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Evaluation of Lactic Acid Bacteria, *Clostridium* and *Paenibacillus* Isolates for 2, 3-Butanediol Production and Process Development using Agro-residues

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A B S T R A C T

2,3-Butanediol (2,3-BD) is one of the promising bulk chemicals due to its extensive industrial application. We need to exploit the innate potential of its microbial producers which also possess delignification and saccharification abilities to facilitate the use of renewable and cheaper agro-residues as raw materials for fermentation. Strains of Lactic acid bacteria, *Paenibacillus* and *Clostridium* were tested for production of 2,3-BD by high through put determination of a pathway intermediate acetoin by Vogues Proskauer test. The VP positive isolates were grown as shaken flask cultures and the concentration of the accumulated 2,3 BD was determined by Gas chromatography. The highest amount of 18.577 g 2,3 BD /L was produced by the LAB isolate LDL-18, followed by other LAB isolates LA-3 and LGr-5 which produced 1.3908 and 1.9893 g/L of 2,3-BD respectively. Both the *Panebacillus* strains showed very less production of 2, 3 BD. Further the possibility of producing 2,3-BDL was tested by using agro-residues *viz*., pineapple pulp waste and delignified paddy straw at different concentrations in batch fermentation. This study is a prelude to an alternate, sustainable energy management strategy to fulfil the increasing demand of this useful chemical and to overcome its expensive chemical synthesis.

Introduction

2,3 Butanediol is an important natural biochemical which finds its application in a wide range of industries; from manufacture of printing inks, perfumes, fumigants, moistening and softening agents, explosives and plasticizers, to its use as a carrier for pharmaceuticals (Li *et al.*, 2013 and Koutinas *et al.*, 2014). 2,3-butanediol is a highly valuable fuel with the burning value of 27198 J/g, which is comparable to other liquid fuels as ethanol (29055 J/g) and methanol (22081 J/g); it can also be converted to methyl-ethyl-ketone by sulfuric acid catalyzed dehydration, which is considered as an effective liquid fuel additive for its higher burning value than ethanol. After combination with methyl-ethyl-ketone and hydrogenation reaction, 2,3-butanediol can be converted to octane, which is used to produce high-quality aviation fuel (Lan *et al.*, 2011).

2,3-BD is known to be produced naturally by a range of sugar (or citrate)-fermenting microbes, including *Bacillus amyloliquefaciens*, *Bacillus subtilis*, *Enterobacter aerogenes Klebsiella pneumoniae*, *Klebsiella oxytoca*, *Lactococcuslactis Paenibacillus polymyxa*, and *Serratia marcescens* (Afschar *et al.*, 1993 and Ji *et al.*, 2011). Some of these are cellulolytic also (Benedict and Jue, 2011). This property can be exploited for utilizing cellulosic substrates for the production of the 2,3 butanediol. The lactic acid bacteria (LAB) are potential alternatives to produce 2,3-BD owing to its safety and possession of a natural 2,3-BD biosynthetic pathway (Gaspar *et al.*, 2011). Attempts have been made with lactic acid bacteria to reroute carbon flux from lactate to the production of other organic compounds via metabolic engineering (Gaspar *et al.*, 2011).

Off late, there is an interesting industrial scale production of 2,3-BD from various agricultural residues as well as logging, pulp and paper, and food industry wastes. Therefore sustainable 2,3 butanediol production employing optimized fermentation process, using natural high producers as inoculants and renewable biowastes as substrates is the need of the hour to support the increasing burden on petrochemical industry. The present study aims at exploring the natural 2,3 BDL producing ability of new lactic acid bacteria, Clostridia and *Paenibacillus* isolates together with exploiting their ability of saccharification of cheap renewable substrates in view of energy management.

Materials and Methods

Bacterial strains and growth conditions

The non-pathogenic lactic acid bacteria (LAB), five *Clostridium* and two *Paenibacillus* isolates which were previously isolated and maintained as pure cultures in the culture bank of Department of Agricultural Microbiology, University of Agricultural Sciences, Dharwad. *Clostridium acetobutylicum* ATCC 824, a solventogenic strain was also included in this study. The Lactic acid bacteria were grown in MRS broth (Hi-Media, Bangalore, India). The Clostridia were maintained under anaerobiosis on P2 medium (Annous and Blaschek 1990). The *Paenibacillus* strains were grown and maintained on Nutrient agar medium.

Qualitative screening of isolates for 2,3-Butanediol production

The cultures were subjected to Vogues Proskauer test in order to determine their ability to produce *acetyl methyl carbinol (acetoin)*, an important intermediate product of a long fermentation pathway leading to the production of 2, 3-butanediol. As the end product is not easily detected, the production of 2,3-Butanediol is thus indirectly detected when the pathway intermediate *acetoin* reacts with reagents to turn red. The medium used is a nutrient broth medium with 0.5% glucose added. An inoculum from a pure culture is transferred aseptically to a sterile tube of VP broth. The inoculated tube is incubated at 35-37 ◌̊ C for 24 hours. After incubation, five drops of Barritt's A are added and the tube is shaken gently to mix the ingredients. Then, five drops of Barritt's B are added. The tube is then slanted to allow maximum oxygen exposure to the reaction mixture and allowed to stand for up to 30 minutes. Development of a red color indicates a positive test.

Analysis of 2,3 Butanediol using Gas Chromatography

Chromatographic standards of 2,3 Butanediol were obtained commercially from Sigma Aldrich. The standard mixture of each solvent

and acids was taken 10 g/l diluted using 20 % orthophosphoric acid as a diluent. 2, 3 Butanediol standards with a concentration of 25ppm was filtered through syringe driven filter nylon 66 of 0.22µ pore size and stored under frozen conditions until use.

Twenty one lactic acid bacterial isolates which showed positive for VP test were subcultured in MRS broth**.** For sample preparation 0.5ml from vigorously growing pure cultures were inoculated into 20 ml T6 broth (Cheng *et al.*, 2016) in 50 ml vials under anaerobic conditions. The concentration of 2,3Butanediol in the fermentation broth was evaluated after (after 96 h at 37^0C) 5^{th} and 6^{th} day at 37^0C. All the samples were filtered through nylon 66 of 0.22µ and stored frozen in sealed vials until use.

The concentration of 2,3-BD produced by the strains was measured using a gas chromatography (LCGC make Agilant model 7260) equipped with a flame ionization detector. Separation took place in 2m DB wax column using nitrogen as the carrier gas. The GC oven temperature was initially controlled at 40^0C for 5 min, and then it was increased at a rate of at ramp 20 per min to a final temperature of 130^0C, which was held for 10 min. The temperature of the injector was set at 280^0C and the detector was set at 280^0C with H_2 flow at 25ml/min, air 100ml/min and N_2 at 25 ml/min. The injection volume was 0.1µl. Data acquisition software was used to integrate the data.

Production of 2,3 BD in batch cultures with agro-residues amendment

Lactic acid bacterial strains LDL18, LA-3 and LGr-5 were found to be high 2,3-BD producers and were used for batch culture studies with amendment of agroresidues. A batch culture medium containing CH_3COONH_4 (2.2g), Yeast Extract (1.5g), K_2HPO_4 (0.5g), KH_2PO_4 (0.5g), $MgSO_4.7H_2O$ (0.2g), $MnSO_4.$ $7H_2O$ (0.01g) and NaCl (0.01g) was prepared in anaerobic environment (N_2 100%).

In a previous experiment, we used citrus fruits wastes with low resultant 2,3 –BD production. Hence in the present study, pineapple pulp waste was used as substrate at 2, 6 and 8% concentration. Cultures were inoculated at 2% in the following treatment combinations.

Treatments
T1: Inoculated with LGr-5 @ 2% substrate concentration
T2: Inoculated with LGr-5 @ 6% substrate concentration
T3: Inoculated with LGr-5 @ 8% substrate concentration
T4: Inoculated with LDL-18 @ 2% substrate concentration
T5: Inoculated with LDL-18 @ 6% substrate concentration
T6: Inoculated with LDL-18 @ 8% substrate concentration

The paddy straw was delignifed using 2% NaOH and autoclaved, washed and dried. The powder form of the substrate having 30-32 % cellulose was treated with cellualse enzyme (@ 0.5 ml per gram of substrate and subjected to 60ºC for 30 mins for obtaining reducing sugars. This substrate was supplemented with yeast extract (1% w/w) and potassium diphosphate (0.5%w/w) as source of nitrogen and phosphorous respectively. Such a substrate was inoculated with the LAB cultures separately for testing for butanediol production. Anaerobic conditions were created by flushing with carbon dioxide and nitrogen

gases using gassing manifold and sealed. The production of 2,3- BD at 5 days of incubation was analysed in GC.

Results and Discussion

Of the total isolates screened for acetoin production, twenty one LAB isolates, two *Paenibacillus* and six Clostridia scored positive for VP test. Kopke *et al.*, (2011) reported 2,3-BD production by three nonpathogenic acetogenic *Clostridium* species—*Clostridium autoethanogenum*, *C. ljungdahlii*, and *C. ragsdalei*—using syngas as sole source of carbon and energy. Strains of *Paenibacillus* have been reported to produce acetoin concentration of 30.98 g L^{-1} under the optimized conditions, with 71.83% of theoretical glucose conversion efficiency (Zhang *et al.*, 2012). Bacteria fermenting sugars *via* the butanediol pathway produce acetoin (i.e., acetyl methyl carbinol or 3-hydroxybutanone) as an intermediate which is then finally reduced to 2,3-butanediol. (2 pyruvate = acetoin + $2CO_2$; acetoin + NADH + H^+ = 2,3-butanediol + NAD^+) by the action of 23BD dehydrogenase or acetoin reductase (Hugenholtz *et al.*, 1993). Hence the VP positive isolates were tested for the production of 2,3-BD in batch fermentation and determined by quantitative Gas chromatography analysis. The concentration of 2,3-BD produced by the LAB isolates ranged from 0.008 to 18.577 g/L.

Table.1 Concentration of 2,3-BD produced by Lactic acid bacterial and *Paenibacillus* isolates in shaken flask cultures

S.No	Isolate Code	Concentration at (gL^{-1})
1	LA-3	1.3908
2	LA-8	0.0013
3	LSP-14	0.0019
4	LGr-5	1.9893
5	LA2	0.0024
6	LA3	0.0008
7	LA8	0.0023
8	LA12	0.0006
9	LABT1	0.0013
10	LABT5	0.0020
11	LBM4(2)	0.0021
12	LBM4	0.0011
13	LDL18	18.577
14	LGN2	0.0012
15	LGN3	0.0020
16	LGN5	0.0016
17	LGN6	0.0013
18	LGN12	0.0014
19	LSP2	0.0010
20	LSP12	0.0009
21	LSP14	0.0008
22	*Paenibacillus*NBF85	0.0003
23	*Paenibacillus*	0.0011

Table.2 Concentration of 2,3-BD produced by *Clostridium* isolates in shaken flask cultures

S.No	Isolate Code	Retention Time (mins)	Concentration (gL^{-1})
1	MR-10-11(3)	10.75	0.0432
2	J-11-99(2)	10.75	0.0453
3	J-11-117(2)	10.75	0.0356
4	J-11-145(1)	10.75	0.0
5	J-11-147(2)	10.75	0.0
6	ATCC 824	10.75	0.0

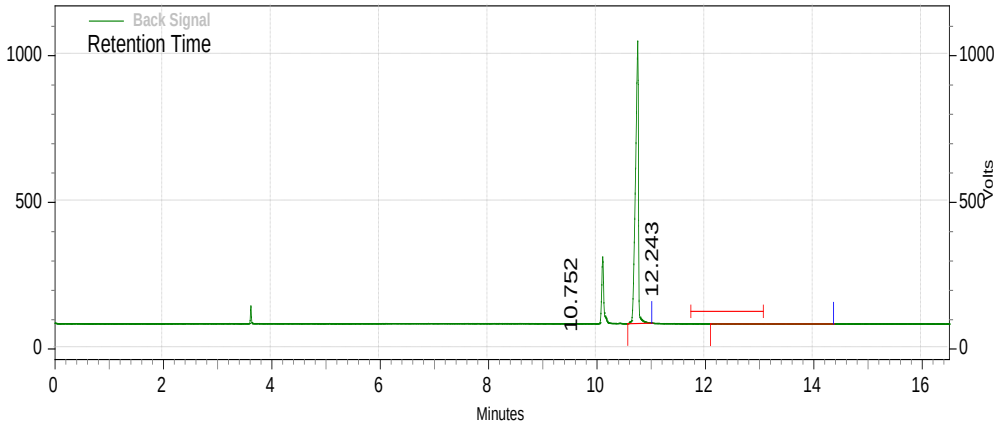
Table.3 Production of 2,3-BD by LAB cultures at various concentrations of pineapple pulp

Treatments	Retention Time	**2**,3, butanediol Concentration ($g\ L^{-1}$)
Inoculated with LGr-5 @2% substrate concentration	10.54	0.084
Inoculated with LGr-5 @ 6% substrate concentration	10.54	0.120
Inoculated with LGr-5 @ 8% substrate concentration	10.54	0.220
Inoculated with LA-3 @2% substrate concentration	10.54	0.234
Inoculated with LA-3 @ 6% substrate concentration	10.54	0.458
Inoculated with LA-3 @ 8% substrate concentration	10.54	0.652

Table.4 Production of 2,3, BD by LAB cultures in delignified paddy straw

TREATMENTS	Retention Time	**2**,3, butanediol Concentration ($g\ L^{-1}$)
Delignifed paddy straw + LGr-5	10.54	0.075
Delignifed paddy straw + LA-3	10.54	0.050
Delignifed paddy straw + LDL18	10.54	0.020

Fig.1 Gas chromatography profile of 2,3-BD produced by strain LDL-18



Strains of lactic acid bacteria, LDL18 showed highest production of 2,3-BD (18.577 g/L) followed by LGr-5 which showed production of 2, 3-BD at 1.98g/L and LA-3 which has shown a concentration of 1.39 g/L (Table 1). As seen in table 2, only three *Clostridium* isolates showed 2,3-BD production. The highest level of 0.0453 g L^{-1} of 2,3Butanediol production was observed by MR-10-5(1). In our study, the reference strain *C. acetobutylicum* ATCC 824 has not shown any 2,3 Butanediol production. The gas chromatography solvent profile of the highest 2,3-BD producing strain LDL-18 is shown in figure 1. Although theoretical yield for 2,3-butanediol was not achieved here, the study has shed light on the natural potential of these putative isolates in 2, 3-BD production and possibility for upscaling and improvement of process technology for maximizing yield by using alternative cheaper substrates.

The highest 2,3-BD producers namely LDL-18, LGr-5 and LA-3 were further evaluated in batch fermentation studies by amendment of renewable agroresidues such as pine apple pulp and delignified paddy straw at different concentrations.

From the results obtained (Table 3 and 4), it is evident that with increase in concentration of the substrate, the production of 2,3-BD also increased. Among the strains tested, LA-3 produced maximum 2,3-BD as compared to LGr-5. Therefore, it is essential to conduct trials on wide range of substrates and the optimize the fermentation variables for maximum bioconversion to 2,3-BD.

In the present study, most Lactic acid bacteria produced only meager concentrations of 2,3-BD. Among the strains, LGr-5 performed better in batch fermentation amended with delignified paddy straw. However the inhibitory compounds present in the alkali pretreated substrate might have restricted the growth of the test isolates. However it is also reported that some of the degradation products viz., furfural, hydroxymethyl furfural formed during pretreatment and hydrolysis of fiber-rich agricultural biomass are in turn stimulatory on the growth of *Clostridium beijerinckii* and butanol production (Ezeji *et al.,* 2007). From the results it is evident that with increasing concentrations of pineapple pulp waste, 2,3-BD production increased with both strains LGr-5 and LDL-18.

Considering the importance of 2,3-BD in wide industrial applications, the present study has attempted to screen microorganisms for

2,3 BDL production. Besides, the use of cheaper agro-residues such as pineapple pulp and paddy straw would surmount the use of costly pure sugars. Other sugar rich substrates could be tried. From the above mentioned results, it can be concluded that the isolates LDL18, LGr-5 and LA-3 are potential platform strains for further refinement of 2,3 BD production process and metabolic engineering.

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