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Optimization of 2,3-Butanediol Production by *Klebsiella pneumoniae* PTCC 1290 Using Taguchi Methodology

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2,3-Butanediol production parameter optimization using *Klebsiella pneumoniae* PTCC 1290 was performed using the design of experiments available in the form of an orthogonal array and a software for automatic design and analysis of the experiments, both based on Taguchi protocol. Optimal levels of physical parameters and key media components namely temperature, pH, inoculum size, agitation, acetic acid and succinic acid were determined. 2,3-Butanediol production obtained from the 18 sets of fermentation experiments performed with the selected factors and levels were further processed with Qualitek-4 software at bigger is better as quality character. The optimized conditions showed an enhanced 2,3-butanediol production of 35.8 % (from 11.856 to 18.459 g L^{-1}). The optimal combinations of factors obtained from the proposed design of experiments methodology was further validated by conducting fermentation experiments and the obtained results revealed an enhanced 2,3-butanediol production of 25.8 %. Taguchi approach of design of experiments resulted in evaluating the main and interaction effects of the factors individually and in combination. This methodology facilitated analysis of the experimental data to establish the optimum conditions for the process, understand the contribution of individual factors and to evaluate the response under optimal conditions.

Key Words: 2,3-Butanediol, Design of experiments, *Klebsiella pneumoniae*, Optimization, Taguchi methodology.

INTRODUCTION

2,3-Butanediol is a colourless and odourless liquid chemical with a high boiling point and low freezing point. It is largely used as a monomer for polymer synthesis. The commercial applications of this diol are not limited in the manufacturing of butadiene or to its use as an antifreeze agent[1]. It is known as 2,3-butylene glycol, a valuable chemical feedstock because of its application as a solvent, a liquid fuel and as a precursor of many synthetic polymers and resins. With a heating value of 27,200 J g^{-1}, 2,3-butanediol compares favourably with ethanol (29,100 J g^{-1}) and methanol (22,100 J g^{-1}) for use as a liquid fuel and fuel additive. Dehydration of 2,3-butanediol yields the industrial solvent methyl ethyl ketone. Further dehydration

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yields 1,3-butanediene, which is the starting material for synthetic rubber and is also an important monomer in the polymer industry. Methyl ethyl ketone can be hydrogenated to yield high octane isomers suitable for high quality aviation fuels. Diacetyl, formed by catalytic dehydrogenation of the diol, is a highly valued food additive. A wide variety of chemicals can also be easily prepared from 2,3-butanediol[2].

Interest in microbial production of 2,3-butanediol has been increasing recently due to the large number of industrial applications of this product[1]. Microbially produced 2,3-butanediol can be converted into 1,3-butadiene, a feedstock chemical currently supplied by the petrochemical industry. 1,3-Butadiene can, in turn, be utilized in the manufacture of plastics, pharmaceuticals, and synthetic rubber[3]. 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[4].

In previous studies have been reported that 2,3-butanediol production is dependent on various variables process[2,4-6]. These studies demonstrated that optimization of media components and culture conditions are important for 2,3-butanediol production. The traditional method of optimization involves varying one factor at a time, while keeping the others constant. This strategy requires a relatively large number of experiments and frequently fails to anticipate the optimal conditions. This essential shortcoming is due to the inability of the approach to consider the effects of possible interactions between factors. The deficiency can be overcome by applying more efficient, statistically based experimental design. In this respect, Taguchi orthogonal design is important tools to determine the optimal process conditions. The advantages of using the Taguchi method are that many more factors can be screened and optimized simultaneously and much quantitative information can be extracted by only a few experimental trials. Therefore, these methods have been extensively applied in parameter optimization and process control[7].

In the present investigation, 2,3-butanediol production fermentation factors optimization was performed using fractional factorial design of orthogonal array of Taguchi methodology. The L-18 experimental array data revealed that different fermentation factors interact with microbial system at individual and in association with other factors at interactive levels and contribute for enhancement of microbial 2,3-butanediol production.

EXPERIMENTAL

Bacterial strain used in this study was *Klebsiella pneumoniae* PTCC 1290, obtained from the Iranian Research Organization for Science and Technology (IROST). The strain was maintained on nutrient agar slants at 4 ºC and sub-cultured monthly. The pre-culture medium was nutrient broth containing (per liter): 2.0 g yeast extract; 5.0 g peptone; 5.0 g NaCl and 1.0 g beef extract sterilized at 121 ºC for 15 min.

Taguchi methodology: Taguchi method of design of experimental involves establishment of large number of experimental situation described as orthogonal

array to reduce experimental errors and to enhance their efficiency and reproducibility of the laboratory experiments[8]. 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, 6 factors having significant influence on the 2,3-butanediol production were selected for the present Taguchi design of experimental study to optimize the submerged culture condition. Six factors (temperature, pH, agitation, inoculum's size, acetic acid and succinic acid) which showed significantly influence on the 2,3-butanediol production[1,9,10], were considered in the present experimental situation (Table-1).

TABLE-1
SELECTED FERMENTATION FACTORS AND THEIR ASSIGNED LEVELS

No.	Factor	Level 1	Level 2	Level 3
a	Temperature (ºC)	28	32	37
b	pH	6.1	6.5	7.5
c	Agitation (rpm)	120	150	180
d	Inoculum size (g L^{-1})	2	5	8
e	Acetic acid (% w/v)	0.1	0.5	1.0
f	Succinic acid (% w/v)	0.5	1.0	1.5

The next step was to design the matrix experiment and to define the data analysis procedure. The appropriate orthogonal arrays for the control parameters to fit a specific study was selected. Taguchi[11] provides many standard orthogonal arrays and corresponding linear graphs for this purpose. In the present case, the 3 levels of factors variation were considered and the size of experimentation was represented by symbolic arrays L18 (which indicates 18 experimental trails). Six factors with 3 levels were used and it is depicted in Tables 1 and 2.

In the design orthogonal array, 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 500 mL Erlenmeyer flasks containing 100 mL of production medium [(g/100 mL of distilled water) glucose 5; yeast extract 1; acetic acid (0.1, 0.5 and 1); succinic acid (0.5, 1.0 and 1.5); KH_2PO_4 0.15; $K_2HPO_4.3H_2O$ 1.14; $(NH_4)_2SO_4$ .3; $MgSO_4.4H_2O$ .024; NaCl .01; EDTA .04; $CaCl_2.2H_2O$ 1.4×10^{-3}; $FeSO_4.7H_2O$ 1×10^{-3}; $ZnSO_4.7H_2O$ $.75 \times 10^{-3}$ and $MnSO_4.4H_2O$ $.28 \times 10^{-3}$ dissolved in 100 mL of distilled water and pH adjusted by adding NaOH or HCl prior to sterilization (15 min, 121 ºC). Glucose was sterilized separately].

Submerged fermentation experiments were performed for 2,3-butanediol production with *Klebsiella pneumoniae* PTCC 1290 employing selected 18 experimental trails (Table-2) in combination with 6 factors at 3 levels (Table-1) and the result obtained from each set as 2,3-butanediol concentration and was shown in Table-2.

TABLE-2
EXPERIMENTAL SETUP (L-18 ORTHOGONAL ARRAY)

Expt. No.	Factor levels						2,3-Butanediol production (g L^{-1})
	a	b	c	d	e	f	
1	1	1	1	1	1	1	8.364
2	1	2	2	2	2	2	14.215
3	1	3	3	3	3	3	10.130
4	2	1	1	2	2	3	12.415
5	2	2	2	3	3	1	12.560
6	2	3	3	1	1	2	11.161
7	3	1	2	1	3	2	14.070
8	3	2	3	2	1	3	9.998
9	3	3	1	3	2	1	13.412
10	1	1	3	3	2	2	15.709
11	1	2	1	1	3	3	7.742
12	1	3	2	2	1	1	9.472
13	2	1	2	3	1	3	12.908
14	2	2	3	1	2	1	11.305
15	2	3	1	2	3	2	11.450
16	3	1	3	2	3	1	11.595
17	3	2	1	3	1	2	13.557
18	3	3	2	1	2	3	13.353

Analysis: Cell concentration of the inoculum was determined by optical density measurement at 620 nm 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 nitrogen as the carrier gas, at 250 ºC injector temperature, 300 ºC detector temperature, and 175 ºC column temperature and using *n*-butanol as the internal standard.

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 2, 3 and 4 levels to each factor. 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.

RESULTS AND DISCUSSION

Submerged fermentation experiments studies with the designed experimental condition showed significant variation in the 2,3-butanediol production (Table-2). Production levels were found to be very much dependent on the culture conditions.

The average affect of the factors along with interactions at the assigned levels on the 2,3-butanediol production by *K. pneumoniae* PTCC1290 was shown in Table-3.

TABLE-3
MAIN EFFECTS OF THE FACTORS AT THE ASSIGNED LEVELS ON 2,3-BUTANEDIOL PRODUCTION

Factors	Level 1	Level 2	Level 3	L2-L1	L3-L2
Temperature	10.938	11.966	12.664	1.027	0.698
pH	12.510	11.562	11.496	-0.949	-0.660
Agitation	11.156	12.763	11.649	1.606	-1.115
Inoculum size	10.999	11.524	13.046	0.524	1.522
Acetic acid	10.910	13.401	11.257	2.490	-2.145
Succinic acid	11.118	13.360	11.090	2.241	-2.270

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 production values from level 1 to 2 or 3. Fig. 1 shows the influence of each individual factor on the 2,3-butanediol production.

Individually at level stage pH has highest affect in level 1 whereas acetic acid and inoculum's size has high affects in level 2 and 3, respectively on 2,3-butanediol concentration. Its clear that the primary factor affecting the substrate utilization rate in natural system is pH. Syu[5] reported the effect of pH, on 2,3-butanediol production. She concluded that the maximum 2,3-butanediol formation was achieved at pH = 5.8 by Fibrobacter succnogenes. In the study the best pH was 6.1

The difference between level 2 and level 1 (L2-L1) of each factor indicates the relative influence of the affect. The larger the difference, the stronger is the influence. It can be seen from Table-3, that among the factors studied, acetic acid showed stronger influence compared to other factors followed by succinic acid, agitation, and temperature in the 2,3-butanediol production.

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[12]. Nakashimada *et al.*[13] found that addition of acetate, propionate, pyruvate and succinate enhanced 2,3-butanediol production. Among the organic acids giving an enhanced 2,3-butanediol concentration, acetate seemed to be the most appropriate additive because it gave the highest 2,3-butanediol production[13]. While acetate at high levels may be inhibitory to *Klebsiella pneumoniae*, low levels of acetate stimulate 2,3-butanediol production[12]. Stormer[14] noted that acetate in it's ionized form induces acetolactate synthase formation and thereby enhances the catalysis of pyruvate to 2,3-butanediol. The production of 2,3-butanediol by *K. oxytoca* NRRL B-199 was enhanced in the presence of low levels (> 8 g L^{-1}) of lactate[15]. *Klebsiella oxytoca* ATCC 8724 grew well on xylose with 10 g L^{-1} succinate and produced additional 2,3-butanediol[16]. The production of 2,3-butanediol by *E. cloacae* NRRL B-23289 was also enhanced by the supplementation of acetate,

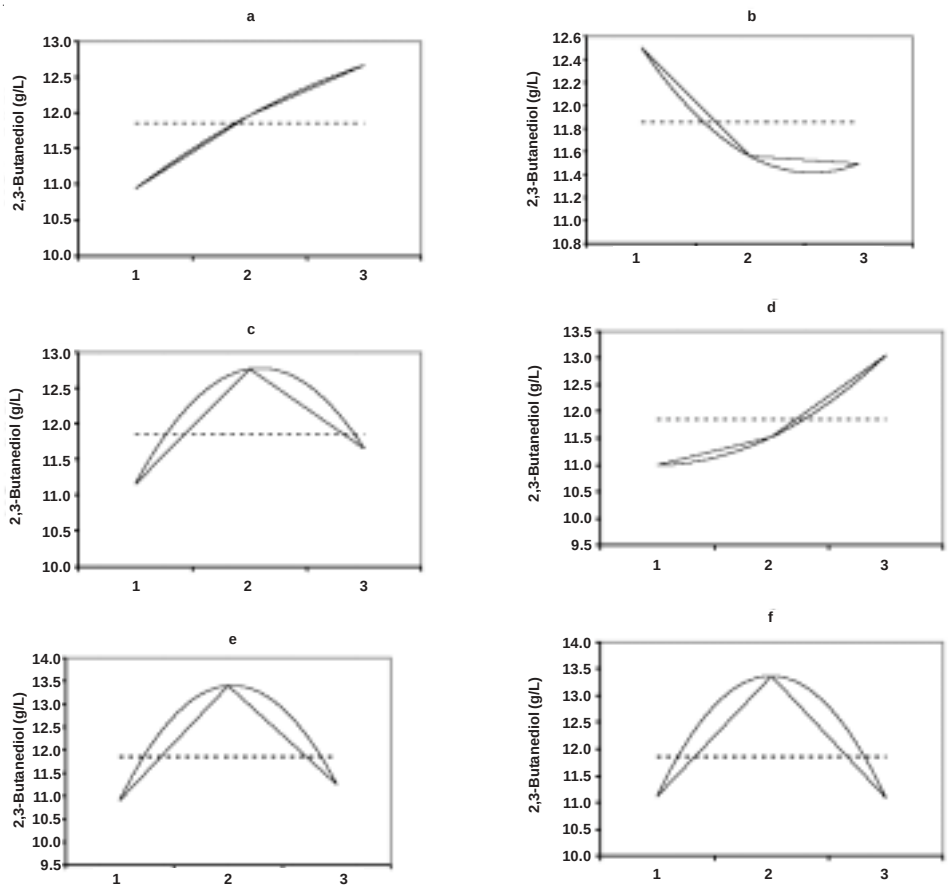

Fig. 1. Impact of selected fermentation-factor-assigned level on 2,3-butanediol production by *K. pneumoniae*. Impact of selected factor assigned levels on 2,3-butanediol production by *K. pneumoniae*. X-axis represents assigned levels of selected factor and Y-axis represents 2,3-butanediol production (g L^{-1}). (a) temperature, (b) pH, (c) agitation, (d) inoculum's size, (e) acetic acid, (f) succinic acid (⋯) indicates average 2,3-butanediol production during experimentation and (—) indicates individual factors contribution 2,3-butanediol production during experimentation

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[13]. In other work on cell-free extracts of *Aerobacter aerogenes* has demonstrated that acetate at low pH (*i.e.*, in the form of acetic acid) serves as an effective inducer for the 3 enzymes involved in the formation of butanediol from pyruvate, *viz.*, pH 6 acetolactate-forming enzyme, acetolactate decarboxylase and diacetyl (acetoin) reductase[12]. Such an induction mechanism probably plays a major role in the enhanced butanediol production of present study, even though the exact

extent of stimulation is not known. Present finding confirm increasing effect of acetic acid on 2,3-butanediol production. In the study 2,3-butanediol production of *K. pneumoniae* at initial substrate concentrations was considerably enhanced by the addition of 0.5 % (w/v) acetic acid to the media.

Increasing of temperature and inoculum's size has resulted in increase 2,3-butanediol production. Perego *et al.*[1] in an optimization study on 2,3-butanediol production by *B. licheniformis* (NCIMB 8059) found that butanediol concentration have a progressive increasing, when temperature was increased from 34 to 37 ºC. Conversely, they all sharply decreased over 37 ºC, 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. On the other hand carbon consumption depends on the culture temperature[6].

The inoculum's size was reported to improve the rate of 2,3-butanediol formation but not its yield on consumed carbon source. An optimization study of glucose fermentation by *B. licheniformis*, likely performed using a factorial experimental design demonstrated that an increase in the inoculum's size had positive effect on the yield as well[17].

Agitation is another important factor for 2,3-butanediol production. Saha and Bothast[2] postulated that aeration may be of value in removing carbon dioxide produced in the process and thus have a stimulatory effect on the fermentation. These results further confirmed that, each studied factor was important in 2,3-butanediol production and the influence of one factor on 2,3-butanediol production was dependent on the condition of the other factor in optimization of 2,3-butanediol production, although they have different influence at their individual levels. Although 2,3-butanediol is a product of anaerobic fermentation, aeration is known to enhance its production[18]. In the case of agitation increase to level 2 resulted in increase and subsequent increase to level 3, showed decrease in 2,3-butanediol concentration. This may be the reason due to the other constitutive effect of culture media. Increasing of pH has reverse effect in 2,3-butanediol production (Fig. 1 and Table-3).

The understanding the interaction between 2 factors gives a better insight into the overall process analysis. Any individual factor may interact with any or all of the other factors creating the possibility of presence of a large number of interactions. This kind of interaction is possible in Taguchi design of experiment. Estimated interaction severity index (SI) of the factors under study helps to know the influence of two individual factors at various levels of the interactions (Table-4). In the Table, the 'columns' represent the locations to which the interacting factors are assigned. Interaction SI presents 100 % of SI for 90 degrees angle between the lines while, 0 % SI for parallel lines. 'Reserved column' shows the column that should be reserved if this interaction effect has to be studied. "Levels" indicate the factor levels desirable for the optimum conditions (based on the first two levels).

TABLE-4
ESTIMATED INTERACTION OF SEVERITY INDEX FOR DIFFERENT PARAMETERS

Interacting factors	Column	SI (%)	Reserved col	Level
Temperature × Succinic acid	(a, f)	55.18	5	(1,2)
Temperature × Acetic acid	(a, e)	51.44	4	(1,2)
Agitation × Inoculum	(c, d)	50.78	1	(2,1)
pH × Acetic acid	(b, e)	1.23	5	(1,2)
Temperature × Inoculum	(a, d)	27.31	7	(3,1)
Temperature × Agitation	(a, c)	26.41	6	(3,2)
pH × Succinic acid	(b, f)	4.55	4	(1,2)
pH × Inoculum	(b, d)	18.57	16	(1,3)
Agitation × Succinic acid	(c, f)	8.52	3	(2,2)
Inoculum × Acetic acid	(d, e)	10.50	3	(3,2)
Agitation × Acetic acid	(c, e)	7.65	2	(2,2)
Acetic acid × Succinic acid	(e, f)	6.92	14	(2,2)
pH × Agitation	(b, c)	5.54	7	(1,3)
Inoculum × Succinic acid	(d, f)	5.02	2	(3,2)
Temperature × pH	(a, b)	2.59	1	(3,3)

In the study, interaction between two selected factors has shown in Table-4. The interaction was measured based on severity index value calculated by software program. This value between two selected factors varied (2.6-55 %) with factor to factor (Table-4). From the table, it can be followed that temperature and succinate (at level 1 and 2, column 5) interactions showed highest interaction SI. (55.18 %) followed by temperature and acetate (at level 1 and 2, column 4) with 51.44 %.

In taguchi approach, analysis of variance (ANOVA) is used to analyze the results of the orthogonal array experiment and to determine how much variation each factor has contributed. From the calculated ratios (F), it can be referred that all factors and interactions considered in the experimental design are statistically significant effects at 95 % confidence limit, indicating that the variability of experimental data explained in terms of significant effects. 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 acetic acid is the most significant factor for the 2,3-butanediol production. Succinc acid and inoculum's size are the next most important significant factors in the 2,3-butanediol production. pH showed least impact among the factors studied with the assigned variance of values. The error observed was very low which indicated the accuracy of the experimentation.

TABLE-5
ANALYSIS OF VARIANCE (ANOVA)

Factors	DOF	Sum of squares (S)	Variance (V)	F ratio (F)	Pure sum (S')	Per cent (P %)
Temperature	2	9.041	4.520	93.406	8.944	11.610
pH	2	3.859	1.929	39.873	3.672	4.884
Agitation	2	8.125	4.062	83.950	8.029	10.422
Inoculum size	2	13.562	6.781	140.118	13.465	17.479
Acetic acid	2	1.847	0.923	225.717	21.751	8.235
Succinic acid	2	0.353	0.178	210.315	0.260	26.299
Other/error	5	0.241	0.048	–	–	1.071
Total	17	7.035	–	–	–	100.00

Table-6 represents the optimum conditions required for the production of maximum 2,3-butanediol by this bacterial strain. Based on software prediction, the average performance of this strain in 2,3-butanediol production was observed to be 11.856 (g L^{-1}). The data also suggested that organic acids play a vital role contributing 46.15 % in butanediol production under the optimized conditions.

TABLE-6
OPTIMAL CONDITIONS AND THEIR PERFORMANCE IN PRODUCTION OF 2,3-BUTANEDIOL

Factors	Level description	Level	Contribution
Temperature	37	3	0.807
pH	6.1	1	0.653
Agitation	150	2	0.906
Inoculum size	8.0	3	1.189
Acetic acid	0.5	2	1.544
Succinic acid	1.0	2	1.503

Total contribution from all factors - 6.603; Current grand average performance - 11.856; Expected result at optimal conditions - 18.459.

The 2,3-butanediol production can be increased from 11.856 to 18.459 (g L^{-1}) *i.e.* overall 35.8 % enhancement in the production can be achieved. Further to validate the proposed experimental methodology, fermentation experiments were performed for 2,3-butanediol production by employing the obtained optimized culture conditions (Table-6). The experimental data showed an enhanced 2,3-butanediol concentration of 15.973 (g L^{-1}) from 11.856 (g L^{-1}) (28.3 % improvement in butanediol production) 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 optimization on any product production and the role of various physico-chemical parameters including organic acids concentration, agitation, 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[19].

Conclusion

Culture conditions and media composition optimization by a conventional one-at-the-approach led to a substantial increase in 2,3-butanediol concentration. 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 orthogonal array 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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