium and cell biomass suspension was prepared by API anaerobe solution. Then, 0.1 ml of suspension containing 9.0 X 10^8 cell/ml was inoculated to API 20A test kit and incubated in

anaerobic work station at 37 °C for 24 hours. Afterwards, various reagents such as, xylene &ehrlich's reagent and bromocresol purple were added to evaluate utilization of indole and various carbohydrates. Subsequently, oxidase, catalase and sporulation tests were conducted and results were then analyzed using *apiweb* identification software (BioMerieux India) for generic level identification (Habu *et al*., 2017).

Bioethanol production by the screened acetogen

The screened acetogen was subjected to bioethanol production in specially designed culture bottle for which, 100 ml of sterilized FT medium was prepared, N_2 gas was purged (0.5LPM/ 6sec) to remove oxygen followed by syngas (60% CO+ 20% CO_2 + 15% H_2+ 5% CH_4) (0.5LPM/ 6sec)as carbon source. The medium was inoculated with 15% inoculum and incubated at 37 °C for 72 hours (Monir *et al*., 2019; Maddipati *et al*., 2011) in anaerobic work station. Following fermentation, fermented liquor was centrifuged in a cooling centrifuge at 3200 g for 20 minutes. The supernatant was collected and boiled in rotary vacuum evaporator at 78 °C to collect distillates. Then, the bioethanol concentration was quantified by GC analysis.

Gas chromatographic (GC) analysis

GC analysis was conducted to quantify bioethanol concentration in the distillate. In the GC (Thermo Scientific, TG-WAXMS, Instrument ID: CEGTH/ INS/C/003) FID and DB-FFAP were used as capillary column, hydrogen as carrier gas (1ml/min) and inlet port temperature was maintained at 150 °C with split ratio of 10:1. For quantification, FID temperature was set at 220 °C with hydrogen and air flow rates at 35 ml/min and 350ml/min. The resulting chromatograms were analyzed using Chem Station data analysis software (Liu *et al*., 2012).

RESULTS AND DISCUSSION

Isolation and screening of Acetogens for bioethanol production

The uni-carbonotrophic acetogens are ubiquitousin nature thus, they are found in chicken manure, compost, sediments, rumen fluid, biogasplant waste etc. On account of that, 07 acetogens were isolated from compost sample out of which, acetogen S1a was screened through PCC assay and depicted 0.69 % of bioethanol production against the slope of line of standard ethanol (Figure 1). Thus, the acetogen S1a has the potential to produce bioethanol. Occurrence and distribution of bioethanol producing acetogensin compost samples were also reported earlier (Zhu *et al*., 2018; Drake *et al*., 2008; Lee *et al*., 2019).

Generic level identification of acetogen

The Gram-positive rod-shaped bioethanol producing acetogen S1a showed positive results to indole, gelatinase, esculin hydrolysis and utilization of various sugars like, D-glucose, D-mannitol, D-lactose, D-maltose, D-xylose, L-arabinose, D-mannose, D-raffinose, D-sorbitol, L-rhamnose & D-trehalose. However, the bacterial isolate S1a was unable to utilize sugars such as, D-sucrose, salicin, glycerol, D-cellobiose and D-melezitose and also

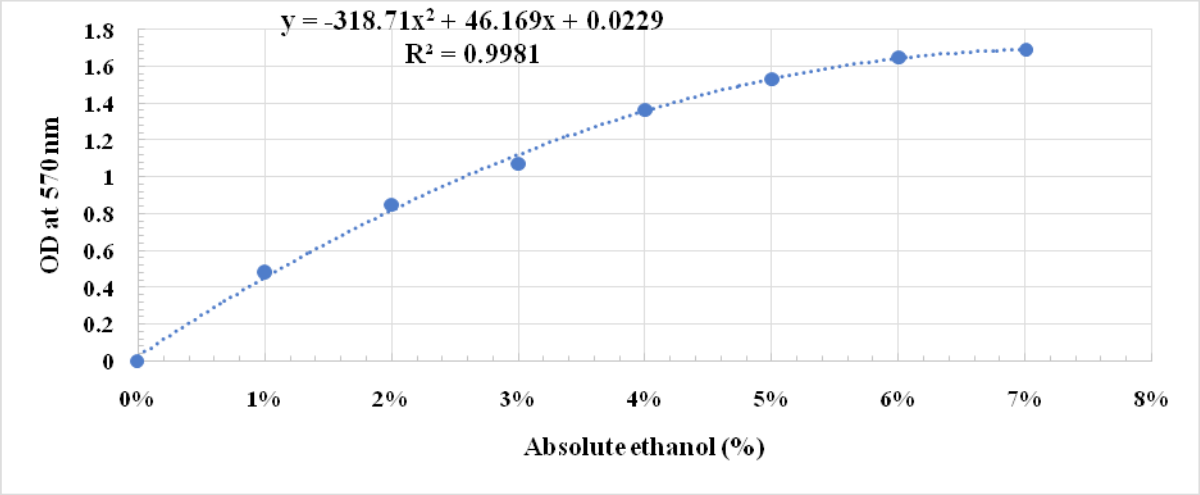

Fig. 1. Slope of standard ethanol

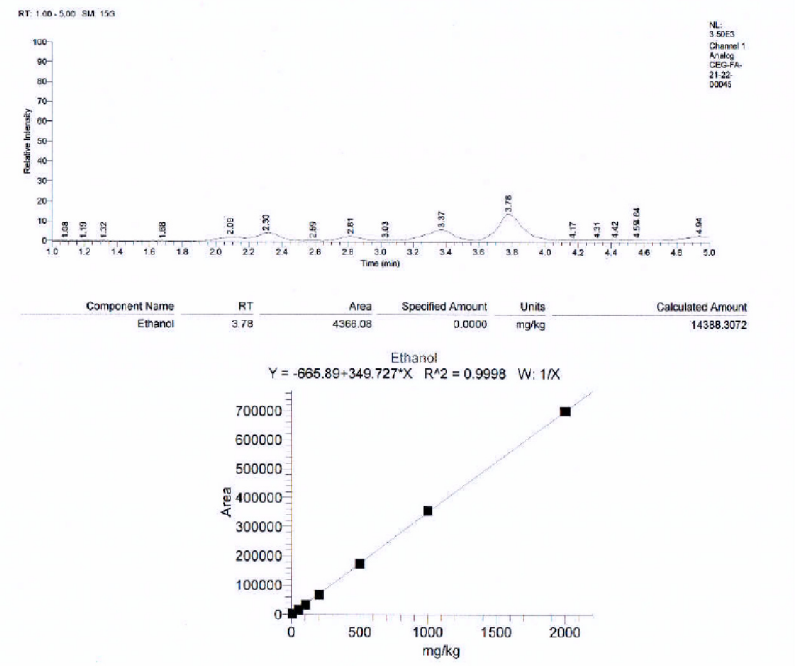

Fig. 2. Quantification of bioethanol through GC analysis

depicted negative results to urease, catalase and sporulation test. Thus, the bioethanol producing acetogen was affiliated to the genera; *Clostridium* sp. S1a as confirmed by morpho-physiological characterization under API 20A system. Similarly, bioethanol producing acetogens such as species of *Clostridium*, *Actinomyces* and *Bifidobacterium* were also identified from different compost and gut samples (Phillips *et al*., 1993; Muller *et al*., 2016; Tun *et al*., 2014; Buruiana *et al*., 2013).

Bioethanol production and quantification

The acetogen *Clostridium* sp. S1a produced 1.44% of bioethanol through syngas fermentation validated through GC analysis (Figure 2). Our result coincides with similar studies, where acetogens *Clostridium ljungdahlii* DSM 13258 and *Clostridium autoethanogenum* produced 0.21 g/l and 352.6 mg/l of bioethanol (Anggraini *et al*., 2018; Abubackar *et al*., 2015) through syngas fermentation. In this process, acetogens reduced uni-carbon components CO and CO_2 to bioethanol through wood-ljungdahl pathway. Moreover, acetogens switch from acetogenesis to solventogenesis to protect themselves from acidic pH and produce bioethanol as a secondary-metabolites during syngas fermentation (Kundiyana *et al*. 2011).

CONCLUSION

In conclusion,the acetogen *Clostridium* sp. S1a produced 1.44% of bioethanol through syngas fermentation in a specially designed culture bottle under unoptimized condition. Thus, further research is highly essential to optimize various bio-parameters regulating large scale syngas fermentation for bioethanol production as well as molecular characterization of the acetogen *Clostridium* sp. S1a.

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Conflict of interest-The authors do not have any conflict of interest to declare.

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