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Enhancement of 2,3-butanediol production by *Klebsiella oxytoca* PTCC 1402

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ABSTRACT

In this study, optimal operating parameters of 2,3-Butanediol production using *Klebsiell aoxytoca* under submerged culture conditions are determined by using Taguchi method. The effect of different factors including medium composition, pH, temperature, mixing intensity and inoculum size on 2,3-butanediol production was analyzed using the Taguchi method in three levels. Based on these analyses the optimum concentrations of glucose, acetic acid and succinic acid were found to be 6, 0.5 and 1.0 (%w/v) respectively. Furthermore, optimum values for temperature, inoculum size, pH and the shaking speed were determined as 37°C, 8 (g/L), 6.1 and 150 rpm respectively. The optimized conditions showed an enhanced 2,3-Butanediol yield of 40%[from 0.539 to 0.897 (g/g)]. The optimal combinations of factors obtained from the proposed DOE methodology was further validated by conducting fermentation experiments and the obtained results revealed an enhanced 2,3-Butanediol yield of 27.63%.

INTRODUCTION

2,3-Butanediol, otherwise known as 2,3-butylene glycol (2,3-BD), is a valuable chemical feedstock because of its application as a solvent, a liquid fuel, and as a precursor of many synthetic polymers and resins[1]. A wide variety of chemicals can also be easily prepared from 2,3-butanediol[2]. Currently, the manufacturing of 2,3-butanediol is still growing by an annual rate of 4-7% due to the increased demand for polybutylene terephthalate resin, γ-butyrolactone, spandex, and their precursors[3].

Interest in microbial production of 2,3-butanediol has been increasing recently due to extensive industrial application of this product[4]. Many bacterial species produce 2,3-butanediol by fermentation, but the best producers seem to be *Klebsiella oxytoca*[5], *Enterobacter aerogenes*[6], *Bacillus polymyxa*[7] and *Bacillus licheniformis*[8].

This work primarily aimed at optimizing the process variables for production of 2,3-butanediol in using statistical optimization technique for multivariable effect. The classical method of optimization involves varying the level of one parameter at a time over a certain range while holding the rest of the test variables constant. This single-factor-at-a-time strategy is generally time consuming and requires a large number of experiments to be carried out. Taguchi's method is based upon an approach, which is completely different from the conventional practices of quality engineering. This methodol-

ogy emphasizes integrating quality into products and processes, whereas usual practice relies upon inspection[9]. In the present study, we optimized 2,3-butanediol production under submerged culture conditions by *Klebsiella oxytoca* PTCC 1402 using Taguchi methodology.

MATERIALS AND METHODS

Microorganism

Bacterial strain used in this study was *Klebsiella oxytoca* PTCC 1402, obtained from the Iranian Research Organization for Science and Technology (IROST). The strain was maintained on nutrient agar slants at 4°C and subcultured monthly. The pre-culture medium was nutrient broth containing 2.0g/l yeast extract, 5.0g/L peptone, 5.0g/L NaCl, and 1.0g/L beef extract, sterilized at 121°C for 15min.

Taguchi methodology

Taguchi method of design of experimental (DOE) involves establishment of large number of experimental situation described as orthogonal array (OA) to reduce experimental errors and to enhance their efficiency and reproducibility of the laboratory experiments[10]. The first step is to determine the various factors to be optimized in the culture medium that have critical effect on the 2,3-butanediol production. Factors were selected and the ranges were further assigned based on the group consensus consisting of design engineers, scientists and technicians with relevant experience. Based on the obtained experimental data, seven factors having significant influence on the 2,3-butanediol production were selected for the present Taguchi DOE study to optimize the submerged culture condition. Seven factors (glucose, acetic acid, succinic acid, temperature, pH, mixing intensity and inoculum size) which showed significantly influence on the 2,3-Butanediol production[1,4,6,11,12] were considered in the present experimental situation (TABLE 1).

The next step was to design the matrix experiment and to define the data analysis procedure. The appropriate OAs for the control parameters to fit a specific study was selected. Taguchi provides many standard OAs and corresponding linear graphs for this purpose[13]. In the present case, the three levels of factors variation was considered and the size of experimentation was represented by symbolic arrays L18 (which indicates 18 experimental trails). Seven factors with three levels were used and it is depicted in TABLE 1 and 2.

In the design OA, each column consists of a number of conditions depending on the levels assigned to each factor. Submerged fermentation experiments were carried out in cotton plugged 500ml Erlenmeyer flasks containing 100 ml of production medium [(g/100 ml of distilled water) glucose(3.0, 6.0 and 9.0), yeast extract 1; acetic acid (0.1, 0.5 and 1); succinic acid (0.5, 1.0 and 1.5); $(NH_4)_2HPO_4$ 2.4; $MgSO_4.7H_2O$ 0.088; KCl 0.18; EDTA 0.051; $FeSO_4.7H_2O$ 2.25×10^{-3}; $ZnSO_4.7H_2O$ 0.75×10^{-3}; $MnSO_4.7H_2O$ 0.28×10^{-3} and sodium citrate 0.0295 dissolved in 100 ml of distilled water and pH adjusted by adding NaOH or HCl prior to sterilization, 15 min; 121°C. Glucose was sterilized separately].

Submerged fermentation experiments were performed for 2,3-butanediol production with *Klebsiella oxytoca* PTCC 1402 employing selected 18 experimental trails (TABLE 2) in combination with 7 factors at three levels (TABLE 1) and the result calculated from each set as 2,3-butanediol yield (g product/g substrate) and was shown in TABLE 2.

Analysis

Cell concentration of the inoculum was determined by optical density measurement at 620nm using a calibration curve to relate this parameter to cell mass dry weight.

2,3-Butanediol concentrations were determined by a Fractovap 4200 gas chromatograph (Carlo Erba, Milan, Italy) using a Chromosorb 101 column (Supelco, Bellefonte, PA) operated with N2 as the carrier gas, at 250°C injector temperature, 300°C detector temperature, and 175°C column temperature, and using n-butanol as the internal standard.

Glucose was assayed through the use of a glucose kit.

Software

Qualitek-4 software (Nutek Inc., MI) for automatic design of experiments using Taguchi approach was used in the present study. Qualitek-4 software is equipped to use L-4 to L-64 arrays along with selection of 2 to 63 factors with two, three and four levels to each fac-

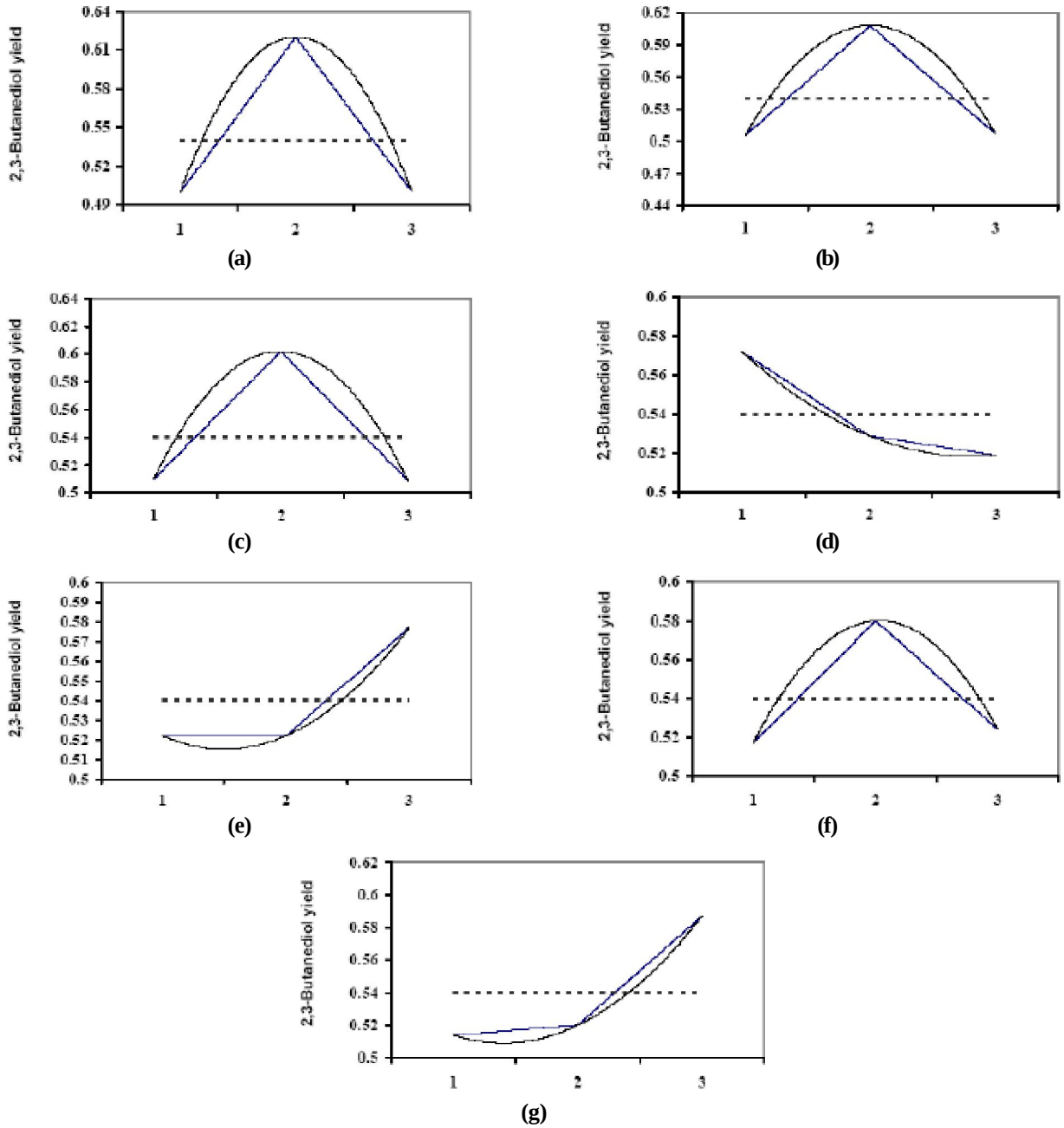

Figure 1 : Impact of selected fermentation-factor-assigned level on 2,3-butanediol yield by *K.oxytoca*. Impact of selected factor assigned levels on 2,3-butanediol yield by *K.oxytoca.* X-axis represents assigned levels of selected factor and Y-axis represents 2,3-butanediol yield. (a) glucose, (b) acetic acid, (c) succinic acid, (d) pH, (e) temperature, (f) mixing intensity, (g) inoculum size (···) indicates average 2,3-butanediol yield during experimentation and (—) indicates individual factors contribution 2,3-butanediol yield during experimentation

tor. The automatic design option allows Qualitek-4 to select the array used and assign factors to the appropriate columns. The obtained experimental data was processed in the Qualitek-4 software with bigger is better quality characteristics for the determination of the optimum culture conditions for the fermentation, to identify individual factors influence on the 2,3-butanediol production and to estimate the performance (fermentation) at the optimum conditions.

RESULT AND DISCUSSION

Submerged fermentation experiments studies with the designed experimental condition showed significant variation in the 2,3-butanediol yield (TABLE 2). Pro-

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TABLE 1 : Selected fermentation factors and their assigned levels

No.	Factor	Level 1	Level 2	Level 3
a	Glucose (%w/v)	3.0	6.0	9.0
b	Acetic acid(%w/v)	0.1	0.5	1.0
c	Succinic acid(%w/v)	0.5	1.0	1.5
d	pH	6.1	6.8	7.5
e	Temperature(ºC)	28	32	37
f	Mixing intensity (rpm)	120	150	180
g	Inoculum size(g/L)	2	5	8

duction levels were found to be very much dependent on the culture conditions. Variation of values in 2,3-butanediol yield at assigned levels by *K.oxytoca* PTCC1402 was depicted in TABLE 3 and figure 1.

The difference between average value of each factor at higher level and lower level indicated the relative influence of the effect at their individual capacities. The positive or negative sign denoted variation of yield values from level 1 to 2 or 3. Glucose (carbon source) and acetic acid showed positive impact with increase in their concentration, while incubation temperature and inoculum size had negligible impact on 2,3-butanediol yield, whereas medium pH had negative influence (Figure 1). Subsector level data denoted that pH factor caused negative influence on 2,3-butanediol yield, while rest of the selected factors showed positive effect with change in fermentation parameter values from level 1 to 2 (TABLE 3). Similarly, further increase in parameter values to level 3 varied the 2,3-butanediol yield (TABLE 3). These data further confirmed that the physiological factor and their concentrations were important in achieving better 2,3-butanediol production. Such variation was also noted with 2,3-butanediol production by other microbes[1,6].

Among the factors studied, glucose showed stronger influence compared to other factors followed by acetic acid, succinic acid and mixing intensity in the 2,3-butanediol yield. Individually at level stage pH has highest affect in level 1 where as glucose and temperature have high affects in level 2 and 3 respectively on 2,3-butanediol yield. With increasing glucose concentration the yield decreased these results show that the fermentation time gradually grows and the conversion yield lowers with increasing the starting substrate level, which is in agreement with what observed for most fermentation processes[6]. To explain such a yield decrease, additional determinations were performed to detect the possible formation of by-products, already observed by Raspoet in various *B. licheniformis* strains[14]. It was demonstrated that, whenever the overall yield of diol lowered, the formations of acetate, ethanol, formate, glycerol and lactate were favored and these by-products became even predominant. These results agree with well-known shifts in fermentation products that occur in many microorganisms under conditions of high availability of the energy source[1].

TABLE 2 : Experimental setup (L-18 Orthogonal Array)

Expt. No.	Factor levels a	b	c	d	e	f	g	2,3-butanediol yield (g product /g substrate)
1	1	1	1	1	1	1	1	0.399
2	1	2	2	2	2	2	2	0.620
3	1	3	3	3	3	3	3	0.483
4	2	1	1	2	2	3	3	0.551
5	2	2	2	3	3	1	1	0.711
6	2	3	3	1	1	2	2	0.582
7	3	1	2	1	3	2	3	0.683
8	3	2	3	2	1	3	1	0.465
9	3	3	1	3	2	1	2	0.355
10	1	1	3	3	2	2	1	0.408
11	1	2	1	1	3	3	2	0.572
12	1	3	2	2	1	1	3	0.523
13	2	1	2	3	1	3	2	0.576
14	2	2	3	1	2	1	3	0.699
15	2	3	1	2	3	2	1	0.601
16	3	1	3	2	3	1	2	0.417
17	3	2	1	3	1	2	3	0.587
18	3	3	2	1	2	3	1	0.501

It is reported that 2,3-butanediol production can be increased by addition of different organic acids, because of they are intermediate metabolites for 2,3-butanediol production[15]. Yutaka et al. found that addition of acetate, propionate, pyruvate, and succinate enhanced 2,3-butanediol production. Among the organic acids giving an enhanced 2,3-butanediol production, acetate seemed to be the most appropriate additive because it gave the highest 2,3-butanediol production[16]. While acetate at high levels may be inhibitory to *Klebsiella oxytoca*, low levels of acetate stimulate 2,3-butanediol production[15]. Stormer noted that acetate in its ionized form induces acetolactate synthase formation, and thereby enhances the catalysis of pyruvate to 2,3-butanediol[17]. The production of 2,3-butanediol by

TABLE 3 : Main effects of the factors at the assigned levels on 2,3-butanediol yield

Factors	Level 1	Level 2	Level 3	L2 – L1	L3 – L2
Glucose	0.500	0.620	0.501	0.119	-0.119
Acetic acid	0.505	0.608	0.507	0.102	-0.101
Succinic acid	0.510	0.602	0509	0.091	-0.093
pH	0.572	0.529	0.519	-0.043	-0.011
Temperature	0.522	0.522	0.577	0.000	0.054
Mixing intensity	0.517	0.580	0.524	0.062	-0.056
Inoculum size	0.514	0.520	0.587	0.006	0.060

K. oxytoca NRRL B-199 was enhanced in the presence of low levels (>8g/l) of lactate[18]. *Klebsiella oxytoca* ATCC 8724 grew well on xylose with 10 g/l succinate and produced additional 2,3-butanediol[19]. The production of 2,3-butanediol by *E. cloacae* NRRL B-23289 was also enhanced by the supplementation of acetate, lactate, and succinate[2]. New finding suggested that some amount of ethanol is formed by acetate reduction. Relative to this, a previous report demonstrated that acetate is converted to butanediol by condensation with pyruvate after the reduction of acetate to acetaldehyde[16]. Our finding confirm increasing effect of acetic acid on 2,3-butanediol yield. In the study 2,3-butanediol yield of *K. oxytoca* at initial substrate concentrations was considerably enhanced by the addition of 0.5% acetic acid to the media.

In the case of succinic acid when the initial concentration of acid was greated, the greater the maximum butanediol yield was greated too. With continiously increasing of succinic acid concentration the yield of butanediol produced as a result of additional succinic acid decreased.

Increasing of temperature and inoculum size has resulted in increase 2,3-butanediol production. Perego etal in an optimization study on 2,3-butanediol production by *B.licheniformis* (NCIMB 8059) found that butanediol production have a progressive increasing, when temperature was increased from 34 to 37°C. Conversely, they all sharply decreased over 37°C, likely due to the well-known thermal inactivation of biosystems at temperature higher than the optimum. Thus supporting the assumption of considering 2,3-butanediol production as a process controlled enzymatically[1]. On the other hand carbon consumption depends on the culture temperature[12].

An optimization study of glucose fermentation by *B. licheniformis*, likely performed using a factorial experimental design demonstrated that an increase in the inoculum size had positive effect on the yield as well[8].

TABLE 4 : Estimated interaction of severity index for different parameters

Interacting factors	Colum✡	SI (%)•	Colλ♠	Opt.○
Mixing intensity * Inoculum	(f*g)	53.31	15	(2,3)
Glucose* Inoculum	(a*g)	49.90	10	(2,1)
Acetic acid* Mixing intensity	(b*f)	40.23	4	(2,1)
Temperature* Mixing intensity	(e*f)	37.70	1	(3,2)
Glucose* pH	(a*d)	37.16	7	(2,3)
Succinic acid* Mixing intensity	(c*f)	33.24	3	(2,2)
Acetic acid *Temperature	(b*e)	30.56	5	(2,2)
pH* Inoculum	(d*g)	29.35	13	(1,3)
Succinic acid* pH	(c*d)	27.40	1	(2,3)
Glucose* Mixing intensity	(a*f)	26.09	5	(2,1)
Temperature* Inoculum	(e*g)	25.44	14	(3,1)
Acetic acid* Inoculum	(b*g)	17.74	11	(2,3)
pH* Mixing intensity	(d*f)	17.53	2	(1,2)
Acetic acid* Succinic acid	(b*c)	13.53	7	(2,2)
Succinic acid* Temperature	(c*e)	10.32	2	(2,3)
Glucose* Acetic acid	(a*b)	8.45	1	(2,2)
Succinic acid* Inoculum	(c*g)	8.40	12	(2,1)
Acetic acid* pH	(b*d)	7.82	6	(2,3)
Glucose* Succinic acid	(b*c)	4.56	6	(2,2)
pH *Temperature	(d*e)	3.65	3	(1,3)
Glucose* Temperature	(a*e)	1.53	4	(2,3)

✡Columns: Represent the column locations to which the interacting factors are assigned. •SI: Interaction severity index (100% for 90° angle between the lines, 0% for parallel lines). ♠Col.: Shows column that should be reserved if this interaction effect were to be studied (2-L factors only). ○Opt.: Indicates the factor levels desirable for the optimum conditions (based strictly on the first two levels)

Mixing intensity is another important factor for 2,3butanediol production. Saha postulate that aeration may be of value in removing carbon dioxide produced in the process and thus have a stimulatory effect on the fermentation[2]. Although 2,3-butanediol is a product of anaerobic fermentation, aeration is known to enhance its production[20]. In the case of mixing intensity increase to level 2 resulted in increase and subsequent increase to level 3, showed decrease in 2,3-butanediol yield. This may be rezoned due to the other constitutive effect of culture media.

TABLE 4 indicates the interaction between two selected factors. The interaction was measured based on

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TABLE 5 : Analysis of variance (ANOVA)

Factors	DOF	sum of squares (S)	variance (V)	F-ratio (F)	pure sum (S')	precent (P%)
Glucose	2	0.056	0.028	492.233	0.056	29.893
Acetic acid	2	0.041	0.020	365.194	0.041	22.162
Succinic acid	2	0.034	0.017	297.380	0.034	18.035
pH	2	0.009	0.004	82.273	0.009	4.945
Temperature	2	0.012	0.006	107.866	0.012	6.503
Mixing intensity	2	0.014	0.007	123.254	0.014	7.439
Inoculum size	2	0.019	0.009	173.59	0.019	10.502
Other/Error	3	-0.001	-0.001			0.521
Total	17	0.185				100

TABLE 6 : Optimal conditions and their performance in production of 2,3-butanediol

Factors	Level description	Level	Contribution
Glucose (%w/v)	6	2	0.079
Acetic acid(%w/v)	0.5	2	0.068
Succinic acid(%w/v)	1.0	2	0.061
pH	6	1	0.030
Temperature(ºC)	37	3	0.036
Mixing intensity (rpm)	150	2	0.037
Inoculum size(g/L)	8	3	0.047

Total contribution from all factors = 0.358, Current grand average performance = 0.539, Expected result at optimalconditions = 0.897

severity index value calculated by software program. This value between two selected factors varied (1-53 %) with factor to factor (TABLE 4).

It is clear that the interaction between two least 2,3-butanediol yield influential factors (at their individual levels) showed the highest severity index and vice versa with two highest influential factors (at their individual levels) (TABLE 4). For example, the severity index between two least impact factors, mixing intensity vs. inoculum size was found to be 53.31%, while the severity index between two higher impact factors, glucose vs. succinic acid, was noted to be only 4.56%. These results further confirmed that, each studied factor was important in 2,3-butanediol yield, and the influence of one factor on 2,3-butanediol yield was dependent on the condition of the other factor in optimization of 2,3-butanediol yield by *K. oxytoca*, although they have different influence at their individual levels.

ANOVA data indicated percentage contribution of selected parameters on 2,3-butanediol yield, which varied with factor to factor. Glucose, acetic acid, succinic acid and inoculum size were observed to be major influential parameters and contributed to more than 80% of total 2,3-butanediol yield (TABLE 5).

By studying the main effects of each of the factors, the general trends of the influence of the factors towards the process can be characterized. The characteristics can be controlled such that a lower or a higher value in a particular influencing factor produces the preferred result. Thus, the levels of factors, to produce the best results can be predicted. ANOVA with the percentage of contribution of each factor with interactions are shown in TABLE 5. It can be observed from the table that glucose is the most significant factor for the 2,3-butanediol yield. Acetic acid and succinc acid are the next most important significant factors in the 2,3-butanediol yield. The least influential factors among selected parameters include pH, incubation temperature and mixing intensity under the studied experimental set up. The error observed (0.521%) was very low which indicated the accuracy of the experimentation (Figure 2).

TABLE 6 represents the optimum conditions required for the maximum 2,3-butanediol yield by this bacterial strain. Based on software prediction, the average performance of this strain in 2,3-butanediol yield was observed to be 0.39 (TABLE 6).

However, fermentation-optimized factors contribution in enhancing the 2,3-butanediol yield was noted to be 0.358. The data also suggested that glucose, acetic acid and succinic acid play a vital role contributing approx. 59% in 2,3-butanediol yield under the optimized conditions (TABLE 6). Temperature, mixing intensity and inoculum size also contributed to the tune of 33.5% in total 2,3-butanediol yield, while the pH of the medium contributed to only 7.5% (TABLE 6) under optimized environment. The total 2,3-butanediol yield under optimized conditions was predicted to be 0.897 by the statistical procedure. The experimental data showed an enhanced 2,3-butanediol yield of 0.746 from 0539 (27.63% improvement in butanediol yield) with the modified culture conditions.

The study of interactive influence of selected factors (TABLE 6) revealed a unique relationship such as showing low influence on product production at individual level and higher severity index at interactive level (TABLE 4), indicating the importance of parameter

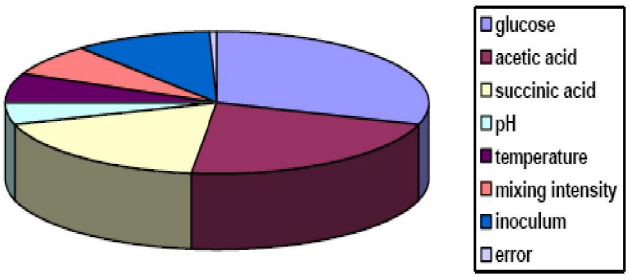

Figure 2 : Relative influence of factors and interaction

optimization on any product production and the role of various physicochemical parameters including carbon source, organic acids concentration, mixing intensity, temperature and pH of the medium in microbial metabolism. Such factor-mediated regulation of microbial fermentation was observed with many microbial species on any product[21].

CONCLUSION

Culture conditions and media composition optimization by a conventional one-at-the-approach led to a substantial increase in 2,3-butanediol yield. However, this approach is not only cumbersome and time consuming, but also has the limitation of ignoring the importance of interaction of various parameters. Taguchi approach of OA experimental design for process optimization, involving a study of given system by a set of independent variables (factors) over a specific region of interest (levels) by identifying the influence of individual factors, establish the relationship between variables and operational conditions and finally establish the performance at the optimum levels obtained. In this methodology, the desired design is sought by selecting the best performance under conditions that produces consistent performance leads to a more fully developed process. The obtained optimal culture condition for the 2,3-butanediol production from the proposed methodology was validated by performance the experiments with the obtained conditions.

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