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Exploration of Acidogenic and Solventogenic *Clostridium* sp. from Habitat of Different Ecosystems

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The acetone-butanol-ethanol as a source of liquid fuel from fermentation process using isolates of the anaerobic bacterium *Clostridium* sp. continues to attract the attention for replacement of conventional liquid fuels. This study aims at isolation and characterization of native anaerobic clostridia from different biodiversity zones of North Karnataka and detection of presence of organic acids by HPLC analysis. As a result, eighty mesophilic, spores forming, anaerobic, butanol producing *Clostridium* isolates were isolated. Based on the morphological, biochemical and physiological characterization, 21 *Clostridium* isolates belonged to the group of clostridia namely *C. acetobutylicum*, *C. beijerinckii* and *Clostridium saccharobutylicum*. The acidogenic strain tentatively identified as *C. acetobutylicum* [MR-10-3(3)] showed maximum production of butyric (121.88 mg/ml), acetic acid (101.40 mg/ml) and least propionic acid (21.01mg/ml) compared to the reference strain *Clostridium acetobutylicum* ATCC 824 (29.62 mg/ml of butyric acid, 33.79 mg/ml of acetic acid and 271.62 mg/ml of propionic acid).

Key words: *Clostridium,* butanol, butyric acid, acetic acid, propionic acid

The last few decades have witnessed dramatic improvements made in the production of fuels and chemicals from biomass and butanol production from agricultural products is no exception. The art of producing butanol from corn that existed during World Wars I and II is no longer seen as an art but rather as science. However, with the incessant fluctuations in oil prices interest in the Acetone-Butanol-Ethanol (ABE) fermentation has resurfaced because of the new global support for the exploitation of biomass as a sustainable source of energy. As an alternate substitute, butanol has advantages over traditional fuel ethanol in terms of energy density and hygroscopicity. Various cheap substrates have been successfully applied in the production of biobutanol, looking to its commercial potential.

The genus *Clostridium* is a heterogeneous collection of gram-positive, spore-forming, rod-shaped bacteria and live in virtually all anaerobic habitats containing organic matter, including soils, aquatic sediments, and anaerobic tissues (such as intestinal tracts of animals). Clostridia contain many saccharolytic species and can easily be isolated from soil samples at the most differing locations[1]. Four distinct species of clostridia were identified among the industrial solventogenic isolates as *Clostridium acetobutylicum*, *Clostridium beijerinckii*, *Clostridium saccharoperbutylacetonicum* and *Clostridium saccharobutylicum*[1]. Due to unique geoclimatic localization, Karnataka is distinguished by the biodiversity of its natural sources. This reflects the occurrence of soil dwelling microorganisms at varying pH, average temperature, humidity, and

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the availability of substrate. To exploit this biodiversity, a study was initiated to isolate native solvent-producing bacteria.

The present study describes the isolation and characterization of native clostridia from different biodiversity zones of North Karnataka and their distribution in different soils. The isolated anaerobic bacteria were also analyzed for presence of organic acids by HPLC.

MATERIALS AND METHODS

Culture and Maintenance

The reference strain *Clostridium acetobutylicum* ATCC 824 (type strain) was procured from National Collection of Industrial Microorganisms (NCIM, PUNE). The culture was maintained in Reinforced Clostridial Medium (RCM).

Collection of samples

The soil samples were collected from paddy field, pond sediment, forest soil and distillery effluent dumped soil from in and around Dharwad district of North Karnataka, India. The samples were drawn from 3' depths below the surface of the earth by drilling with clean auger and brought in sterile pregassed serum bottles for isolation.

Properties of soil samples

The pH was observed using pH meter (Ezodo pL-600) and organic carbon content of the soil was determined by wet oxidation method[2]. Based on % organic carbon content it was rated as low (<0.5), medium (0.5 to 0.75 %) and high (>0.75).

Media preparation

The anaerobic Reinforced Clostridial Medium (RCM) containing the following ingredients in 1 L of distilled water: 10.0 g beef extract, 10.0 g peptone, 3.0 g yeast extract, 1.0 g starch, 5.0 g sodium chloride, 3.0 g sodium acetate, 0.5 g cysteine hydrochloride and 5 g glucose, pH 7.0 of 10 ml was dispensed in glass vials and flushed with mixture of nitrogen and carbon dioxide (80:20) gases using gassing manifold (fabricated by Mossi agency, Coimbatore) followed by sealing with rubber septa and aluminum crimps and sterilized. Similarly, RCM agar media was also prepared using agar at 2.2 %.

Isolation of solvent producing bacterial isolates by enrichment and roll tube technique

One gram of each soil sample was suspended in 10 ml of sterilized RCM medium in above vials separately. The inoculated vials were incubated in water bath at 70°C in order to select the spore formers and also to inactivate the vegetative cells. The vials were enriched by incubating for 5 days at 37°C and checked daily for growth and gas production[3, 4]. The enriched samples of 0.5 ml were transferred individually using sterilized syringe in 5 ml of RCM agar medium and immediately rolled on cold ice to spread the inoculum along with media uniformly till it solidified. After solidification the vials were incubated at 37°C for 5-6 days and the colonies were observed and recorded. The typical colonies representing characters of *Clostridium* were isolated with the help of glass loop and transferred to presterilized oxygen free RCM broth to enrich their growth.

Endospore staining

Endospore staining was done by Malachite green stain method[5]. The slide was examined under microscope (Motic model) and micro photography was done with 100x magnification.

Biochemical characterization

Biochemical characterization of the native anaerobic *Clostridium* isolates was done according to Anaerobic laboratory Manual[3,4]. The fresh grown cultures in RCM media containing 1×10^6 CFU/ ml were used in all the experiments.

Indole production test

The 24 h fresh grown culture was inoculated in 5 ml tryptone yeast glucose (TYG) medium containing the following ingredients in 1 L of distilled water: 16 g/l tryptone, 5 g/l yeast extract, 5 g/l sodium chloride, 5 g/l glucose, 0.5 g/l cysteine HCl and incubated for 24 to 28 h at 37°C. Once the growth was observed, 1 ml of the kovac's reagent was added to the culture and agitated gently and observed for the formation of red ring.[3]

Gelatin liquefaction test

A fresh inoculum of 1 ml was transferred to a vial containing 10 ml TY medium (similar to TYG but instead of glucose, gelatin was used as carbon source) and incubated for 16 h at 37°C. The vials were cooled to 4°C for 8 hrs to observe liquefaction. Liquefaction of gelatin indicated positive[6]result.

Catalase activity[1]

The culture was grown in 5 ml TYG and

incubated overnight at 37ºC. The culture of 1 ml was taken to a cavity slide and ten drops of 3% hydrogen peroxide were added. The culture having catalase activity was able to degrade H_2O_2 and form O_2 and H_2O with bubbling and the absence of bubbling indicated lack of catalase indicating it as an obligate anaerobe.

Physiological characterization

Physiological tests were done for differentiating solventogenic *Clostridium* sp. as per the methods[6].

Curd formation and production of riboflavin

Twenty ml fresh milk and 0.05 mg resazurin (pH 7.1) was flushed with 100% N_2 for 12 min and sterilized at 121°C. One ml of the 24 h grown culture was inoculated into 20 ml milk medium and incubated at 37°C. Results were considered positive when the milk coagulated and curd was formed within 24 h. For riboflavin production the above stock cultures were incubated at 37°C for 5 days and then stored at 4°C for 10 days. The results were considered positive for riboflavin production if the media turned yellow.

Rifampicin sensitivity test

TYG agar plates were flooded with the overnight cultures. After air drying, filter paper discs containing 10 and 100 ng rifampicin were placed onto the agar surface separately using anaerobic glove box (Imset, Pune). Sensitivity to rifampicin was detected by inhibition of growth around the disks[7].

Analysis of organic acids by High performance liquid chromatography[8]

Sample preparation

Based on biochemical and physiological characterization, 21 *Clostridium* isolates were selected for detection of organic acids by HPLC. 0.5 ml of 24 hr grown culture was inoculated in 10 ml of TYG broth and incubated for 90 h in order to reach acidic phase. The fermented TYG broth was centrifuged at 6000 rpm for 5 min and the supernatant was used for organic acid estimation. Chromatographic standards of acetic acid (99.9%), propionic acid (99.9%) and butyric acid (99.9%) were obtained commercially from Sigma Aldrich, Bangalore.

The analysis was performed in HPLC (Waters Ltd.) equipped with 515 pumps and UV dual detector (Waters » dual 2187). Separation of compounds was done with C18, symmetry column (4.6x150mm, 0.45µm pore size). For analysis, the wavelength of UV- detector was set at 210 nm and the mobile phase was a mixture of phosphate buffer solution (15 mmol/L, pH 2.1) and methanol (85:15, V/V) and the flow rate was 1 ml/min. Before injecting into HPLC the standards as well as samples were filtered through 0.45 µm membrane filter. Mobile phase was also filtered through 0.25 µm membrane filter. Standard of 20 µl was injected to obtain the peaks followed by samples. Integrated data was collected on Empower ver. 3.0 in system.

RESULTS AND DISCUSSION

Eighty mesophilic, spore forming butanol producing anaerobic *Clostridium* isolates were isolated from thirty different soil samples from paddy field, pond sediment, forest soil and distillery effluent dumped soil (Table 1) by Hungate's Roll tube technique.

Among the samples, pond sediment showed good number of isolates. A total of 31 isolates from 13 pond sediment samples while only 10 isolates could be isolated from 4 samples of paddy field soil (Table 1). Fifteen isolates of anaerobic *Clostridium* were found from distillery effluent dumped sites and slightly higher number of 21 isolates from 7 forest samples was recorded. Similar study was conducted in specific provinces of Colombia and totally 178 spore-forming, anaerobic solvent producing *Clostridium* sp. were obtained from 155 agriculture soil samples[2]. It is also stated that there exists a correlation between kind of crop or source of sample and the occurrence of the *Clostridium* sp.[3]The enriched samples which produced gas within 24 h were found better sources for isolation of solvent-producing bacteria than those samples which grew in more than 48 h. During enrichment good quality of inoculums was indicated by the obvious smell of solvents and frothing caused by gas production. There are number of media for isolation of anaerobic bacteria but RCM medium served as enrichment medium and inhibited the growth of non butanol producing bacteria[9].

The cells of all isolates depicted straight rods with rounded ends, arranged in pairs with subterminal endospores and initially phase dark, gram positive, became gram negative in older

cultures. Towards the end of exponential growth, the rod shaped cells accumulate granulose forming swollen cigar shaped clostridial form[10]. These morphological changes are typically associated with metabolic shift from acidogenesis to solventogenesis[10]. According to the microbiological criteria these isolates are mesophilic, spore forming, anaerobic and belonged to the genus *Clostridium.*[7]

Based on the morphological characterization, the 80 anaerobic *Clostridium* isolates were employed for biochemical characterization *viz.* indole, gelatin and catalase tests (Table 2). Among 80/21 isolates were found positive for liquefaction of gelatin and indole production. Gelatin liquefaction test detects the ability of an organism to produce an exoenzyme, called gelatinase that hydrolyzes gelatin[1]. *C. saccharoperbutylacetonicum* is differentiated from *C. beijerinckii* by its ability to hydrolyze gelatin[6]. Indole test is a qualitative test for determination of organism's ability to split indole from tryptophan molecule. Tryptophan is an amino acid that is oxidized by bacteria to form indolic metabolites by

Table 1. The sources of soil samples along with their properties and number of isolates of *Clostridium* sp. isolated

S. No	Place / Sample No.	Sample used	pH	Organiccarbon (%)	No. ofcolonies isolated
1	Niralagi (MR-10-01)	Paddy Soil	6.8	1.8	02
2	Niralagi (MR-10-02)	Pond sediment	6.6	0.4	-
3	Nakapur (MR-10-03)	Pond sediment	6.7	0.5	04
4	Hangal (MR-10-04)	Pond sediment	6.6	0.3	01
5	Urabanahalli (MR-10-05)	Paddy Soil	7.1	0.4	04
6	-do- (MR-10-06)	Paddy field	6.5	0.6	02
7	Sourab (MR-10-07)	Paddy soil	6.6	0.6	02
8	Sourab (MR-10-10)	Forest Soil	6.7	3.0	04
9	Niralgi (MR-10-11)	Pond sediment	7.3	0.9	04
10	Near Hulkoppa (MR-10-12)	Pond sediment	7.2	0.9	02
11	Joida (MR-10-16)	Pond sediment	7.5	0.7	01
12	Shivanagudi (MR-10-17)	Pond sediment	7.2	1.2	01
13	Near Ulavi (MR-10-18)	Pond sediment	7.1	1.3	02
14	Near Ulavi (MR-10-19)	Pond sediment	6.9	1.2	02
15	Near Ulavi (MR-10-20)	Pond sediment	6.7	0.6	05
16	Near Dandeli (MR-10-21)	Forest Soil	6.7	1.2	02
17	Near Marda (MR-10-22)	Forest Soil	6.6	1.8	05
18	Near Joida (MR-10-23)	Forest Soil	6.9	4.4	04
19	Near Dandeli (MR-10-21	Forest soil	6.7	1.4	05
20	Tadas (MR-10-26)	Pond sediment	6.8	0.3	03
21	Mundagod (MR-10-27)	Pond sediment	7.1	0.9	03
22	Near Mundagod (MR-10-28)	Forest soil	7.3	1.8	02
23	Near Mundagod (MR-10-29)	Forest soil	7.0	1.8	02
21	Near Dasankoppa (MR-10-32)	Pond sediment	7.2	0.6	03
25	Renuka sugar factory, Munavalli (J-11-35)	Distilleryeffluent dumpedsoil	6.3	3.3	02
26	Renuka sugar factory, Munavalli (J-11-36)	Distilleryeffluent dumpedsoil	6.4	3.5	01
27	Renuka sugar factory, Munavalli (J-11-37)	Distilleryeffluent dumped soil	6.7	1.9	03
28	Renuka sugar factory, Munavalli (J-11-38)	Distilleryeffluent dumped soil	6.5	3.0	03
29	Renuka sugar factory, Munavalli (J-11-39)	Distilleryeffluent dumped soil	6.6	4.5	03
30	Renuka sugar factory, Munavalli (J-11-40)	Distilleryeffluent dumped soil	6.2	5.1	03

Table 2. Biochemical characterization of native *Clostridium* isolates

S. No	Isolate code	Gelatin liquefaction	Indole production	Catalase activity
1	MR-10-1(2)	+	-	-
2	MR-10-3(2)	+	+	-
3	MR-10-3(3)	+	+	-
4	MR-10-4(1)	+	-	-
5	MR-10-5(1)	+	-	-
6	MR-10-5(2)	+	-	-
7	MR-10-5(3)	+	-	-
8	MR-10-5(4)	+	+	-
9	MR-10-10(1)	+	-	-
10	MR-10-10(2)	+	+	-
11	MR-10-10(3)	+	-	-
12	MR-10-12(2)	+	-	-
13	MR-10-18(1)	+	-	-
14	MR-10-21(1)	+	-	-
15	MR-10-21(2)	+	-	-
16	MR-10-23(1)	+	-	-
17	MR-10-27(2)	+	-	-
18	MR-10-27(3)	+	-	-
19	MR-10-28(2)	+	-	-
20	J-11- 35(1)	+	-	-
21	J-11-39(1)	+	-	-
22	ATCC 824	+	+	-

Table 3. Physiological characterization of native *Clostridium* isolates

S. No	Isolate code	Curd formation	Riboflavin production	Rifampicin Sensitivity	Tentative classification
1	MR-10-1(2)	+	-	S	*Clostridium saccharobutylicum*
2	MR-10-3(2)	+	+	S	*Clostridium acetobutylicum*
3	MR-10-3(3)	+	+	S	*Clostridium acetobutylicum*
4	MR-10-4(1)	+	-	S	*Clostridium saccharobutylicum*
5	MR-10-5(1)	+	-	R	*Clostridium beijerinckii*
6	MR-10-5(2)	+	-	S	*Clostridium saccharobutylicum*
7	MR-10-5(3)	+	-	S	*Clostridium saccharobutylicum*
8	MR-10-5(4)	+	+	S	*Clostridium acetobutylicum*
9	MR-10-10(1)	+	-	S	*Clostridium saccharobutylicum*
10	MR-10-10(2)	+	+	S	*Clostridium acetobutylicum*
11	MR-10-10(3)	+	-	S	*Clostridium saccharobutylicum*
12	MR-10-12(2)	+	-	R	*Clostridium beijerinckii*
13	MR-10-18(1)	+	-	R	*Clostridium beijerinckii*
14	MR-10-21(1)	+	-	S	*Clostridium saccharobutylicum*
15	MR-10-21(2)	+	-	S	*Clostridium saccharobutylicum*
16	MR-10-23(1)	+	-	S	*Clostridium saccharobutylicum*
17	MR-10-27(2)	+	-	S	*Clostridium saccharobutylicum*
18	MR-10-27(3)	+	+	S	*Clostridium acetobutylicum*
19	MR-10-28(2)	+	-	S	*Clostridium saccharobutylicum*
20	J-11-35(1)	+	-	S	*Clostridium saccharobutylicum*
21	J-11-39(1)	+	-	S	*Clostridium saccharobutylicum*
22	ATCC 824	+	+	S	*Clostridium acetobutylicum*

Note: R= resistant, S= sensitive to rifampicin

tryptophanase. The indole splits from the tryptophan molecule detected by Kovac's reagent that produced a distinct color. Red ring formation was seen after addition of Kovac's reagent to the growing culture indicated the indole production. The catalase activity of the solvent producing *Clostridium* isolates was compared with aerobic *Bacillus* sp. and reference *C. acetobutylicum* ATCC 824. All the 80 butanol producing *Clostridium* isolates employed for the catalase activity found anaerobic as they revealed the absence of bubbles which indicated that they were inefficient to breakdown hydrogen peroxide ($H_2O_{2)}$ and liberate oxygen (O_2) unlike strictly aerobic *Bacillus* sp.

Consequently, physiological tests have been done to distinguish the four different groups of solvent-producing clostridia with curd formation from milk, riboflavin production, and rifampicin susceptibility: group I, *Clostridium acetobutylicum* (-, -, s); group II, *Clostridium saccharobutylicum* NCP 262 (-, -, s); group III, *C. saccharoperbutylacetonicum* (-, -, r); group IV, *C. beijerinckii* (-,-, r)[6]. The formation of bright yellow color in milk cultures was the basic criteria for differentiation of *Clostridium* sp[1]. Based on these criteria, all the 21 isolates were found positive for curd formation, 13 isolates were tentatively identified as *Clostridium saccharobutylicum*, 5 isolates as *C. acetobutylicum* and 3 as *C. beijerinckii* (Table 3). From native isolates of Colombian sources it has been classified about six butanol producing as C. *acetobutylicum*, three as *C. beijerinckii*, and three as *Clostridium* NCP 262[3]. Based on the biochemical and physiological characterization 21 efficient *Clostridium* isolates were analyzed for production of organic acids by High Performance Liquid Chromatography (Table 3). The results were compared with the retention time of respective standards (Fig 3). The strain tentatively identified as *C. acetobutylicum* [MR-10-3(3)] found to produce maximum butyric (121.88 mg/ml) and acetic acid (101.40 mg/ml) compared to reference strain. The strain [MR-10-3(3)] was isolated from pond sediment of pH 6.7. In general, soil with slightly acidic pH favored the occurrence of maximum number of butanol producing bacteria[3]. Production of solvents, especially butanol, is manifestly influenced by butyric acid. As, the

Table 4. Estimation of organic acids of butanol producing anaerobic *Clostridium* isolates by HPLC

S. No	Isolate code	Butyric acid (mg/ml)	Acetic acid (mg/ml)	Propionic acid (mg/ml)
1	MR-10-1(2)	0	117.76	22.43
2	MR-10-3(2)	0	97.19	27.40
3	MR-10-3(3)	121.88	101.40	21.01
4	MR-10-4(1)	0.00	14.23	21.90
5	MR-10-5(1)	5.72	38.03	108.18
6	MR-10-5(2)	0.00	43.72	110.18
7	MR-10-5(3)	0.84	56.72	108.74
8	MR-10-5(4)	39.71	38.71	104.30
9	MR-10-10(1)	13.29	44.83	12.13
10	MR-10-10(2)	3.58	24.70	69.25
11	MR-10-10(3)	11.82	55.18	75.97
12	MR-10-12(2)	0.33	22.10	114.19
13	MR-10-18(1)	12.44	29.39	74.67
14	MR-10-21(1)	52.21	33.56	113.76
15	MR-10-21(2)	41.49	32.96	73.87
16	MR-10-23(1)	10.59	37.91	64.24
17	MR-10-27(2)	45.76	35.52	109.50
18	MR-10-27(3)	44.36	25.72	107.23
19	MR-10-28(2)	25.39	74.83	105.87
20	J-11-35(1)	0.00	56.68	114.42
21	J-11-39(1)	0.00	85.00	121.19
22	ATCC 824	29.62	33.79	271.62

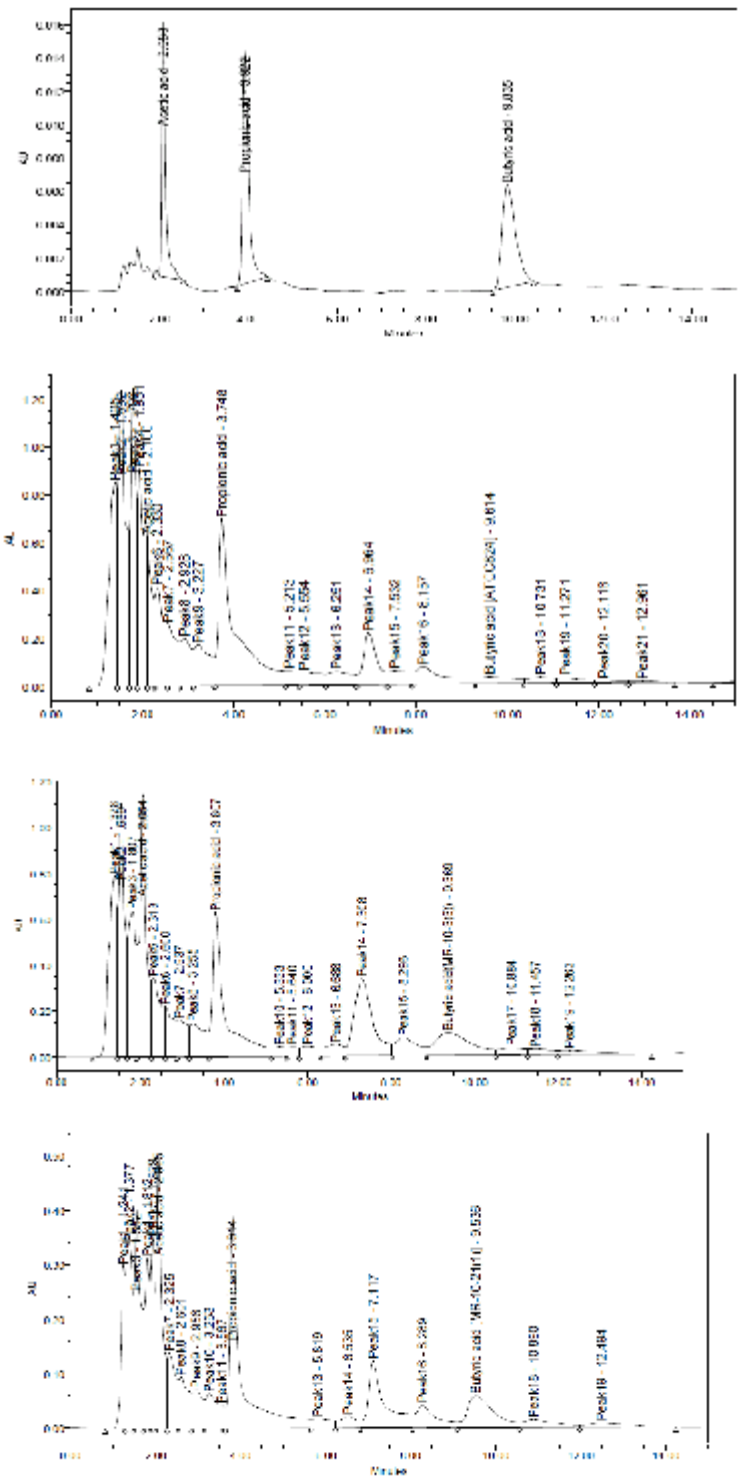

Fig. 1. HPLC profile of organic acids produced by native anaerobic *Clostridium* isolates. A- Standard acetic acid, propionic & butyric acid, B- *Cl. acetobutylicum* ATCC 824, C- native strain MR-10-3(3) & D- native strain MR-10-21(1)

organic acids produced in the fermentation broth activate the solvents production[11-15]. Similarly J-11-39(1) showed the highest propionic acid production. MR-10-21(1) showed second highest butyric acid (52.21 mg/ml) and (33.56 mg/ml) of acetic acid. The isolates MR-10-5(4), MR-10-21(2), MR-10-27(2), MR-10-27(3) and MR-10-28(2) were medium in the production of butyric acids as well as acetic acids and MR-10-5(1), MR-10-5(2), MR-10-5(3), MR-10-5(4), MR-10-12(2), MR-10-27(2), MR-10-27(3) and MR-10-28(2) were found medium producers of propionic acid.

Least production of butyric acid and acetic acid was observed in MR-10-5(1), MR-10-5(3), MR-10-10(1), MR-10-10(2), MR-10-10(3), MR-10-12(2), MR-10-18(1), and MR-10-23(1). While, the isolates MR-10-1(2), MR-10-3(2), MR-10(3), MR-10-4(1) and MR-10-10(1) found to produce less propionic acid. The remaining isolates MR-10-1(2), MR-10-3(2), MR-10-4(1), J-11-35(1) and J-11-39(1) were inefficient to produce butyric acid (Table 4). The reference strain produced (29.62 mg/ml) butyric acid, (33.79 mg/ml) acetic acid and (271.62 mg/ml) propionic acid. The results of organic acids (Fig. 1) production prove that these isolates are solvent producing as these are intermediates of ABE and it is noted that acids produced during the acidogensis were utilized for solvent production. So the isolates with highest organic acids may have improved results in productivity and yield.

CONCLUSIONS

As a result of the renewed interest in the solvent-producing bacteria over past few years, the present investigation oriented on isolation of efficient anaerobic butanol producing *Clostridium* isolates from various habitats of North Karnataka. Obtained results showed that the biochemically and physiologically characterized 21 *Clostridium* isolates were belonged to three species of solvent-producing clostridia, namely *C. acetobutylicum*, *C. beijerinckii*, *Clostridium saccharobutylicum*. The *C.acetobutylicum* [MR-10-3(3)] produced maximum butyric acid (121.88 mg/ml) and acetic acid (101.40 mg/ml) compared to the reference strain *Clostridium acetobutylicum* ATCC 824 (33.79 mg/ml) of acetic acid, (29.62 mg/ml) of butyric acid and (271.62 mg/ml) of propionic acid. Thus, these results have prompted for further characterization of isolates from other sources.

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