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SHORT COMMUNICATION

A novel thin-layer chromatography method to screen 1,3-propanediol producers

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Abstract To date, there is no established protocol for the screening of 1,3-propanediol producers. The proposed method has a wide applicability to harness the commercial potential of microorganisms which produce 1,3-propanediol as the end product. Glycerol fermentation broth of 50 bacteria spotted on thin-layer chromatography plates and run by appropriate solvent systems followed by colour development using vanillin reagent gave different coloured spots with most of the compounds present in the fermentation broth. The appearance of a purple-coloured spot of 1,3-propanediol with a retention factor (R_f) of 0.62 forms the basis for the selection of 1,3-propanediol producers. Apart from being a rapid detection system the proposed method is pH independent and its authenticity was reconfirmed by HPLC.

Keywords Thin-layer chromatography · 1,3-Propanediol · Glycerol · Fermentation · Vanillin

Introduction

1,3-Propanediol has now become one of the most promising bulk chemicals which has attracted worldwide attention owing to its enormous range of applications in the polymer, cosmetics, food, adhesives, lubricant, laminate, solvent, antifreeze and pharmaceutical industries [7]. In 2008, the huge market for 1,3-propanediol for use in polymers was estimated to be worth over US$6.0 billion [6]. In this context, the production of 1,3-propanediol through biological routes has turned out to be a boon for the plastics industry [11].

1,3-Propanediol has been reported to be produced and accumulated by bacteria under anaerobic condition as the major end product of glycerol fermentation. The available reports reveal that quantitative estimation of 1,3-propanediol in fermentation broth is carried out only by two methods: high-performance liquid chromatography (HPLC) equipped with an Aminex HPX-87H column [3] and gas chromatography (GC) equipped with a glass column packed with Chromosorb 101 [5]. Here, GC already has a drawback because the same column can not detect glycerol (substrate for 1,3-propanediol production) present in the fermentation broth. HPLC can detect 1,3-propanediol and glycerol along with other by-products (lactic acid, acetic acid, butyric acid, 2,3-butanediol, ethanol) simultaneously; however, the process is expensive, time consuming, tedious and uneconomical and together with the limited column lifetime it fails to meet the criteria necessary to become a rapid screening procedure.

There are two main hurdles in the development of a rapid screening procedure for 1,3-propanediol producers. First, 1,3-propanediol does not have a specific absorbance peak in the visible–ultraviolet range; thus, a spectrophotometric method cannot be designed [4]. Second, the complexity of the fermentation broth due to the presence of several compounds having similar functional groups (–OH and –COOH) to the target analyte, e.g. triol (glycerol), diols (1,3-propanediol, 2,3-butanediol), monoalcohols (ethanol, butanol) and carboxylic acids (lactic acid, acetic acid, butyric acid), potentially interferes when designing a specific protocol [1].

Since it is hard to escape the general perception that screening acts as the foundation for microbial production

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of any biomolecule, it is of the utmost important to have an efficient, authentic, reliable and inexpensive screening procedure to hunt for the most potent producer/s. Wittlich et al. [12] selectively screened thermophiles to find a strain able to use the hot effluents from fat cleavage plants as a source of glycerol. Du et al. [4] described a novel redox potential (oxidoreduction potential [ORP])-based screening strategy for the isolation of mutants of *Klebsiella pneumoniae* which have an increased ability to produce 1,3-propanediol. However, the determination of the preferred range of ORP of wild type is required to screen mutants making this process un-useful for general screening. Similarly, Ringel et al. [9] developed a screening procedure based on the 1,3-propanediol tolerance, crude glycerol tolerance and utilization, and exclusion of pathogenic microorganisms.

Therefore, in the present investigation, the objective is to design a simple and rapid protocol for 1,3-propanediol detection with wide applicability to harness the undoubted commercial potential of microorganisms to produce 1,3-propanediol as the major end product.

Materials and methods

Materials

1,3-Propanediol, glycerol, lactic acid, acetic acid, butyric acid, 2,3-butanediol, 1,2-propanediol and butanol were purchased from Sigma Chemicals (St. Louis, USA). TLC plates (Silica gel 60 F_{254}) were purchased from E. Merck Ltd., Germany.

Fermentation

The fermentation broths used in this investigation were derived from glycerol fermentation by 50 bacterial cultures, isolated from rumen and gut of the ruminants. The bacteria were cultivated under an anaerobic conditions in 500-ml anaerobic bottles containing 200 ml production medium with the following composition (g l^{-1}): glycerol, 25.0; Tryptone 10.0; $K_2HPO_4{\cdot}3H_2O$ 5.0; KH_2PO_4, 3.48; $MgCO_3$, 150 mM; $CaCl_2{\cdot}2H_2O$, 0.20; $CoCl_2{\cdot}6H_2O$, 0.004, $MgCl_2{\cdot}7H_2O$, 0.40 and $Na_2S{\cdot}9H_2O$, 0.02 at pH 7.0 ± 0.2. The details of fermentation are described in Anand and Saxena [2]. The samples obtained after 96 h of fermentation were centrifuged at 10,000×*g* for 20 min to pellet the cells, followed by filtration using syringe filters (pore size 0.22 μm, Mdi filters, India).

Thin-layer chromatography

One-dimensional chromatography on pre-activated silica gel TLC plates was carried out in saturated tanks. Samples were spotted 1.5 cm from the lower edge of the plate and at least 1.0 cm from the lateral border. The chromatograms were developed by the ascending technique with the desired mobile phase. The solvent front was drawn 10.0 cm from the application line.

Analytical methods

The concentration of 1,3-propanediol was determined by the HPLC method described by Anand and Saxena [2].

Results and discussion

Selection of developing reagent

Selection of the best developing reagent plays an important role in the development of thin-layer chromatography procedures. Here, 10 μl of standard solution (50 mg ml^{-1}) of 1,3-propanediol, glycerol, lactic acid, acetic acid, butyric acid, 2,3-butanediol, 1,2-propanediol, butanol and ethanol, which are reported to be present in the fermentation broth [1], were spotted separately on TLC plates. Air-dried plates were sprayed with three different developing reagents separately: (1) bromocresol green, (2) bromophenol blue and (3) vanillin. Subsequently, the plates were heated at 100 °C for 5 min to develop the spots of the compounds.

It is evident from the Table 1 that the developing reagents bromocresol green and bromophenol blue gave yellow-coloured spots on TLC plates with only carboxylic acids, whereas vanillin reagent gave different coloured spots with most of the compounds e.g. carboxylic acids, 1,3-propanediol, 1,2-propanediol and 2,3-butanediol gave pale white, purple, pink and blue-coloured spots, respectively. Here, acetic acid, ethanol and butanol did not give any spot. Although 1,2-propanediol has never been reported to be produced in glycerol fermentation [10], it is used in the present investigation owing to its similar characteristics with 1,3-propanediol. The distinct purple spot of 1,3-propanediol gave an opportunity to design a rapid method for the detection of 1,3-propanediol producers; therefore, vanillin reagent was selected for further investigation.

Selection of solvent system

The choice of best mobile phase and the optimization of its composition are very important because the chromatographic separation is difficult to achieve [8]. In the present investigation, the next hurdle was to separate all these compounds on the TLC plate if present in the mixture. To achieve this, 10 μl of standards of these compounds (50 mg ml^{-1}) were spotted individually (for reference) and

Table 1 Appearance of standard compounds on TLC plates when developed with three different reagents

Compounds	Appearance on TLC plates		
	A	B	C
1,3-Propanediol	−	−	+ (purple)
Glycerol	−	−	+ (pale white)
Lactic acid	+ (yellow)	+ (yellow)	+ (pale white)
Acetic acid	+ (yellow)	+ (yellow)	−
Butyric acid	+ (yellow)	+ (yellow)	+ (pale white)
2,3-Butanediol	−	−	+ (blue)
1,2-Propanediol	−	−	+ (pink)
Butanol	−	−	−
Ethanol	−	−	−

A TLC plate developed with bromocresol green (0.04 % in ethanol), *B* TLC plate developed with bromophenol blue (0.4 % in ethanol, pH 6.7), *C* TLC plate developed with vanillin (1.0 gm vanillin + 2.0 ml conc. H_2SO_4 + 98.0 ml ethanol), + colour appeared, − no colour appeared

Table 2 R_f values of standard compounds in three different solvent systems

Compounds	R_f values in three different solvent systems		
	A	B	C
1,3-Propanediol	0.52 ± 0.07	0.71 ± 0.02	−
Glycerol	0.45 ± 0.0001	0.32 ± 0.03	−
Lactic acid	0.55 ± 0.015	0.65 ± 0.011	0.40 ± 0.018
Acetic acid	−	−	0.51 ± 0.014
Butyric acid	0.45 ± 0.02	0.64 ± 0.01	0.33 ± 0.017
2,3-Butanediol	−	0.81 ± 0.25	−
1,2-Propanediol	0.53 ± 0.012	0.72 ± 0.015	−
Butanol	−	−	−
Ethanol	−	−	−
Mixture[a]	Smear	Smear	Smear

A benzene/methanol/glacial acetic acid 45:8:11, *B* chloroform/methanol 70:30, *C* ethanol/ammonium hydroxide (25 %)/water 25:4:3, − no spot

[a] Mixture of standards

Table 3 R_f values of standard compounds run in three different ratios of the solvent system (chloroform/methanol)

Compounds	R_f values in different ratios of chloroform/methanol		
	A	B	C
1,3-Propanediol	0.65 ± 0.015	0.62 ± 0.018	0.53 ± 0.017
Glycerol	0.28 ± 0.015	0.25 ± 0.021	0.18 ± 0.015
Lactic acid	0.61 ± 0.014	0.54 ± 0.013	0.50 ± 0.017
Acetic acid	−	−	−
Butyric acid	0.60 ± 0.010	0.52 ± 0.013	0.47 ± 0.011
2,3-Butanediol	0.78 ± 0.027	0.75 ± 0.01	0.70 ± 0.019

A 75:25, *B* 80:20, *C* 85:15, − no spot

as a mixture on three TLC plates. These plates were then run in three different solvent systems, separately, which were (a) benzene/methanol/glacial acetic acid 45:8:11; (b) chloroform/methanol 70:30; (c) ethanol/ammonium hydroxide (25 %)/water 25:4:3. The solvents were allowed to run up to two-thirds of the plate height followed by the similar procedure of colour development. It is clearly evident from the observations presented in Table 2 that with the solvent system C, except carboxylic acids, none of the compounds moved on to the TLC plates. A smear was observed with the solvent system A. Although with the solvent system B, each standard spotted individually moved a certain distance on the TLC plate, the retention factor (R_f) values were not very distinct from each other. Therefore, the mixture of these standards produced a smear due to overlapping. Here also acetic acid, butanol and ethanol do not gave any spot as was observed previously.

To further maximize the efficiency of the procedure, the resolution of different standards was achieved by using different ratios of the selected solvent system i.e. chloroform/methanol. Three different ratios, (A) 75:25, (B) 80:20, (C) 85:15, of the chloroform and methanol solvent system were used under the same conditions. The R_f values of each compound obtained are presented in

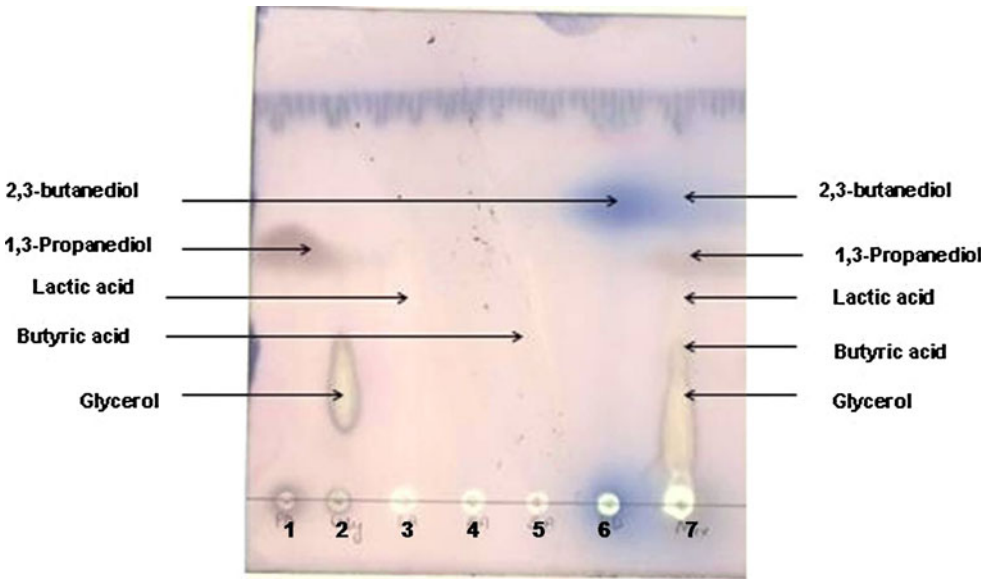

Fig. 1 TLC analysis of different standard compounds run individually and as a mixture in the solvent system chloroform/methanol 80:20 (v/v): *1* 1,3-propanediol, *2* glycerol, *3* lactic acid, *4* acetic acid, *5* butyric acid, *6* 2,3-butanediol and *7* mixture

Table 3. It is clearly evident that with the ratio of (A) 75:25, no distinct resolution of standards was achieved. Only one distinct spot corresponding to R_f 0.28 was observed. Other compounds formed a smear at around R_f 0.62. With the ratio (C) 85:15, it was observed that 1,3-propanediol, lactic acid and butyric acid gave similar R_f values. Finally, with the ratio (B) 80:20, all the standards from the mixture were separated and the different R_f values obtained correspond to each of their standards (Fig. 1).

Test of sensitivity of the TLC method

Efficiency of any screening method depends upon the sensitivity of the method. To examine the critical concentration of 1,3-propanediol required to give a visible spot on TLC plates, 20 µl of different concentrations (10, 20, 30, 40, 50, 60, 70, 80, 90, 100 mg ml^{-1}) of 1,3-propanediol was spotted on TLC plates and run in chloroform/methanol (80:20) followed by a similar process of colour development. The results obtained are presented in Fig. 2 which shows that even at 10 mg ml^{-1}, a distinct purple spot appeared.

Influence of pH on the colour development

In the majority of studies, the optimum pH value for the production of 1,3-propanediol is 7.0 [3, 5]. However, 1,3-propanediol production is coupled to the production of organic acids (lactic acid, acetic acid, butyric acid and succinic acid) via an oxidative pathway which acidifies the medium during the fermentation process. Therefore, we performed an experiment examining the influence of pH on our proposed detection method. Herein samples of 1,3-propanediol were adjusted to a broad range of pH from 2.0 to 10.0 (with an interval of 1.0) and were subsequently spotted on TLC plates. The proposed TLC process was then followed. The results are presented in Fig. 3 which clearly shows that change of pH value has no influence on

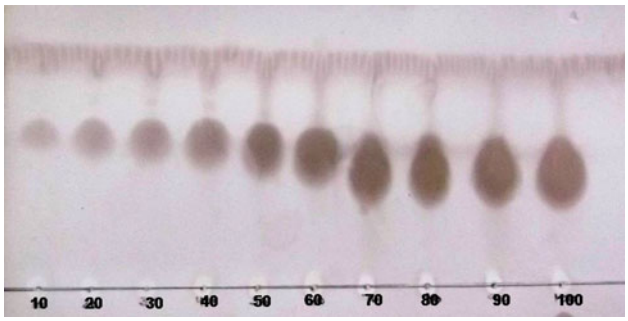

Fig. 2 Sensitivity test for different concentrations of 1,3-propanediol run in the solvent system chloroform/methanol 80:20

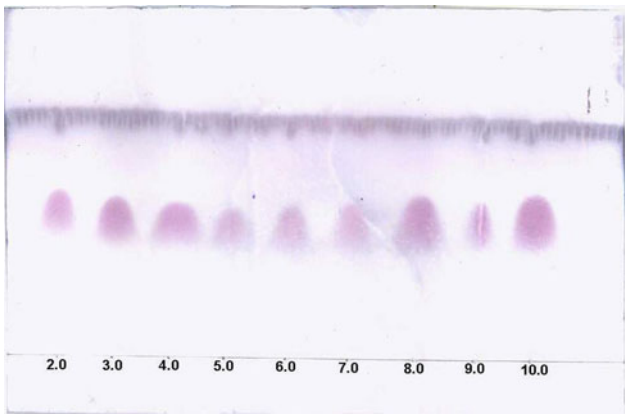

Fig. 3 Influence of pH on the colour development of 1,3-propanediol

Table 4 Analysis of the bacterial samples for 1,3-propanediol on TLC plates and by HPLC method

Isolate no.	TLC	HPLC (g/l)[a]	Isolate no.	TLC	HPLC (g/l)[a]	Isolate no.	TLC	HPLC (g/l)[a]
CB-1	+	2.5	B-8	+	1.5	B-25	−	−
CB-2	−	−	B-9	−	−	B-26	+	1.3
CB-3	−	−	B-1	+	2.1	B-27	−	−
CB-4	+	1.8	B-11	+	2.3	B-28	+	1.7
CB-5	−	−	B-12	−	−	B-29	−	−
CB-6	+	1.5	B-13	+	1.5	B-30	−	−
CB-7	+	1.2	B-14	−	−	B-31	−	−
CB-8	+	2.2	B-15	+	1.5	B-32	−	−
CB-9	−	−	B-16	−	−	B-33	−	−
CB-10	−	−	B-17	+	2.0	B-34	−	−
B-1	−	−	B-18	−	−	B-35	+	1.0
B-2	+	1.2	B-19	+	1.9	B-36	−	−
B-3	−	−	B-20	−	−	B-37	+	1.6
B-4	−	−	B-21	−	−	B-38	−	−
B-5	+	1.6	B-22	+	2.1	B-39	−	−
B-6	+	1.7	B-23	+	1.2	B-40	−	−
B-7	−	−	B-24	−	−			

+ Purple spot appeared after concentrating the sample up to 10 times, − purple spot did not appear after concentrating the sample up to 10 times

[a] Estimation of 1,3-propanediol (g/l) produced by bacterial isolates by HPLC without concentrating the samples

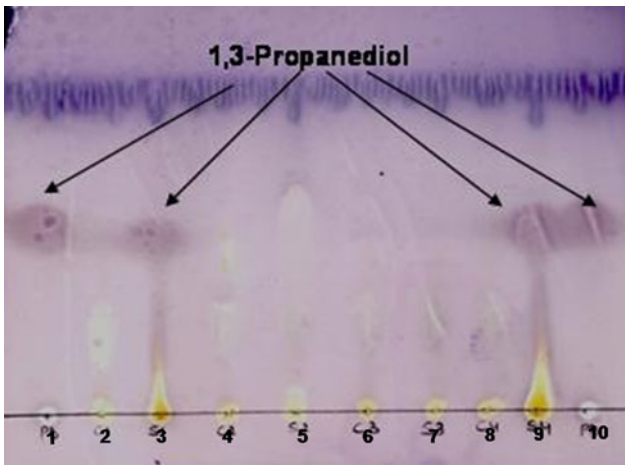

Fig. 4 TLC analysis of the concentrated samples obtained from four bacteria for 1,3-propanediol production: *1*, *10* standard 1,3-propanediol; *2*, *4*, *6* and *8* culture broth of isolates CB1, CB2, CB3 and CB4 after 0 h of incubation; *3*, *5*, *7*, *9* culture broth of isolates CB1, CB2, CB3 and CB4 after 96 h of incubation

the colour development as all the samples gave a purple spot and no colour difference was observed among the samples.

Evaluation of TLC procedure for screening 1,3-propanediol producers using fermentation broth

To authenticate the efficiency of the proposed TLC method, a total of 50 randomly selected bacterial cultures were used. For each culture, fermentation broth obtained at 0 and after 96 h of incubation was used. Five millilitres of each of these culture supernatants (0 and 96 h) was concentrated to 0.5 ml using a Thermo Scientific speed vacuum concentrator (Sawant SC210A). It also resulted in the complete loss of ethanol (boiling point 78 °C). Among organic acids, only acetic acid (boiling point 118 °C) evaporated during concentration. Now, each of these concentrated samples (20 µl) with the standard 1,3-propanediol as a reference was spotted and resolved on the TLC plates. The plates were developed under the same conditions described. The results of TLC are listed in Table 4 which shows that out of 50 only 21 were 1,3-propanediol producers. To further authenticate the accuracy and efficiency of the proposed procedure, the samples were analysed by HPLC (Table 4). The results of prominent 1,3-propanediol producers along with a few negative ones are presented in Fig. 4 which shows that in two of the concentrated culture filtrates (after 96 h), a purple-coloured spot corresponding to the R_f value of standard 1,3-propanediol (0.62 ± 0.018) was observed. Two other cultures that were non-1,3-propanediol producers did not show any purple spot corresponding to 1,3-propanediol even after concentration. Results obtained were coherent with the HPLC data. On the basis of the results, it can be concluded that the proposed method is very rapid and reliable and such a fast method has not been reported so far for screening of 1,3-propanediol producers. This TLC procedure will allow even hundreds of concentrated samples to

be analysed rapidly as it requires only 30 min for each TLC run. Moreover, the method is economical as no special equipment or chemicals are required.

Conclusions

This study establishes a new TLC-based method characterized by simple reaction conditions, high sensitivity, pH independence and reproducibility for detecting 1,3-propandiol in fermentation broths. The presence of 1,3-propandiol is characterized by the appearance of a purple spot at R_f 0.62 ± 0.018 on TLC using a solvent system comprising chloroform and methanol (80:20) and vanillin as the developing reagent. Owing to the importance of 1,3-propanediol and its application in polymer industries, such a screening method for the rapid detection of 1,3-propanediol producers is of immense importance.

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