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Development of a Highly Sensitive, Fast and Efficient Screening Technique for the Detection of 2,3-Butanediol by Thin Layer Chromatography

Saran S[1], Yadav S[2] and Saxena RK[1]*

[1]Technology Based Incubator, University of Delhi South Campus, Biotech Centre, Benito Juarez Road, New Delhi-110021, USA
[2]Department of Microbiology, University of Delhi South Campus, Benito Juarez Road, New Delhi-110021, USA

Abstract

The interest in microbial production of 2,3-butanediol is based on the awareness that 2,3 butanediol is a promising bulk chemical due to its extensive industrial applications. Here, we report a novel method for the detection of 2,3-butanediol. The proposed method has a wide applicability to harness the commercial potential of microorganisms which produce 2,3-butanediol as the end product. Experimentally 32 bacterial strains were screened for 2,3-butanediol production. After 72 h the samples were spotted on thin-layer chromatography plates and ran in a solvent system comprising of hexane:ethyl-acetate:glacial acetic acid in the ratio of 70:30:1.5, followed by colour development using vanillin reagent. The appearance of a blue-colored spot of 2,3-butanediol with a retention factor (R_f) of 0.68 corresponds to the R_f value of the standard 2,3-butanediol which forms the basis for the selection of 2,3-butanediol producers. Apart from being a rapid detection system the proposed method is highly sensitive as it was able to detect a concentration as low as 1.0 mg/ml and its authenticity was reconfirmed by GC and GC-MS. Low cost of this method provides an effective support for 2,3-butanediol detection at any scale.

Keywords: 2,3-butanediol, Thin-layer chromatography, Rapid detection

Introduction

Continuous depletion of petroleum fuel-reserves is one of the prime concerns in this era. Despite of oil reserve availability, current estimates show that accessing them will become extremely difficult in a few decades [1,2]. Due to this, the bio-refinery systems that integrate biomass conversion processes and equipment to produce fuels, power, and chemicals from annually renewable resources are at the stage of worldwide development [3]. Many chemicals that could only be produced by chemical processes in the past can now have the potential to be generated biologically using renewable resources. Microbial production of 2,3-butanediol (2,3-BD) is one such example [4-6]. Interest in this bioprocess has increased remarkably because 2,3-BD has a large number of industrial applications, and microbial production will alleviate the dependence on oil supply.

2,3-BD can be produced efficiently *via* mixed acid fermentation with prokaryotes such as *Klebsiella pneumonia Klebsiella oxytoca Enterobacter aerogenes Serratia*, and *Bacillus polymyxa*. In these bacteria, pyruvate is first converted into α-acetolactate by acetolactate synthase. In anoxic state, α-acetolactate decarboxylase catalyzes the conversion of α-acetolactate into acetoin. 2,3-Butanediol is resulted from the reduction of acetoin by butanediol dehydrogenase. It exists in three stereoisomers, the dextro- (d-), levo- (l-) and meso-forms. The available reports reveal that quantitative estimation of 2,3-BD in fermentation broth is carried out only by two methods: high-performance liquid chromatography (HPLC) equipped with an Aminex HPX-87H column or by Alltech IOA-2000 organic acid column [7,8] and gas chromatography (GC) equipped with a glass column packed with Chromosorb 101 [9-11]. Although HPLC can detect 2,3-butanediol along with other by-products (1,4-propanediol, lactic acid, butyric acid, acetic acid, acetoin, 1,3-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. Therefore it was of utmost important to have an efficient, authentic, reliable and inexpensive screening procedure to hunt for the most potent 2, 3-butanediol producer/s. Therefore, in the present investigation, the objective is to design a simple and fast protocol for 2,3-butanediol detection with wide applicability to harness the undoubted commercial potential of microorganisms to produce 2,3-butanediol as the major end product.

Materials and Methods

Materials

2, 3-butanediol, 1, 4-propanediol, 1, 3-butanediol, lactic acid, butyric acid, acetic acid, acetoin and ethanol were purchased from Sigma Chemicals (St. Louis, USA). TLC plates (Silica gel 60 F254) were purchased from E. Merck Ltd., Germany.

Fermentation conditions

Thirty two bacterial cultures, including different strains of *Klebsiella pneumoniae* were procured from the laboratory stock culture collection, Department of Microbiology, University of Delhi South Campus, New Delhi, India and screened for 2,3-butanediol production. The growth medium consisting of glucose 20 g/l, $(NH_4)_2HPO_4$ 5 g/l, $MgSO_4$ 0.3 g/l, KCl 1.0 g/l, pH 7.0 was used to grow and maintain the bacterial cultures.

The bacteria were grown in 250 ml Erlenmeyer flasks containing 50 ml production medium with the composition (g/L): glycerol, 25.0; Tryptone 10.0; $K_2HPO_4 \bullet 3H_2O$ 5.0; KH_2PO_4, 3.48; $MgCO_3$, 150 mM; $CaCl_2 \bullet 2H_2O$, 0.20; $CoCl_2 \bullet 6H_2O$, 0.004, $MgCl_2 \bullet 7H_2O$, 0.40 and $Na_2S \bullet 9H_2O$, 0.02 at pH 7.0 ± 0.2. Incubation was carried out at 37°C,

***Corresponding author:** Saxena RK, Technology Based Incubator, University of Delhi South Campus, Biotech Centre, Benito Juarez Road, New Delhi-110021, Tel: 91-11-24116559; E-mail: rks.micro2012@gmail.com

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150 rpm for 72 h. The samples obtained after 72 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). The pellet thus obtained was used for growth estimation and supernatant was used for 2, 3-butanediol estimation.

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 2,3-butanediol and other by-products was determined by the GC method described by [9].

GC–MS analysis

GC–MS analysis was performed with the Shimadzu QP-2010 Plus with Thermal Desorption System. Compounds were separated on a capillary column, the injector temperature was 260°C, and 1 µL samples were injected in split mode. The split ratio was 100; the total flow was 125.20 mL/min. The oven temperature was maintained at 40°C for 1 min after injection, then programmed at 10°C min-1 to 200°C, which was held for 4 min, and then at 10°C min^{-1} to 200°C, which was held for 4 min. 2,3-Butanediol was identified by the use of standard database of mass spectra.

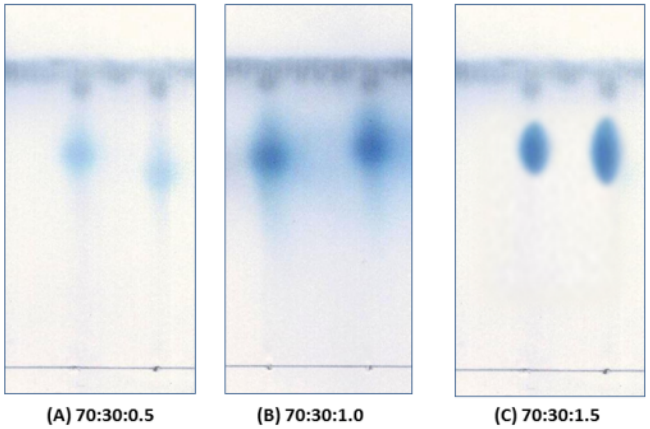

Figure 1: Influence of solvent system on the colour development of 2,3-butanediol.

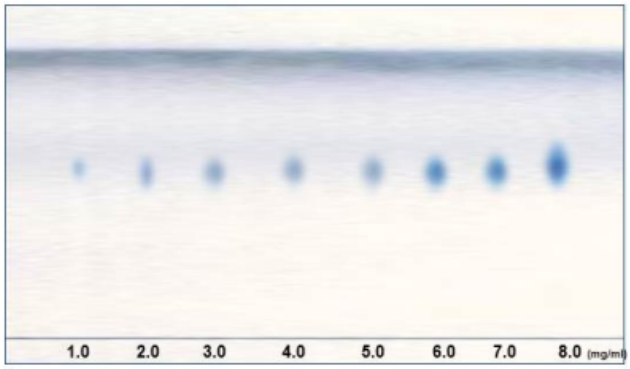

Figure 2: Sensitivity test for different concentrations of 2,3-butanediol run in the solvent.

Results and Discussion

Selection of solvent system

The choice of best solvent system and the optimization of its composition are very important because the chromatographic separation is difficult to achieve. In the present investigation, 10 µl of standards of 2, 3-butanediol, 1, 4-propanediol, 1, 3-butanediol, lactic acid, butyric acid, acetic acid, acetoin and ethanol (50 mg/ml) were spotted individually and as a mixture on two TLC plates. These plates were then run in two solvent systems, separately, which were (a) chloroform:methanol 90:10 (b) hexane: ethyl acetate: glacial acetic acid 70:30:1. The solvents were allowed to run up to two-thirds of the plate height followed by its development using vanillin reagent. It was observed that with the solvent system 'b', except 2,3-butanediol, none of the compounds moved on to the TLC plates, however, a smear was observed with the solvent system 'a' and each standard spotted were not distinct and the mixture of these standards produced a smear due to overlapping. To further maximize the efficiency of the procedure and to enhance the resolution of 2,3-butanediol, different permutation combination of mobile phase were tested. Three different ratios, (A) 70:30:0.5, (B) 70:30:1.0, (C) 70:30:1.5, of the hexane: ethyl acetate: glacial acetic acid was used under the same conditions. It is clearly evident from the Figure 1 that with the ratio of (A) 70:30:0.5, no distinct resolution was achieved. Only one distinct spot corresponding to R_f 0.68 was observed. With the ratio (B) 70:30:1.0, a bit cleaner plate was observed. Finally, with the ratio (C) 70:30:1.5, much clearer plates were obtained with distinct 2,3-butanediol spots. The above edge of this solvent system is that the procedure is specific for 2,3-butanediol and other by-products were not detected.

Test of sensitivity of the TLC method

A detection method is only reliable if it is able to detect even a minimal quantity of the desired product. Here, to examine the critical concentration of 2,3-butanediol required to give a visible spot on TLC plates, 10µl of different concentrations (1.0, 2.0, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0 mg/ml) of 2,3-butanediol was spotted on TLC plates and run in hexane: ethyl acetate: glacial acetic acid (70:30:1.5) followed by a similar process of colour development. The results obtained are presented in Figure 2 which shows that even at 10 mg ml^{-1}, a distinct blue spot appeared. Therefore, it can be inferred that this process is highly sensitive and can be efficiently used for the screening of 2,3-butanediol producers.

Screening of 2,3-butanediol producers using TLC procedure

To authenticate the efficiency of the proposed TLC method, a total of 32 randomly selected bacterial cultures were used. For each culture, fermentation broth obtained at 72 h of incubation was used. Ten microliters of each of these culture supernatants (72 h) with the standard 2,3-butanediol 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 1) which shows that out of 32 only 12 were 2,3-butanediol producers. To further authenticate the accuracy and efficiency of the proposed procedure, the samples were analysed by GC (Table 2). Among the 12 strains producing 2,3-butanediol, strain no. UDSC 6 was found to be a potent producer resulting in 2.97 g/L of 2,3-butanediol production as detected by GC. The results of prominent 2,3-butanediol producers along with a two negative ones are presented

Isolates No.	2,3-Butanediol production		Isolates No.	2,3-Butanediol production	
	24 hrs.	48 hrs.		24 hrs.	48 hrs.
UDSC 1	+	+	UDSC 17	+	+
UDSC 2	+	+	UDSC 18	-	-
UDSC 3	-	-	UDSC 19	+	+
UDSC 4	-	-	UDSC 20	-	-
UDSC 5	-	-	UDSC 21	-	-
UDSC 6	++	+++	UDSC 22	+	++
UDSC 7	+	++	UDSC 23	-	-
UDSC 8	+	+	UDSC 24	-	-
UDSC 9	-	-	UDSC 25	-	-
UDSC 10	-	-	UDSC 26	+	+
UDSC 11	-	-	UDSC 27	-	-
UDSC 12	+	++	UDSC 28	-	-
UDSC 13	-	-	UDSC 29	-	-
UDSC 14	-	-	UDSC 30	-	-
UDSC 15	-	-	UDSC 31	+	+
UDSC 16	+	+	UDSC 32	-	-

−blue spot not appeared, + Faded blue spot appeared, ++ Visible blue spot appeared, +++ Prominent dark blue spot appeared

Table 1: Analysis of the bacterial samples for 2,3-butanediol on TLC plates.

S.No	Isolates no.	48 hrs.	2,3-butanediol yield g/L
1.	UDSC 1	+	0.85
2.	UDSC 2	+	0.16
3.	UDSC 6	+++	2.97
4.	UDSC 7	++	2.68
5.	UDSC 8	+	0.73
6.	UDSC 12	++	1.60
7.	UDSC 16	+	0.56
8.	UDSC 17	+	0.14
9.	UDSC 19	+	0.65
10.	UDSC 22	++	1.26
11.	UDSC 26	+	0.89
12.	UDSC 31	+	0.94

+ Faded blue spot appeared, ++ Visible blue spot appeared, +++ Prominent dark blue spot appeared

Table 2: Evaluation of positive 2,3-butanediol producers on TLC plates and by GC method.

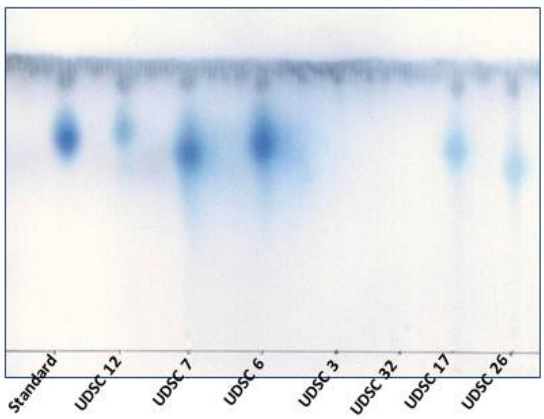

Figure 3: TLC analysis of the concentrated samples obtained from seven bacteria for 2,3- butanediol production after 72 h of incubation.

in Figure 3 which shows that in five of the culture filtrates (after 72 h), a blue coloured spot corresponding to the R_f value of standard 2,3-butanediol (0.68 ± 0.02) was observed. Two other cultures that were non 2,3-butanediol producers did not show any blue spot corresponding to 2,3-butanediol. Results obtained were coherent with the GC 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 2,3-butanediol producers. This TLC procedure will allow even hundreds of samples to be analysed rapidly as it requires only 15 min for each TLC run. Moreover, the method is economical as no special equipment or chemicals are required.

GC–MS analysis

2,3-butanediol scrapped from the TLC plates was confirmed by GC–MS in split mode. The mass spectrum showed it was 2,3-butanediol.

Conclusions

This method is simple, reliable and well suited for the routine laboratory assay of a large number of samples. Using this protocol, hundreds of samples can be analyzed in a day's time. Moreover, this method is economical as no special equipments or chemicals are required. Realizing the importance of 2,3-butanediol and its application in different industries, devising the methods for rapid screening of 2,3-butanediol producers is of great importance.

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References

1. Lin L, Cunshan, Z, Vittayapadung S, Xiangqian S, Mingdong D (2010) Opportunities and challenges for biodiesel fuel. Appl Energy 88: 1020-1031.
2. Li L, Chen C, Li K, Wang Y, Gao C (2014) Efficient Simultaneous Saccharification and Fermentation of Inulin to 2,3-Butanediol by thermophilic Bacillus licheniformis ATCC14580. Appl Environ Microbiol 80: 6458-6464.
3. Ji XJ, Huang H, Li S, Du J, Lian M (2008) Enhanced 2,3-butanediol production by altering the mixed acid fermentation pathway in *Klebsiella oxytoca.* Biotechnol Lett 30: 731-734.
4. Biebl H, Zeng AP, Menzel K, Deckwer WD (1998) Fermentation of glycerol to 1,3-propanediol and 2,3-butanediol by *Klebsiella pneumoniae.* Appl Microbiol Biotechnol 50: 24–29.
5. Perego P, Converti A, Del Borghi A, Canepa P (2000) 2,3-Butanediol production by *Enterobacter aerogenes*: selection of the optimal conditions and application to food industry residues. Bioprocess Eng 23: 613-620.
6. Ji XJ, Huang H, Li S, Du J, Lian M (2008) Enhanced 2,3-butanediol production by altering the mixed acid fermentation pathway in *Klebsiella oxytoca.* Biotechnol Lett 30: 731-734.
7. Kim SJ, Seo SO, Jin YS, Seo JH (2013) Production of 2,3-butanediol by engineered Saccharomyces cerevisiae. Bioresour Technol 146: 274-281.
8. Kopke M, Mihalcea C, Liew F, Tizard JH, Ali MS (2011) 2,3-Butanediol Production by Acetogenic Bacteria, an Alternative Route to Chemical Synthesis, Using Industrial Waste Gas. Appl Environ Microbial 77: 5467-5475.
9. Wang Y, Li L, Ma C, Gao C, Tao F, Ping Xu P (2013) Engineering of cofactor regeneration enhances (2S,3S)-2,3-butanediol production from diacetyl. Scientific Reports 3: 2643
10. Xiao Z, Wang X, Huang Y, Huo F, Zhu X (2012) Thermophilic fermentation of acetoin and 2,3-butanediol by a novel *Geobacillus* strain. Biotechnol Biofuels 5: 88.
11. Wang X, Lv M, Zhang L, Li K, Gao C (2003) Efficient bioconversion of 2,3-butanediol into acetoin using Gluconobacter oxydans DSM 2003. Biotechnol Biofuels 6: 155.