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Research Article

Characterization of *Clostridium* strains and analysis of organic acids production by HPLC

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ABSTRACT : Based on the morphological, physiological characterization and utilization of different carbon sources, out of 47 isolates, the efficient 15 isolates were selected for detection of organic acids by High Performance Liquid Chromatography (HPLC). The highest production of butyric acid 3.49 mg/ml was found in CL 12(2) which was isolated from sugarcane rhizosphere soil where as standard strain *Clostridium acetobutylicum* ATCC 824 was produced 6.85 mg/ml of butyric acid.

KEY WORDS : Organic acids, HPLC, *Clostridium* strain

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INTRODUCTION

India's proven oil resources are currently estimated at about five million barrels. A strong growth on oil demand has resulted in increased petroleum consumption by 75 per cent of the since last decade. India and China are expected to account for 17 per cent of global liquid fuel consumption surpassing total consumption of entire Europe by 2025. The rate of fuel consumption at present results in depletion of the natural reserves. An alternate energy resource is essential to meet the ever growing demands of energy (Gehlhar *et al.*, 2010).

Biobutanol is an important source of biofuel and *Clostridial* ABE (acetone, butanol and ethanol) fermentation could be an alternative for dwindling petrol chemicals knowledge regarding. *C. acetobutylicum* was cultivated on an industrial scale nearly a century ago, although the desired compound in that process was the fermentation co-product acetone. Chaim Weizmann led the British effort to produce acetone using *C. acetobutylicum*, a process that met the war needs of England (Dixon, 1997). This process played a crucial part in both World War I and II. With the re-emergence of interest in producing fuels from biomass, there is once again a focus on strain improvement and the use of alternative feedstock e.g. biomass made the bacterial solvent production competitive again. Biobutanol has gained considerable attention in recent years, compared with the traditional biofuel ethanol, because it has a higher energy content; can be applied in pure form or blended in any concentration with gasoline or diesel; can be used in any automobile engine without modifications; is non-hygroscopic, has a lower vapour pressure and less corrosive (Durre, 2007). This paper describes the efficiency of Butanol producing isolates for production of organic acids *viz.*, acetic acid, butyric acid and propionic acid by HPLC.

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EXPERIMENTAL METHODS

Based on the morphological characterization, the *Clostridium* isolates were employed for biochemical characterization *viz.* indole, gelatin and catalase tests (Dolly *et al.*, 2000). 47 efficient isolates were selected for physiological characterization *viz.*, rifampicin and curd formation tests were done for differentiating solventogenic *Clostridum* as described by Johnson *et al.* (1997).

Based on the relevant tests done (morphological and biochemical characterization), the *Clostridium* isolates were selected and screened for organic acids HPLC method as it was feasible and used for analysing intermediates (acetic acid, butyric acid and propionic acid) of A-B-E by the promising isolates. Among these 47 isolates 15 were selected for estimation of intermediate organic acids (acetic acid, butyric acid and propionic acid) by HPLC for production of Acetone, Butanol and Ethanol (A-B-E)

Analysis of Organic acids by high performance liquid chromatography :

The operating conditions of HPLC system (Waters) equipped with gradient pump (Waters 515) manual injector fitted with a 20- µL loop, a UV detector (ë dual 2487) set to 210 nm, column symmetry C_{18} (4.6 × 150 mm,0.5µm pore size). Data was collected on Empower Ver 3.0 in system. The analysis was performed by gradient mode with mobile phase phosphate buffer pH 2.1 and methanol in ratio 80:20 at flow rate 1ml/min. All the reagents were prepared in Millipore water and filtered through a 0.45 mm membrane filter (Zhao *et al.*, 2001).

Chromatographic standards were obtained commercially of Sigma Aldrich, and the working standard solution of acetic acid 30 mg/ml, butyric acid 60 mg/ml and propionic acid 30 mg/ml was used. The specific gravity of each solvent was considered while preparing standards. Acetic acid had 1.05 g/ml, butyric acid 0.96 g/ml and propionic acid 0.997 g/ml. Initially the standards acetic acid, butyric acid and propionic acid of HPLC grade (Sigma) were fed to the HPLC. While injecting, the same volume of standard as well as sample of 20 µl were used.

Sample preparation was done by inoculating the strains in T_6 broth and incubated @ 37^0 C for 92 h. Then the broth was centrifuged (10000 x 10min) to separate the aqueous phase containing organic acids .The supernatant was collected and was filtered through a 0.45mm Nylon 99 membrane and finally it was injected directly into the Chromatograph. The corresponding peaks eluted at similar retention time of that of standard indicated the respective acids produced by the isolates.

EXPERIMENTAL RESULTS AND ANALYSIS

The results obtained from the present study have been discussed in detail under following heads :

Analysis of organic acids produced by *Clostridium* spp.:

Based on the relevant tests as mentioned above (morphological and biochemical characterization), out of 47 isolates, the efficient 15 were selected for detection of organic acids by High performance liquid chromatography.

A total of 47 isolates were tentatively identified as *Clostridium* spp producing butanol. Among these 47 isolates 15 were selected for estimation of intermediate organic acids (acetic acid, butyric acid and propionic acid) by HPLC for production of Acetone, Butanol and Ethanol (A-B-E).

The results were compared with that of retention time of respective standards (Table 1) The highest production of Acetic acid 37.11 mg/ml was detected in CL- 26(3) which was isolated from maize rhizosphere soil, whereas, 28.59 mg/ml, 28.44mg/ml, 27.74 mg/m was observed in CL- 15(2), in CL- 12(2) and CL-15(3), respectively. (Table 2). The isolates CL-42(1), CL-20(2), CL-13(1), CL-9(2), CL-22(1), CL-40(4), CL-20(1) and CL-31(2) were found to produce medium amount of acetic acid of 24.35 mg/ml, 22.53 mg/ml 12.10 mg/ml, 9.66 mg/ml, 9.56 mg/ml, 7.21 mg/ml, 6.27 mg/ml and 6.27 mg/ml, respectively (Table 3). The least production of acetic acid of 4.88 mg/ml, 4.88 mg/ml and 4.07 mg/ml was observed in the isolates CL-11(3), CL-37(2), CL-42(3), respectively. The reference strain *C. acetobutylicum* ATCC 824 produced 30.80 mg/ml of acetic acid (Fig. 1).

The highest production of butyric acid 3.49 mg/ml was found in CL 12(2) which was isolated from sugarcane rhizosphere soil (Fig. 2). The isolates CL 40(4), CL 15(2), CL 11(3), CL 37(2), CL 15(3) and CL 22 (1) was found to produce medium amount of butyric acid 2.26 mg/ml, 1.74 mg/ml, 1.60 mg/ml, 1.60 mg/ml, 1.16 mg/ml and 1.014 mg/ml, respectively (fig 2). Whereas, the least production of butyric acid 0.012 mg/ml, 0.064 mg/ml, 0.61 mg/ml, 0.950 mg/ml and 0.950 mg/ml was observed in the isolates CL 9(2), CL 13(1), CL 42(1), CL 20(1) and CL 31(2), respectively (Fig. 2). The reference strain *C. acetobutylicum* ATCC 824

Table 1: HPLC analysis of standard acetic acid, butyric acid and propionic acid

Sr. No.	Standards	Concentration (mg/mL)	Retention time (RT) min	Peak area
1.	Acetic acid	30	2. ±05	18613764
2.	Butyric acid	60	9. ±52	38284315
3.	Propionic acid	30	3. ±88	16976739

Table 2 : Production of different organic acids by *Clostridium* isolates as analyzed through HPLC

Sr. No.	Isolates code	Concentration of acids (mg/ml)		
		Acetic acid	Butyric acid	Propionic acid
1.	CL 9(2)	9.66	0.012	11.58
2.	CL 11(3)	4.88	1.60	7.56
3.	CL 12(2)	28.44	3.49	5.44
4.	CL 13(1)	12.10	0.064	12.29
5.	CL 15(2)	28.59	1.74	7.97
6.	CL 15(3)	27.74	1.16	5.05
7.	CL 20(1)	6.27	0.950	8.44
8.	CL 20(2)	22.53	0	4.53
9.	CL 22 (1)	9.56	1.014	13.70
10.	CL26(3)	37.11	0	0.64
11.	CL 31(2)	6.27	0.950	8.44
12.	CL 37(2)	4.88	1.60	7.56
13.	CL 40(4)	7.21	2.26	30.69
14.	CL 42(1)	24.35	0.61	3.96
15.	CL 42(3)	4.07	0	5.38
16.	*Clostridium. acetobutylicum*	30.80	6.85	0.61

was produced 6.85 mg/ml of butyric acid (Table 2).

The highest production of propionic acid was found in CL-40(4) which exhibited followed by 30.69 mg/ml, CL-22(1) 13.70 mg/ml, CL-13(1) 12.29 mg/ml, and CL-9 (2) of 11.58 mg/ml (Table 4). The isolates CL-20(1), CL-31(2), CL-11(3), CL-37(2), CL-12(2), CL- 42(3) and CL- 15(3) were found to produce medium amount of propionic acid of 8.44 mg/ml, 8.44 mg/ml, 7.56 mg/ml, 7.56 mg/ml, 5.44 mg/ml, 5.38 mg/ml, 5.05 mg/ml, respectively . Whereas, the least production of propionic acid was 4.53 mg/ml, 3.96 mg/ml and 0.64 mg/ml observed in the isolates CL-20(2), CL-42(1) and CL-26(3), respectively.The reference strain *C. acetobutylicum* ATCC 824 produced 0.61 mg/ml of propionic acid (Table 2).

The wide variation in the production of organic acids among the isolates indicates genetic variability and variation in the metabolic pathway adopted by the isolates, since generally, butyric acid producing isolates show more of propionic acids when compared to the reference strain. Further optimization of parameters may maximize the Butanol production. Majority of the isolates belonged to *C.acetobutylicum* followed by *C.beijernckii.* The other isolates which were either positive for gelatin liquefaction or indole production may have other special functional activity which needs further characterization.

Conclusion:

Total of 15 were screened for estimation of intermediate organic acids (acetic acid, butyric acid and propionic acid) by HPLC for production of Acetone, Butanol and Ethanol (A-B-E). The highest production of butyric acid 3.49 mg/ml was found in CL 12(2) which was isolated from sugarcane rhizosphere soil. This indicates the relation between kind of crop and the number of solvent producing *Clostridium*.

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