

Production of 2, 3-Butanediol from Sugarcane Molasses Using *Bacillus subtilis*

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ABSTRACT:

2,3-butanediol can be easily converted to 1,3-butadiene, a low priced bulk chemical. A sustainable industrial fermentation process for the bulk production of 2,3-butanediol needs to be economically viable. The focus of this study is to develop a low-cost fermentation medium where an inexpensive sugar source like sugarcane molasses and a non-pathogenic microbe, *Bacillus subtilis*, can be used for the production of 2,3-butanediol. Various industrial sources of nitrogen were evaluated to replace yeast extract that is an expensive media component. Soybean meal was found to be the most effective. Several medium supplements were evaluated that could enhance the fermentation process. Potassium chloride proved to be the most beneficial. The developed medium was tested in both shake-flask and fermenter scales which showed peak 2,3-butanediol titers of 50 g/l. Thus a low cost fermentation medium was developed that could be industrially used for the bulk production of 2,3-butanediol.

Keywords: 2,3-butanediol, *Bacillus subtilis*, sugarcane molasses, medium optimization, industrial production.

[I] INTRODUCTION

2,3-butanediol is a four-carbon molecule that has several industrial applications. It is used in the production of printing inks, perfumes, moistening and softening agents, pharmaceuticals and explosives [7]. It can also be used for the production of methyl ethyl ketone which is a solvent and a useful fuel additive. The other

potential important application of 2,3-butanediol is its use in the manufacture of 1,3-butadiene. 1,3-butadiene is an industrially important chemical which is used in the synthesis of synthetic rubber [2,7]. Microbial fermentation routes can be used for 2,3-butanediol production [2]. Several microbes

such as *Serratia marcescens* [29], *Paenibacillus polymyxa* [6], *Klebsiella oxytoca* [8] and *Klebsiella pneumoniae* [15] have been studied for 2,3-butanediol production with reports of high titers. However, pathogenicity of these microbes has been one of the major issues for industrial scale bulk production of 2,3-butanediol [1]. *Bacillus subtilis* is a gram positive microorganism. It is a biosafety level 1 microbe and has been given GRAS (Generally Regarded As Safe) status by the US Food and drug administration. *Bacillus subtilis* naturally produces 2,3-butanediol [1]. It is a well studied organism and is amenable to genetic manipulations. It can therefore be used as an industrial host for the microbial production of 2,3-butanediol.

The use of biomass derived sugars can facilitate the development of a sustainable process for the production of 2,3-butanediol [7]. Sugarcane molasses, a dark syrupy byproduct of the sugar extraction process from sugarcane juice, is rich in sugars and contains a high amount of sucrose. It is available at a low price and thus is an economically viable carbon source for the production of a bulk chemical through a fermentation route [9].

In this study, the development of a cost effective fermentation medium composition was intended that is suitable for the industrial production of 2,3-butanediol, using sugarcane molasses and *Bacillus subtilis*. Various media compositions have been reported in literature for the production of 2,3-butanediol using *Bacillus subtilis* but not from sugarcane molasses. Firstly, these medium compositions were tested and based on the best performance on sugarcane molasses fermentation, a basal medium composition was selected. However, the cost of this medium would not be feasible at the industrial scale and this medium was taken forward for industrial optimization.

A fermentation medium used in an industrial process needs to be effective with respect to the cost of its components as well as its fermentation

performance. Superior fermentation performance is obtained by yielding high product formation in a short time [22]. The composition of a fermentation medium can influence product titers and yields [11]. To aid in the selection of useful medium components, a mathematically calculated performance index was used which cumulatively considered the contribution of useful fermentation parameters such as consumed sugar, yield and sugar consumption rate.

Industrialization of seed medium was also undertaken. It is reported that an inoculum which is adapted to the fermentation conditions helps in improving the fermentation performance [5, 17]. A comparison was made between the use of synthetic sugars and sugarcane molasses in the seed medium. A study was carried out to look at the effect of these changes in the inoculum preparation on the fermentation performance.

Apart from the sugar source, the important constituents of a nutrient medium are the nitrogen sources. For fermentative 2,3-butanediol production, the nitrogen source and its concentration influences product yield [24]. A common nitrogen source in the fermentation media compositions is yeast extract. However, the high cost of yeast extract is inhibitory for its use on an industrial bulk scale. A range of cost-effective nitrogen sources were assessed in this study to identify alternatives for yeast extract.

Various supplements have been included in media compositions and are reported to enhance 2,3-butanediol production [7]. The medium supplements such as iron, phosphate and manganese stimulate 2,3-butanediol production in *Paenibacillus polymyxa* fermentations [13]. The addition of sodium salt of ethylene diamine tetra acetic acid is reported to increase cell permeability and enhance mass transfer during fermentation. This supplement has shown to increase the conversion of glucose to 2,3-butanediol in *Klebsiella pneumoniae* fermentations [21]. Also, potassium is reported to stimulate 2,3-butanediol formation in

Paenibacillus polymyxa fermentations [10]. In this study, these supplements were tested in the medium formulations and the best supplement was identified and its concentration was optimized for the production of 2,3-butanediol using *Bacillus subtilis* from sugarcane molasses. Finally, a comparative study of the developed industrial medium versus the basal medium was done at both the shake flask level and at the fermenter level. The fermentation performance of the developed industrial medium was at par with the basal medium. The maximum titer of 2,3-butanediol attained in this study by using the developed medium was 50 g/l from sugarcane molasses. The work done here shows the economic potential of sugarcane molasses fermentation for the production of 2,3-butanediol using *Bacillus subtilis*.

[II] MATERIALS AND METHODS

2.1. Microorganism and screening methods

Bacillus subtilis 1A1 was obtained from Bacillus Genetic Stock Center (BGSC), Ohio, USA. Various media compositions were tested as mentioned in Table 1. The selected basal medium for further studies composed of yeast extract 5 g/l, corn steep liquor 15 g/l, ammonium sulfate 5 g/l and manganese sulfate 0.05 g/l. Sugars were maintained at approximately 100 g/l. pH of the medium was set to 6.5. The seed medium composition was sugar 60 g/l, yeast extract 5 g/l, corn steep liquor 20 g/l, ammonium sulfate 5 g/l and potassium dihydrogen phosphate 1 g/l. The inoculum concentration used for fermentation studies was 5% v/v. Various nitrogen sources were tested at concentrations as mentioned in the text. The role of different medium supplements were also investigated at concentrations as mentioned in the text. All the shake-flask studies were carried out at 34 °C and 150 rpm.

2.2. Fermentation studies

Bench scale batch fermentations were carried out in 1L fermenters (New Brunswick, BioFlo®/Celligen® 115, USA). The pH and

dissolved oxygen was monitored by pH and dissolved oxygen probes (Metler-Toledo, India). Sugarcane molasses was used as a feedstock with an initial sugar concentration of 150 g/l. Initially, high agitation and aeration rates were maintained at 400 rpm and 400 lpm, respectively for 11 hours to stimulate rapid cell growth. After this stage, microaerophilic conditions were maintained as it is suitable for 2,3-butanediol formation [1]. For the next 13 hours, the agitation and aeration rates were reduced to 350 rpm and 40 lpm respectively. Thereafter the agitation was reduced further to 250 rpm. The pH and temperature were maintained at 6.0 and 34 °C respectively, throughout the fermentation. The seed medium composition was as above and the fermentation medium compositions were as mentioned in the text.

2.3. Screening data analysis

Three parameters that affect the process economics are sugar consumed, sugar consumption rate and the yield of product. A performance index based on these variables was calculated for each of the cases during screening. Evaluation and comparison of these cases was carried out by ranking them on the basis of a greater performance index. For calculating the performance index, all the parameters were normalized to specific normalization factors, multiplied by the weightage factors and then summed up. Mathematically, it is represented by

$$\text{Performance index} = \sum_{i=1}^n \frac{y_i}{f_i} \cdot w_i$$

where, y_i are parameters, f_i are normalization factors and w_i are weightage factors.

The consumed sugar was normalized to the initial sugar concentration present at the start of the fermentation. Sugar consumption rate was normalized to 2 g/l/h, the maximum sugar consumption rate observed in the lab (unpublished data). Product yield was normalized to 0.526 g/g of sugar, the theoretical maximum yield attainable. The product yield was

based on the sum of 2,3-butanediol and acetoin titers. This was done because in a fermentation process, 2,3-butanediol can be reversibly converted to acetoin at low residual sugar concentrations in fermentation broth [30]. In such cases, it is difficult to track the attained peak concentration of 2,3-butanediol as a part of it could be converted to acetoin. Hence, the total concentration of acetoin and 2,3-butanediol present in the broth at the end of fermentation was considered for determining the product yield. Also, in this study equal weightages were assigned to all the three parameters.

2.4. Analytical methods

The major sugar components in sugarcane molasses are sucrose, glucose and fructose. These sugars in the fermentation broth were analyzed by high performance liquid chromatography (Agilent HPLC, 1200 series, Santa Clara, USA) equipped with RI detector at 40°C. Their concentrations were determined by using Aminex 87 HPX-N (300 x 7.8mm) at 85°C and 0.01M disodium hydrogen phosphate as mobile phase. The flow rate of both mobile phases was 0.6 ml/min. Total sugar was calculated by summing up the concentrations of sucrose, glucose and fructose. The analysis of acetoin, 2,3-butanediol and ethanol was done by Gas chromatography method. The fermentation broth was diluted with N, N Dimethyl formamide (DMF) with addition of isoamyl alcohol as internal standard and then quantified using a GC system (Agilent 7890 A, Santa Clara, USA) equipped with flame ionization detector and a 60 m ATTM-Wax capillary column (0.53 mm internal diameter, 1.0 µm film thickness; Grace, USA). The operating conditions were as follows: helium used as carrier gas; the injector temperature and detector temperature were 220°C and 240°C respectively; and the column oven temperature was maintained at 80°C for 4 min, and then raised to 200°C at the rate of 10 °C /min. The software Chemstation B03.02 was used for data acquisition and evaluation. The concentration of products was

determined using response factor with respect to the internal standard.

The protein content of the nitrogen sources was analyzed by the Kjeldahl method. 0.5 gm of the sample was digested in the presence of concentrated sulphuric acid and 30% w/w hydrogen peroxide in a HACH digester at the temperature of 440°C, till the solution becomes clear. The digest was cooled and then steam distilled in a LABCONCO steam distillation assembly with 50% w/w sodium hydroxide solution into 0.1N sulphuric acid containing methyl red indicator. The distillate was titrated with 0.1N sodium hydroxide until the end point was attained. A blank determination was carried out simultaneously. The nitrogen content was determined from the titer value, which was then multiplied by a predetermined factor to obtain the total protein content of the sample.

[III] RESULTS

3.1. Medium screening for sugarcane molasses fermentation to 2,3-butanediol

Acetoin is an intermediate substrate in the 2,3-butanediol pathway [1]. The criteria considered for selecting a reported fermentation medium was its use in fermentative production of either acetoin or 2,3-butanediol by *Bacillus subtilis* as it would favor the carbon flow through the 2,3-butanediol pathway. Three such medium compositions were selected as described in Table 1. These media compositions were tested for sugarcane molasses fermentation using *Bacillus subtilis* 1A1.

The performance indices were calculated for each of the medium performances as shown in Table 2. Xu medium and Fan medium had similar performance indices. The product yield in the case of Xu medium was much higher and therefore led to Xu medium's higher ranking over Fan medium. Yang medium turned out to be the lowest ranked due to low sugar consumption. Therefore, among the three medium compositions, Xu medium was selected as the

basal medium composition and taken forward for further improvement.

3.2. Effect of seed medium sugar source on sugarcane molasses fermentation to 2,3-butanediol

Following the selection of an appropriate basal medium, inoculum development was studied in order to evaluate the best fermentation performance that can be attained by the basal medium. *Bacillus subtilis* 1A1 was cultivated in two kinds of seed medium – one having synthetic sucrose as the sugar source and the other having sugarcane molasses as the sugar source. These two different kinds of inoculum were used for fermentation of sugarcane molasses in the basal medium.

Performance indices were calculated for both the conditions (Table 3). The ranking of sugarcane molasses based seed medium was higher. There was a substantial 10% increase in the performance index reflecting an improvement in all the three parameters, consumed sugar, product yield and sugar consumption rate. Thus, a positive effect on fermentation performance was seen by using an adapted inoculum and hence inoculum preparation was done by using sugarcane molasses in the seed medium for the successive studies.

3.3. Replacement of nitrogen source in the basal medium

The nitrogen sources in the basal medium are corn steep liquor, ammonium sulfate and yeast extract. Corn steep liquor has been reported as an essential component for 2,3-butanediol formation using *Bacillus subtilis* [28] and was seen in this study as well (unpublished results). The presence of ammonium sulfate as a nitrogen source in the medium was first questioned. To minimize the amount of additional yeast extract added to the medium, the nitrogen content of ammonium sulfate was substituted by protein content provided by yeast extract. Performance indices (Table 4) show a comparable fermentation profile. It was therefore concluded that increase

in yeast extract to 6.5 g/l could lead to an elimination of ammonium sulfate from the medium composition.

Yeast extract is an expensive component and cannot be used at an industrial scale for a bulk chemical production that requires minimal processing costs [5, 7]. Alternatives for yeast extract were explored. The concentration of the substitutes was based upon an equivalent amount of protein as provided by yeast extract. Soybean meal (9.5 g/l), cotton seed meal (10 g/l), rice dry distillers' grain with solubles (12 g/l), corn steep liquor (increased from 15 g/l to 33.5 g/l) were tested as alternatives to yeast extract. Performance indices were calculated for each of the cases and ranked accordingly (Table 4). Although the product yields were similar, sugar consumption varied extensively. Based on the performance index, soybean meal was ranked the highest among the alternatives. Since the fermentation performance of soybean meal was similar to yeast extract, soybean meal was considered as a suitable substitute to yeast extract. Therefore, corn steep liquor (15 g/l) and soybean meal (9.5 g/l) were maintained as nitrogen sources in the developing medium.

3.4. Effect of medium supplements on sugarcane molasses fermentation to 2,3-butanediol

Medium supplements have been reported to have an enhancing effect on 2,3-butanediol formation [5, 7]. To test this, supplements were removed from the basal medium (with soybean meal) that led to a drastic decrease of 8% in the performance index (Table 5). Hence, for better fermentation performance, supplements are a necessary component of the fermentation medium. Basal medium contained manganese sulfate as a supplement. Previous studies have shown that there is reduction in butanediol yield in the presence of sulfate salt [5]. Hence other reported medium supplements [10, 13, 21] for 2,3-butanediol production were evaluated on sugarcane molasses fermentation using *Bacillus*

subtilis. The working concentrations of these supplements (Table 5) were selected from literature, except for potassium chloride whose molar concentration was kept the same as that of potassium dihydrogen phosphate.

Performance indices were calculated for each of the cases (Table 5). Ethylene diamine tetraacetic acid (EDTA) and ferric chloride did not show incremental effects on sugarcane molasses fermentation to 2,3-butanediol. Manganese sulfate, potassium dihydrogen phosphate and potassium chloride were equally ranked at the top. Also, noteworthy is the fact that the supplements not only have an effect on product yield but also on sugar consumption. As compared to manganese sulfate, potassium dihydrogen phosphate and potassium chloride led to higher consumed sugar characteristics, and therefore were selected for optimization.

Potassium dihydrogen phosphate and potassium chloride were tested as supplements at concentrations of 50% higher and lower than the above. Performance indices were calculated for each of the cases and were ranked accordingly (Table 6). It was seen that reduction in the concentrations of these two salts had a more beneficial effect on fermentation performance. These are in comparison with both the basal medium (with soybean meal) and the respective reported concentrations (above). Potassium chloride (2.9 g/l) resulted in the highest ranking and was selected as the most effective supplement and replaced manganese sulfate in the medium composition.

3.5. Fermentation performance comparison between the basal medium and the developed medium at shake flasks

The new medium containing corn steep liquor (15 g/l), soybean meal (9.5 g/l) and potassium chloride (2.9 g/l) was referred to as the developed medium. The developed medium with a

performance index of 89 (highest in this study) showed an improvement not only over the basal medium (with soybean meal) but also over the initially selected basal medium (Xu medium) that had a performance index of 88. Fermentation profile is shown in Fig 1. The performance of the developed medium matched with that of the basal medium. The final titer of 2,3-butanediol obtained by using the developed medium was 44 g/l in 72 hrs of sugarcane molasses fermentation and using an initial sugar concentration of 112 g/l. Acetoin concentration was at 9 g/l.

3.6. Bench scale fermentation performance of the basal and the developed medium:

The scalability of developed fermentation medium was evaluated at bench scale in 1 liter fermenters. The initial concentration of total sugars in the media from sugarcane molasses was set to 150 g/l. A comparison of fermentation performance was done of the developed medium and the basal medium. Fermentation profiles are shown in Fig. 2. It was seen that almost complete sugar utilization was seen at 96 hrs in the developed medium, whereas it took about 144 hrs for complete sugar utilization in the basal medium. Initially the developed medium did not show favorable sugar consumption and no 2,3-butanediol was seen for the initial 24 hrs, however the fermentation improved rapidly later resulting in complete sugar utilization and attainment of peak product titers by 96 hrs. A peak 2,3-butanediol concentration of 50 g/l was obtained at this time point. In the case of basal medium a peak 2,3-butanediol concentration of 52 g/l was obtained at 144 hours.

The performance indices were calculated for developed medium at 96 hours and for the basal medium at 96 and 144 hours (Table 7). For the case of basal medium, at 96 hrs, in addition to lower sugar consumption, the product yield on

consumed sugar is also lower. However, by 144

Yang medium [28]	Fan medium [4]	Xu medium [26]
Corn steep liquor: 50 g/l Ammonium citrate: 5 g/l K ₂ HPO ₄ : 3 g/l MgSO ₄ ·7H ₂ O: 0.3 g/l FeSO ₄ ·7H ₂ O: 0.05 g/l Succinic acid: 0.6 g/l	Yeast extract : 5 g/l Corn steep liquor: 10 g/l Urea: 2 g/l MnSO ₄ : 0.05 g/l	Yeast extract: 5 g/l Corn steep liquor: 15 g/l Ammonium sulphate: 5 g/l MnSO ₄ : 0.05 g/l

hours the sugar consumption and the product yield caught up with that of developed medium (96 hours) as seen by their contributions to the performance index. Leaving apart the sugar consumption rate, the performance of the developed medium and the basal medium was the same at bench scale. The increased sugar consumption rate seen with developed medium is a significant aspect that leads to better process economics.

Table 1: Medium compositions used for medium screening

The initial concentration of sugars were set to 100 g/l using sugarcane molasses as the sugar source

Media type	Parameters used for performance index calculation						Performance index	Rank
	Consumed sugar		Product yield		Sugar consumption rate			
	Normalized value	Contribution to performance index	Normalized value	Contribution to performance index	Normalized value	Contribution to performance index		
Xu medium	0.88 ± 0.01	29	0.84 ± 0.03	28	0.69 ± 0.00	23	80	1
Fan medium	0.89 ± 0.00	30	0.76 ± 0.00	25	0.72 ± 0.00	24	79	2
Yang medium	0.65 ± 0.02	22	0.71 ± 0.03	24	0.54 ± 0.02	18	64	3

Table 2: Comparison of different media compositions for cane molasses fermentation using *Bacillus subtilis* 1A1*

*Errors represent standard deviations of at least three replicates.

Sugar source in seed medium	Parameters used for performance index calculation						Performance index	Rank
	Consumed sugar		Product yield		Sugar consumption rate			
	Normalized value	Contribution to performance index	Normalized value	Contribution to performance index	Normalized value	Contribution to performance index		
Sugarcane molasses	0.98 ± 0.01	33	0.90 ± 0.00	30	0.75 ± 0.01	25	88	1
Sucrose	0.88 ± 0.01	29	0.84 ± 0.03	28	0.69 ± 0.00	23	80	2

Table 3: Effect of sugar source used in the seed medium on cane molasses fermentation using *Bacillus subtilis* 1A1*

* Errors represent standard deviations of at least three replicates.

Nitrogen source	Parameters used for performance index calculation						Performance index	Rank
	Consumed sugar		Product yield		Sugar consumption rate			
	Normalized value	Contribution to performance index	Normalized value	Contribution to performance index	Normalized value	Contribution to performance index		
Basal medium (control)	0.98 ± 0.01	33	0.90 ± 0.00	30	0.75 ± 0.01	25	88	1
Yeast extract	0.98 ± 0.00	33	0.79 ± 0.02	26	0.78 ± 0.00	26	85	2

Soybean meal	0.89 ± 0.04	30	0.90 ± 0.02	30	0.70 ± 0.03	23	83	3
Rice Dry Distillers' grain with soluble	0.74 ± 0.02	25	0.87 ± 0.03	29	0.59 ± 0.01	20	74	4
Cotton seed meal	0.62 ± 0.03	21	0.90 ± 0.01	30	0.49 ± 0.02	16	67	5
Corn steep liquor	0.61 ± 0.01	20	0.86 ± 0.03	29	0.46 ± 0.01	15	64	6

Table 4: Performance of different nitrogen sources on cane molasses fermentation using *Bacillus subtilis* 1A1*

*Errors represent standard deviations of at least three replicates.

Supplement	<u>Parameters used for performance index calculation</u>						Performance index	Rank
	Consumed sugar		Product yield		Sugar consumption rate			
	Normalized value	Contribution to performance index	Normalized value	Contribution to performance index	Normalized value	Contribution to performance index		
KH ₂ PO ₄ (10.6 g/l) [13]	0.96 ± 0.03	32	0.77 ± 0.00	26	0.76 ± 0.02	25	83	1
Basal medium (with soybean meal)	0.89 ± 0.04	30	0.90 ± 0.02	30	0.70 ± 0.03	23	83	1
KCl (5.8 g/l) (Molarity is equivalent to KH ₂ PO ₄)	0.93 ± 0.03	31	0.86 ± 0.03	29	0.68 ± 0.02	23	83	1
MnCl ₂ (0.3 mg/l) [13]	0.88 ± 0.01	29	0.87 ± 0.03	29	0.68 ± 0.01	23	81	4
FeCl ₃ (6.4 mg/l) [13]	0.83 ± 0.03	28	0.82 ± 0.03	28	0.67 ± 0.03	22	78	5
Control w/o supplements	0.80 ± 0.04	27	0.89 ± 0.02	30	0.61 ± 0.03	20	77	6
EDTA [#] (0.5 g/l) [21]	0.64 ± 0.05	21	0.73 ± 0.00	24	0.47 ± 0.03	16	61	7

Table 5. Effect of medium supplements on cane molasses fermentation using *Bacillus subtilis* 1A1*

*Errors represent standard deviations of at least three replicates.

Ethylene diamine tetraacetic acid

Variation	<u>Parameters used for performance index calculation</u>						Performance index	Rank
	Consumed sugar		Product yield		Sugar consumption rate			
	Normalized value	Contribution to performance index	Normalized value	Contribution to performance index	Normalized value	Contribution to performance index		
KCl	0.99 ± 0.00	33	0.91 ± 0.01	30	0.77 ± 0.00	26	89	1

(2.9 g/l)								
KH ₂ PO ₄ (5.3 g/l)	0.98 ± 0.00	33	0.85 ± 0.01	28	0.70 ± 0.00	23	84	2
KH ₂ PO ₄ (10.6 g/l)	0.96 ± 0.03	32	0.77 ± 0.00	26	0.76 ± 0.02	25	83	3
KCl (8.7 g/l)	0.93 ± 0.02	31	0.86 ± 0.01	29	0.69 ± 0.01	23	83	3
KH ₂ PO ₄ (15.9 g/l)	0.97 ± 0.02	33	0.78 ± 0.02	26	0.73 ± 0.02	24	83	3
KCl (5.8 g/l)	0.93 ± 0.03	31	0.86 ± 0.03	29	0.68 ± 0.02	23	83	3

Table 6: Comparison of KCl and KH₂PO₄ concentrations on cane molasses fermentation using *Bacillus subtilis* 1A1*
*Errors represent standard deviations of at least three replicates.

Variation	Parameters used for performance index calculation						Performance index	Rank
	Consumed sugar		Product yield		Sugar consumption rate			
	Normalized value	Contribution to performance index	Normalized value	Contribution to performance index	Normalized value	Contribution to performance index		
Developed medium-96 h	0.98	33	0.71	24	0.74	25	82	1
Basal medium-96 h	0.86	29	0.66	22	0.68	23	74	2
Basal medium-144 hrs	0.98	33	0.72	24	0.52	17	74	2

Table 7: Comparison of developed medium and basal medium on cane molasses fermentation using *Bacillus subtilis* 1A1* at bench scale

*Errors represent the range of variation from the mean value of trials in duplicates.

[IV] DISCUSSION

One of the major challenges in developing an industrially feasible process for the production of 2,3-butanediol is the reduction in fermentation costs [11, 15]. In order to reduce fermentation costs, inexpensive agro byproducts such as sugarcane molasses have been widely used in fermentation medium [7, 9]. Sugarcane molasses has many advantages such as - the presence of fermentable sugars, abundant nutritional content apart from carbon source, easy availability and its' lower price as compared to synthetic carbohydrates [9, 25]. The other major cost contributor to the fermentation process economics is the nutrient requirements. Nutrient requirements not only control the product concentrations but also the product yields [11]. This study is mainly focused on developing a cost-effective

fermentation process for the production of 2,3-butanediol using a non-pathogenic microbe, *Bacillus subtilis*.

Industrial fermentation economics are driven by three major factors -the amount of sugar consumed the rate at which the sugar is consumed and the product yield of the process. While developing a cost-effective fermentation medium, all the three factors are important to judge the performance of the medium in a fermentation process. However, in the commonly used medium optimization techniques [11], the evaluation of the medium components is based on the effect of these on the response of only one of the performance factors. It could either be product yield or sugar consumption. In this study, a method was developed to concurrently evaluate

the performance of the fermentation process based on all the three factors, cumulatively. A performance index was calculated by summing up the weighted and normalized performance factors. Ranking was based on the higher values of the performance index. To simplify the study, synergistic effect of variables were not considered. A step by step selection of useful attributes based on their performance indices helped in gradual development of a fermentation medium that was cost effective.

The first step in this study was to evaluate fermentation media reported in literature. Nutrient medium requirements for each micro-organism are highly specific. Different media compositions lead to alterations in the intracellular metabolic activities that finally may lead to different fermentation products. Therefore, media compositions considered in this study were based on reported literature where *Bacillus subtilis* has been tested either for 2,3-butanediol or acetoin fermentations [1, 4, 23, 25, 26, 28, 30]. However some of these media compositions suffer from the disadvantage of low titers that are not advantageous for bulk production. One such medium is Luria Bertini medium which is used for 2,3-butanediol and acetoin fermentations [1, 23]. It was seen that this medium is very poor in driving sugar consumption and facilitating product formation (unpublished data). Therefore, only three media compositions were taken forward for further investigation – Yang medium, Fan medium and Xu medium as described in Table 1.

Performance indices were determined for each of the medium compositions for fermentation of cane molasses to 2,3-butanediol using *Bacillus subtilis* 1A1. Corn steep liquor has been reported to be a source of essential microbial nutrients to stimulate cell growth as well as the production of 2,3-butanediol and acetoin [28]. Despite the high concentrations of corn steep liquor (50 g/l) in Yang medium, it resulted in the lowest performance index. This could be because of the lack of rich nutritional load provided by yeast

extract that is well documented for its ability to stimulate 2,3-butanediol fermentations [5]. Extension of fermentation time could have led to complete sugar consumption in Yang medium, however this could affect the productivity of fermentation process remarkably, and hence Yang medium was not considered further in the study. Xu medium and Fan medium, on the other hand, showed better performances. Since they had similar medium compositions their performance indices were comparable. Finally, Xu medium was selected as the basal medium based on its slightly better performance and significantly better product yield. .

A small but significant contribution of fermentation economics is also the cost involved in inoculum preparation. Development of fermentation inoculum from a cryopreserved stock culture of bacteria was done to attain a higher cell density of metabolically active cells. Further, usage of cane molasses as a substitute for sucrose in the seed medium had the advantages such as unaltered effect on cell density, generation of adapted inoculum and reduction in inoculum preparation cost. A favorable impact on fermentation rates was seen which was consistent with the documented findings [5, 17].

Nitrogen is a macronutrient whose content and type influences fermentation process performance and economics [24, 27]. It can be supplied in the fermentation medium as an inorganic source in the form of ammonia gas, ammonium salts or nitrates or in organic form such as amino acids or urea [22]. *Bacillus subtilis* has been shown to assimilate urea, nitrates, and ammonium salts [3, 4, 18]. Its ability to produce proteases [20] provides a good source of free amino acids from organic nitrogen sources that can be used as a source of nitrogen. It is reported that free amino acid glutamine is the preferable nitrogen source used by *Bacillus subtilis* and ammonium is utilized as an alternative in its absence [3]. On this basis, for this study, it was hypothesized that ammonium sulfate could be replaced effectively

by any organic nitrogen source that are rich in amino acids. Ammonium sulfate was replaced by additional yeast extract to substitute its nitrogen by protein content of yeast extract. As expected, the replacement did not have any negative impact on sugar uptake; however the product yield was reduced to some extent (Table 4).

Next, replacement of yeast extract was vital since it is an expensive medium component. A range of inexpensive nitrogen substitutes such as cotton seed meal, soybean meal, corn steep liquor and rice dry distillers' grains with solubles' were tested. Variability was seen in the performance indices of these cases. Among these, the performance index of soybean meal was the closest to the yeast extract case and was selected as its replacement. The reason for efficient fermentation performance using soybean meal could be due to its rich amino acid profile and easy digestibility [25]. As for the other nitrogen sources, the greatest impact was seen in the sugar consumption. This could be due to differences in the nature of proteins provided by each of these sources or the poor digestibility of proteins present in these sources. Compositional analysis of these sources could be useful in understanding their impact on fermentation. In addition, a noticeable point is that the source of nitrogen did not affect the product yield (Table 4) and also did not make any remarkable changes in cell growth (data not shown). One may draw a conclusion that in this study nitrogen only affects the sugar uptake.

Mineral supplements are required for microbial growth and metabolism [22]. Although organic carbon and nitrogen sources comprise of mineral salts, extra addition is essential to impart product stimulation in fermentation. Various supplements have been reported to stimulate 2,3-butanediol production – phosphate ions stimulate the entire cell metabolism and improve the sugar consumption [13], manganese acts as an essential cofactor for 2,3-butanediol pathway enzymes [13], ferric acts as a growth supporting nutrient in bacteria as well as helps in increasing sugar

consumption [13, 16], potassium and chlorides are osmolytes and important effectors of cell metabolism [12,19], EDTA affects the cell wall composition and alters the mass transfer characteristics of the cells [21]. All of these supplements were tested individually, for their effect on the fermentation process. In comparison to the “control without supplements”, except for EDTA, all the supplements had a positive impact on the fermentation (Table 5). The negative response of EDTA could be due to its chelating property that would have reduced the activity of the essential extracellular carbohydrate and protein degrading enzymes. The positive effect of the other supplements was mostly seen through increase in sugar consumption. Optimization of the potassium salts showed that decrease in the concentrations further improved the fermentation performance. This impact could be for the reason that higher concentrations of cations in fermentations result in higher osmotic pressures that affect cell growth and essential enzyme activities [14]. A peak performance index, highest among all the compositions tested in this study, was obtained by using potassium chloride at its lowest tested concentration.

The performance of the developed, low cost medium was the same as that of the basal medium, both at the shake flask and fermenter levels at the point of complete sugar utilization. It has thus, been shown that sugarcane molasses can be used for the industrially scalable production of 2,3-butanediol with the use of *Bacillus subtilis* and the low cost medium developed in this study.

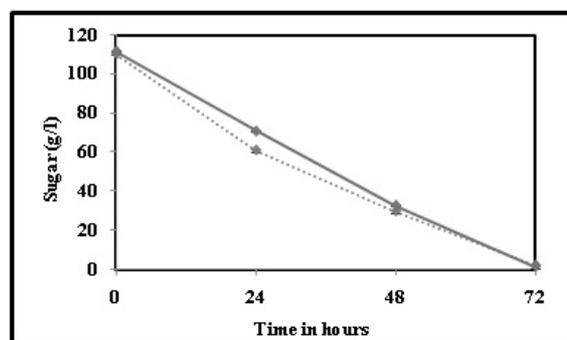


Fig: 1 (A)

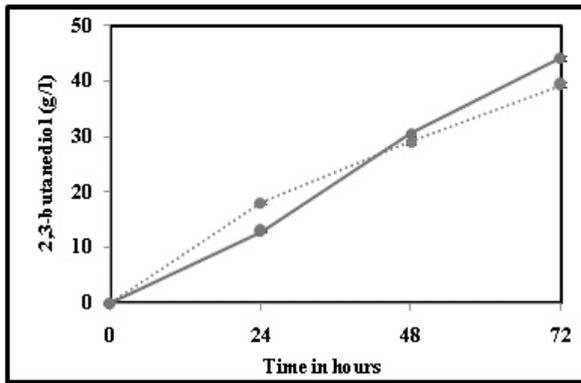


Fig. 1 (B)

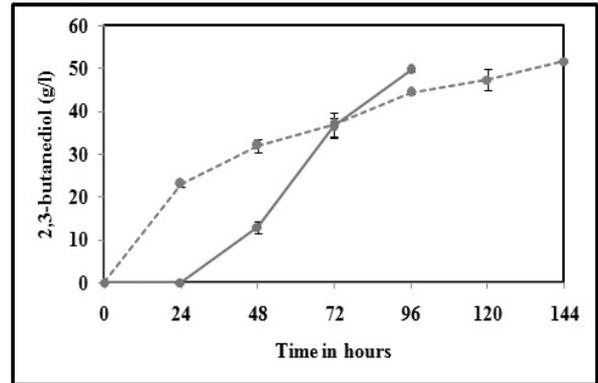


Fig. 2(B)

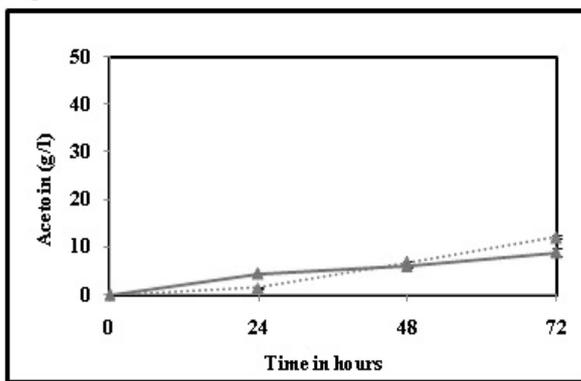


Fig. 1 (C)

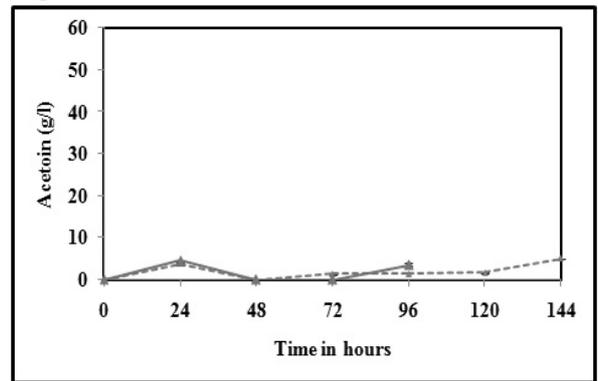


Fig. 2(C)

Fig. 1 Comparison of cane molasses fermentation profiles of the developed medium and the basal medium using *Bacillus subtilis* 1A1 at shake flasks.

(A) Sugar consumption (B) 2,3-butanediol (C) Acetoin. Similar fermentation performances were seen by both of the media. Key: (—, solid line) Developed medium, (-----, dashed line) Basal medium, (◆, diamond) Sugar consumption, (●, circle) 2,3-butanediol, (▲, triangle) acetoin. The error bars represent standard deviations of 3 trials.

Fig.2 Comparison of cane molasses fermentation profile of the developed medium and the basal medium using *Bacillus subtilis* 1A1 at bench scale.

(A) Sugar consumption (B) 2,3- butanediol (C) Acetoin. The developed medium exhibited enhanced productivity in terms of sugar consumption rate and led to a faster fermentation than the basal medium. Key: (—, solid line) Developed medium, (-----, dashed line) Basal medium, (◆, diamond) Sugar consumption, (●, circle) 2,3-butanediol, (▲, triangle) Acetoin. The error bars represent the range of variation from the mean value of trials in duplicates.

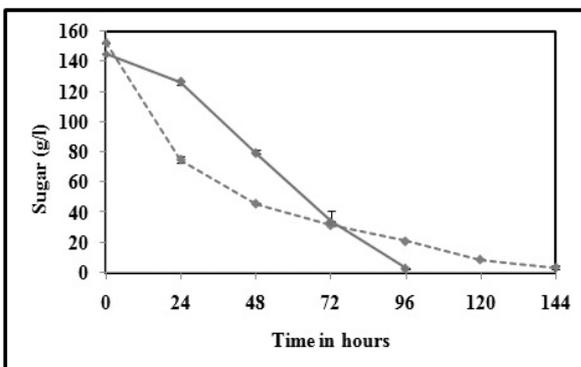


Fig.2 (A)

[V] CONCLUSION

A systematic study for development of a low-cost fermentation medium was carried out. The focus of the study was to reduce the fermentation costs and attempt for effective utilization of sugarcane molasses sugars for the microbial production of 2,3-butanediol using a non-pathogenic microbe, *Bacillus subtilis*. A new mathematical method was illustrated for the concurrent evaluation of

fermentation performance based on industrially relevant factors. Initially, the study was started by selecting a basal medium among reported medium compositions that gave the best fermentation performance. Inoculum optimization helped in improving the fermentation performance. Soybean meal was selected as the replacement for yeast extract among various industrial sources of nitrogen. Potassium chloride turned out to be the most effective medium supplement among the various supplements tested. Successful scalability of the developed medium was seen at the bench scale with respect to utilization of sugars at elevated concentrations. Further developments can be carried out by using a statistical medium optimization study that combines the classical methods with the industrially relevant, performance index. The study of a synergistic effect of two or more supplements may further improve the fermentation performance. Detailed changes in the compositional analysis of the broth during fermentation could give clues to additional enhancements. Optimization of fermentation conditions would also be beneficial. Finally, improvements in the microbe either through classical mutagenesis methods or through targeted metabolic engineering methods could lead to the industrialization of 2,3-butanediol production.

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