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Comparison study on production of 2, 3-butanediol using batch flask fermentation from two different microorganisms

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Abstract

Currently, several researchers were focused on the production of 2,3-butanediol for its use in various applications. Our research has mainly aimed in the production of 2,3-butanediol from two different strains such as Bacillus subtilis and Bacillus cereus. Since these are GRAS microorganisms, we have chosen these for our production. Mostly anaerobic species are only used for the production of 2,3-buatnediol. But we have used aerobic species for this project. Here the Bacillus cereus is the new species used for this production. From this we conclude that Bacillus cereus is also producing 2,3-butanediol. To our research, we summarize that the microbial production of 2,3-butanediol by Bacillus species which shows an efficientsynthesis of 2,3-butanediol on a laboratory scale.

Keywords: *Bacillus subtilis*, *Bacillus cereus*, 2,3-butanediol, GRAS microorganism, aerobic species.

Introduction

The biological process is major interest in the face of inadequacy of biofuels. 2,3-BD is a pale, fragrance-free liquid with a huge industrial applications and one of the valued biofuel having 27.2kJ g^{-1} as heating value, comparatively satisfactory to used fuels being used, such as ethanol and methanol[1]. It can be enriched by dehydrating with methyl-ethyl-ketone, which is an industrial solvent or to 2,3-BD, an essential solvent for producing synthetic rubber[2]. Now-a-days, interest for its production by microbial method is increasing. Besides, 2,3-BD is used in pharmaceuticals as carrier and also in the production of diacetyl, an additive agent in foodstuffs[3]. The microbial production of 2,3-BD is more eco-friendly and economically feasible technique. Presently, many studies are focusing on 2,3-BD production through sugar fermentation[4]. Regardless of the effective productions achieved through the glucose conversion, the comparatively high cost of sugar substrates is still a major concern during 2,3-BD production[5]. Hence the cheaper and best alternative biomass based production of 2,3-BD is of high importance. The most important precursor of 2,3-BD is Acetoin (AC). It isproduced in bacteria by the action of enzyme α-acetolactate synthase from pyruvate, which catalyze the condensation reaction of two pyruvate molecules involving a decarboxylation to provide α-acetolactate, and α-acetolactate decarboxylase that carboxylates to acetoin through catabolic pathway[6]. Various isomeric types of 2,3-BD can be formed by reduction reaction with different acetoinreductase with diverse stereo specificity or by a cascade of reactions called "butanediol cycle" the presence of which has been testified in diverse bacteria[7]. Since simple oil reserves become gradually scarce, bio-fuel systems that participate in conversion of biomass process and machinery to yield biofuels, energy, and chemical products from yearly renewable sources are in the phase of global development[8]. Numerous chemical products that could only be produced by chemical process in the old time can now have the capability to be created through bio based methods spending renewable resources. Additionally, BD has impending applications in the industry which manufactures inks for printing, fragrances, fumigant, moisturizing agents, food items, and pharmaceutical products. Batch flask fermentation is a simple process for the fermentation of microorganism in a lab scale process. It is a inexpensive and modest procedure. Most commonly Erlenmeyer flask is used, and it is suitable for low volume short term fermentations[9]. We can have a more cultured shaker in a precisely defined environment. We can increase and decrease the speed of the shaker and make lots of trial and error.

Objective: To produce 2,3-butanediol using batch flask fermentation and analyze this by using TLC (Thin layer chromatography) and HPLC (High performance liquid chromatography) and comparison of yields obtained from two different strains.

Materials and methods

Chemicals required: The chemicals used were of glucose, peptone, yeast extract, beef extract, NaCl, dihydrogren potassium phosphate, soya bean meal, urea, ferrous sulphate, magnesium sulphate, potassium hydroxide, alpha napthol, ethanol, hexane, ethyl acetate, glacial acetic acid, silica gel.

Selection of strains: *Bacillus subtilis, Bacillus cereus* are aerobic strains used for this project. *Bacillus subtilis* were

collected from Bharathidhasan University and *Bacillus cereus* were collected from Anna University, trichy. They are considered as GRAS microorganisms for this production of 2,3-butanediol. Here we are using new strain of *B.cereus* for this production.

Microbes used: *Bacillus subtilis* is a Gram-positive, catalase-positive bacterium, found in soil. It is a rod shaped microbe which forms a tough protective endospore and tolerates at extreme environmental conditions[10,11]. It also produces protective endospores.

Medium preparation: The batch flask fermentation contains 50ml of seed medium with glucose in an Erlenmeyer flask which was incubated at 37^0c on a rotary shaker for 16 hours. Then the starter culture was now taken for the production process.

Production medium: Fermentation flask (250ml), Erlenmeyer flask with (150ml) was taken and fermentation medium were inoculated with starter culture and it wascultivated on a shaker at 180rpm for about 4days.After the fermentation process the medium was taken for centrifugation at 9000rpm at 20minutes. Then the supernatant was taken for the next process for both the cultures. Periodical analysis of both the samples was performed to view.

Sample separation: After fermentation process both production medium was taken for centrifugation process at 8000rpm for 15 minutes. The supernatant containing 2,3-butanediol with crude was taken for further analysis process and the pellet and cell mass debris was discarded.

Analysis of 2,3-Butanediol: The cell mass concentration was determined by measuring the OD at 600nm in a UV-visible spectroscopy system. The qualitative test of sample was determined by using TLC (Thin layer chromatography) and quantitative test was carried out using HPLC (High performance liquid chromatography).

Comparison of yields: Based on HPLC, we quantified the amount of yield obtained from the two different strains. From this interpretation we have confirmedthat *Bacillus cereus* was also producing 2,3-butanediol. To our research, we summarize that the microbial production of 2,3-butanediol by *Bacillus sp*ecies which shows thehighly efficientsynthesis of 2,3-butanediol on a laboratory scale[12].

Results and discussion

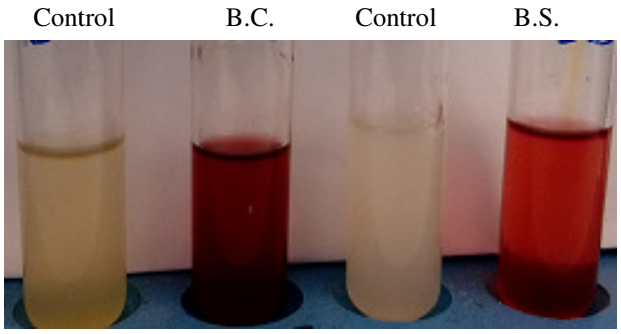
Confirmation test for 2,3-butanediol: MRVP Results: It is a type of imvic test. These organisms used mixed acid pathway to produce acid end products such as lactic acid and formic acid. If methyl red is added and immediate colour change has appeared then our organism is fermented in acid environment. Both species *Bacillus cereus* and *Bacillus subtilis* have turned to red colours which indicate that the bacteria are stable and able to survive in acid environment.

Qualitative test using TLC: Result for TLC: By TLC these plates shows the positive test results by presence of 2,3-butanediol. The blue colour change indicates that occurrence of 2,3-butanediolin both the organisms and hence the result is positive.

Quantitative test for HPLC: The schematic of an HPLC instrument typically includes a sampler, pumps, and a detector. The sampler brings the sample mixture into the mobile phase stream which carries it into the column. Through HPLC was quantified the amount of 2,3-butanediol from the two different strains. The mobile phase was mixture of buffer and methanol (90:1) at the flow rate of 0.75mL/min. 20µl of standards and samples were injected. The quantification was analysed for the amount of 2,3-butanediol on *Bacillus subtilis* and *Bacillus cereus.*

Bacillus cereus Bacillus subtilis.

Figure-1: two different strains.



Bacillus cereus ***Bacillus subtilis***

Figure-2: Confirmation test by voges-proskauer

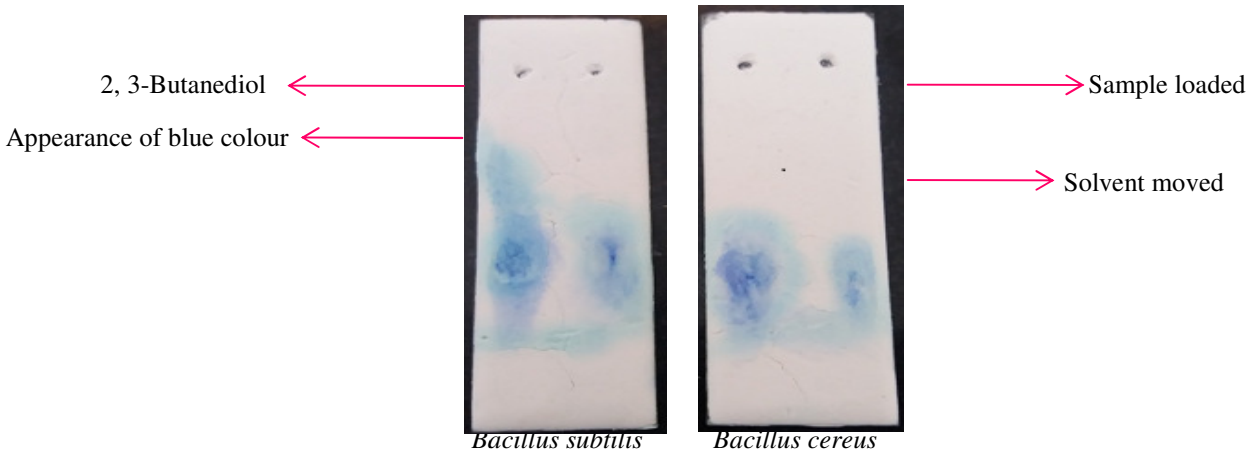


Figure-3: Qualitative test using TLC

The composition of the fermentation broth of sample *(B.subtilis, B.cereus*) was analysed by high Performance liquid chromatography using refractive index detector (RID).

Column	5µm ODS hypersil, 4.6mmx10.0mm
Detector	Refractive index detector
Nebulizer gas temp.	350°C
Nebulizer gas flow	13L/min
Nebulizer pressure	55psig
Capillary voltage	5kV
Flow	0.75mL/min
Injection Volume	20µL
Buffer	1% acetic acid
Mobile Phase	Buffer:Methanol (90:1)

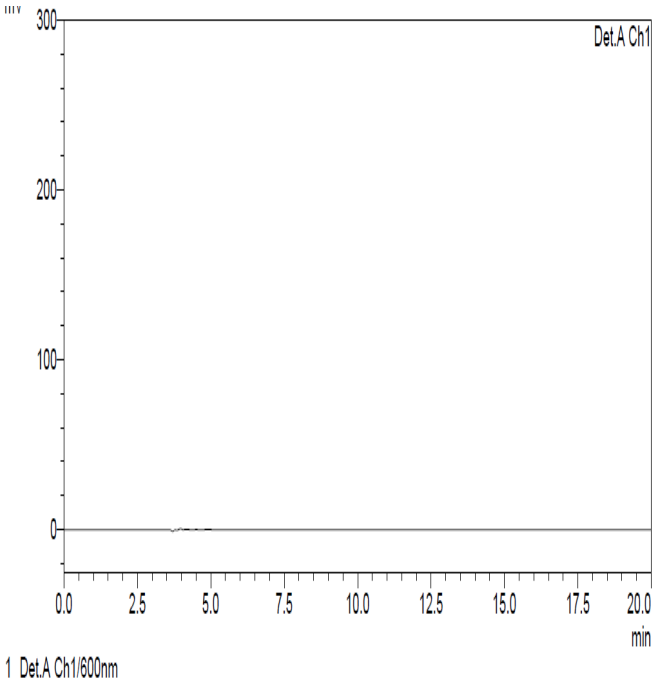
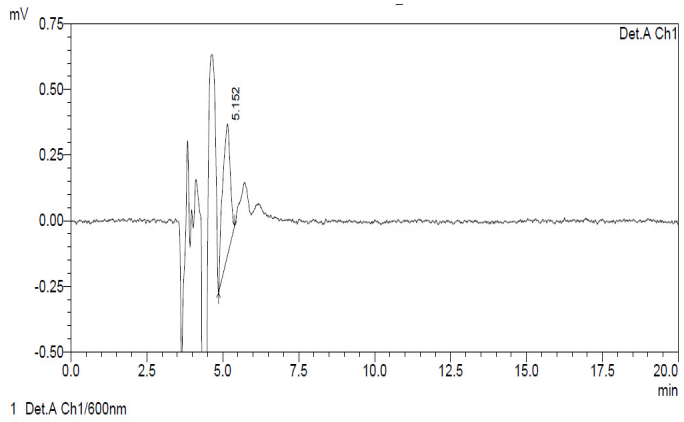


Figure-4: HPLC for blank.

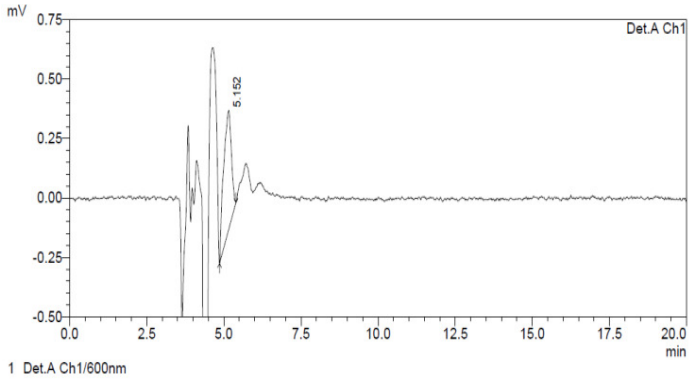


PeakTable

Detector A Ch1 600nm

Name	Ret. Time	Area	Area %	Tailing Factor
Standard	5.152	9051	100.000	0.870

Figure-5: HPLC for standard.

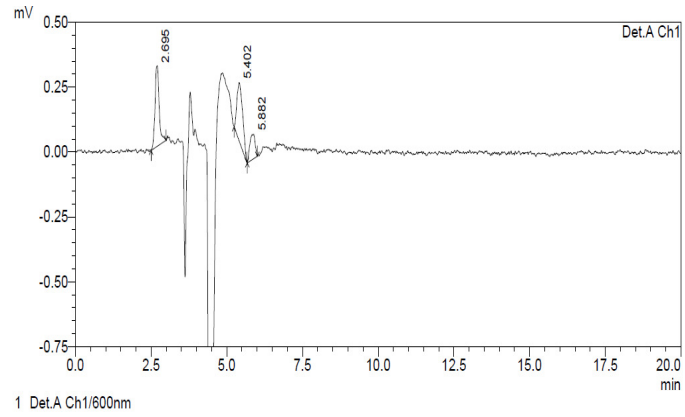


PeakTable

Detector A Ch1 600nm

Name	Ret. Time	Area	Area %	Tailing Factor
Standard	5.152	9051	100.000	0.870
		9051	100.000	

Figure-6: HPLC for sample 1 *(Bacillus subtilis).*



PeakTable

Detector A Ch1 600nm

Name	Ret. Time	Area	Area %	Tailing Factor
Peak_1	2.695	2970	41.853	1.314
Peak_2	5.402	2958	41.682	1.287

Figure-7: HPLC for sample 2 *(Bacillus cereus).*

Based on HPLC, we quantified the amount of yield obtained from the two different strains. Yield obtained from *Bacillus subtilis*: 1.28ml (150ml) and yield obtained from *Bacillus cereus*: 1.06ml (150ml)

Therefore the *Bacillus subtilis* is giving more yield than the *Bacillus cereus.* Therefore, from this interpretation we have confirmed that *Bacillus cereus* is producing 2,3-butanediol.

Discussion: Reverse phase HPLC of flavones was performed using aquapore column using linear elution gradient. Isoflavones was detected using absorbance at 262nm. Time ranging from 10-30minutes was left till 100% solvent was obtained. The column was equilibrated in solvent prior to chromatography. Eluted isoflavones were detected at 262nm[15]. The composition of the cultures was determined by using HPLC. According to thin layer chromatography in the early stage trimer was formed by a subsequent condensation with succinate to give higher oligomers. In this TLC analysis was performed on silicate-coated sheets using a mixture of petroleum ether and ethyl acetate as eluant. The spots were visualized by exposing to iodine vapour[16]. The distance travelled by the substance in the mobile phase with respect to time is determined in our paper. In the other paper they have used HPLC to check the purity of hard segment. The hard segments of various lengths show different retention times and uv absorptivity of each hard segment is found to be approximately the same. So, they concluded the purity of hard segments is better than 90% according to HPLC traces[17].

Conclusion

Finally, we have conclude that the 2, 3-butanediol production was achieved by two different aerobic microorganisms such as *Bacillus subtilis* and *Bacillus cereus* on laboratory scale fermentation with suitable medium and culture condition. The 2, 3-butanediol production was confirmed by using TLC (Thin layer chromatography) and quantitative test was carried out using HPLC (High performance liquid chromatography). During our research, we summarize that the microbial production of 2, 3-butanediol by *Bacillus sp*ecies which shows an efficient synthesis of 2, 3-butanediol on a laboratory scale.

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