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Process Biochemistry 41 (2006) 547–551

PROCESS BIOCHEMISTRY

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Short communication

Medium optimization by orthogonal array designs for urease production by *Aspergillus niger* PTCC5011

M.R. Bakhtiari [a,*], M.G. Faezi [b,c], M. Fallahpour [a], A. Noohi [b], N. Moazami [a], Z. Amidi [a]

[a] *Department of Biotechnology, Iranian Research Organization for Science and Technology (IROST), No. 71, Forsat St., IROST, Tehran 15819, Iran*

[b] *Department of Microbiology, Faculty of Science, Tehran University, Tehran, Iran*

[c] *Department of Microbiology, Lahijan Azad University, Lahijan, Iran*

Received 24 April 2005; received in revised form 31 July 2005; accepted 6 September 2005

Abstract

This paper describes medium optimization for urease production by *Aspergillus niger* PTCC5011 by one-factor-at-a-time and orthogonal array design methods. The one-factor-at-a-time method was used to study the effects of carbon and nitrogen sources on urease production. Among various carbon and nitrogen sources used, sucrose and yeast extract were the most suitable for urease production, respectively. Subsequently, the concentration of sucrose, yeast extract and mineral sources were optimized using the orthogonal array method in two stages. The effects of nutritional components for urease production by *A. niger* PTCC5011 in the first and second stages were in order of urea > $NiSO_4$ > sucrose > KH_2PO_4 > K_2HPO_4 > $CaCl_2$ > yeast extract > $MgSO_4$ and yeast extract > sucrose > K_2HPO_4 > KH_2PO_4 > urea > $CaCl_2$ > $NiSO_4$, respectively. The optimal concentrations of nutritional components for improved urease production were determined as 20 g l^{-1} sucrose, 0.85 g l^{-1} urea, 3.4 g l^{-1} yeast extract, 0.03 g l^{-1} $NiSO_4{\cdot}6H_2O$, 0.5 g l^{-1} $MgSO_4{\cdot}7H_2O$, 0.04 g l^{-1} $CaCl_2$, 5.5 g l^{-1} KH_2PO_4 and 0.35 g l^{-1} K_2HPO_4. These results showed that urea, $NiSO_4$, yeast extract and sucrose had significant effect on urease production by *A. niger* PTCC5011. Tween 80 and $MgSO_4$ had negligible effect on urease production by this strain. The subsequent confirmation experiments determined the validity of the models. Maximum urease activity in optimized media by one-factor-at-a-time and orthogonal array methods were about 1.41 and 2.74 times greater than with the basal medium, respectively.

Keywords: *Aspergillus niger* PTCC5011; Optimization; Orthogonal array; Production; Urease

1. Introduction

Urease (urea amidohydrolase, EC.3.5.1.5) is a nickel-containing enzyme that catalyzes the hydrolysis of urea to yield ammonia and two molecules of carbon dioxide. Urease has many industrial applications, such as in diagnostic kits for determination of urea in blood serum, in alcoholic beverages as a urea reducing agent [1–6], and in biosensors of haemodialysis systems for determining blood urea [7].

Amongst the filamentous fungi, *Aspergillus niger* has been used for commercial production of many enzymes, e.g. pectinase, glucose oxidase, glucoamylase, hemicellulase, glucanase, acid proteinase and catalase [8–10]. Many investigators have attempted to obtain optimal submerged culture conditions for these enzymes produced by *A. niger*, but less attentions have been paid to determine optimum nutritional requirements for urease production by *A. niger*.

In the conventional one-factor-at-a-time method for optimizing fermentation medium conditions, (i.e. nutrients, temperature, pH, etc.), one independent variable is changed while all others are held at definite levels. This one-dimensional evaluation is tedious and time-consuming, especially for a large number of variables, and usually does not lead to the determination of optimal conditions per se, mainly due to ignoring interactions. On the other extreme, there are full-factorial experimental designs that consider all variables and their interactions at once. But this dedicates huge numbers of trials, which have to be done, most probably beyond the time and budget capabilities. As a solution, fractional factorial experimental designs, including Placket-Burman design, orthogonal array and response surface methodology (RSM) designs have been introduced, reducing the number of the tests while giving reliable results [11–13]. These methods have been successfully applied to optimize variables of fermentation

* Corresponding author. Tel.: +98 21 66028141; fax: +98 21 88838350.
E-mail address: bakhtiari@irost.ir (M.R. Bakhtiari).

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doi:10.1016/j.procbio.2005.09.002

processes for the production of primary and secondary metabolites [14–17].

The purpose of this study was to optimize fermentation medium nutrients to improve urease production by *A. niger* PTCC5011 using one-factor-at-a-time and a statistics based orthogonal array designs. At first, the effects of carbon and nitrogen sources on urease production were investigated by one-factor-at-a-time. Then, the concentration of medium components was optimized using taguchi method as a fractional factorial design in two stages.

2. Materials and methods

2.1. Microorganism

Fungal strain of *A. niger* obtained from the Persian Type Culture Collection (PTCC, Tehran, Iran) was used for urease production throughout this study.

2.2. Culture conditions

The strain was cultivated on the slants of potato dextrose agar (PDA) and incubated at 30 °C for 5 days in order to obtain conidia. The basal medium was a chemically defined medium developed in our laboratory consisting: urea, 1.3 g; glucose, 20 g; $MgSO_4{\cdot}7H_2O$, 0.5 g; KH_2PO_4, 13.3 g; K_2HPO_4, 0.346 g; $CaCl_2$, 0.3 g; $NiSO_4{\cdot}7H_2O$, 0.032 g and distilled water up to 1 l (pH 5.5). For submerged fermentation, the conidia suspension of *A. niger* was inoculated in amounts to give 10^5 conidia ml^{-1} in 250 ml-Erlenmeyer flasks containing 50 ml of medium. The initial pH was adjusted to 5.5. The temperature and agitation rate were 30 °C and 180 rpm during the fermentation processes, respectively.

2.3. Preparation of homogenized mycelia

The fungal mycelia were filtered by filter paper (Whatman #42). Mycelia recovered by filtration were washed three times with 0.1 M potassium phosphate buffer (pH 7.0). Preparation of homogenized mycelia was achieved by disrupting the washed mycelia in 0.1 M potassium phosphate buffer (pH 7.0) with a blender and resulting suspension was used for urease assay [18].

2.4. Enzyme assay

The urease activity was measured by Weatherburn method [19]. The reactions were done in micro tubes containing 100 μl of sample, 500 μl of 50 mM urea and 500 μl of 100 mM potassium phosphate buffer (pH 8.0) in a total volume of 1.1 ml. The reaction mixture was incubated at 37 °C for 30 min in a shaking water bath. The reaction stopped by transferring 50 μl of reaction mixture to the tubes containing 500 μl of phenol–sodium nitroprusside solution (0.05 g sodium nitroprosside + 1 g phenol/100 ml distilled water). Five hundred microliters of alkaline hypochlorite (3.56 g Na_2HPO_4 + 1 ml sodium hypochlorite + 100 ml distilled water) was added to the tubes, and incubated at room temperature for 30 min. Finally, the optical density of the color complex was measured at 630 nm against the blank (500 μl phenol nitroprusside sodium + 500 μl sodium hypochlorite + 50 μl distilled water) with a spectronic 20D+ spectrophotometer and compared to a standard curve prepared with $(NH_4)_2SO_4$. Controls used for the enzyme reactions were reaction mixture without substrate and reaction mixture without incubation. One unit of urease activity was defined as the amount of enzyme liberating 1 μmol NH_3 from urea per minute, under the above assay conditions [7].

2.5. Orthogonal arrays for medium optimization

To examine the interactions among nutritional components of production medium and optimize their concentrations for urease production, L_{18} orthogonal arrays were used in two steps: at first an L_{18} ($3^7\&2^1$) orthogonal array was selected to examine the effects of seven 3 level and one 2 level factors on urease production (Table 1). At the second step another L_{18} ($3^7\&1^2$) orthogonal array was used to adjust the concentrations of effective factors and to test the influence of Tween 80 on urease activity by this strain (Table 4). The orthogonal arrays, data analysis and ANOVA were obtained using Qualitek-4 (2000) software based on taguchi method.

3. Results and discussion

3.1. One-factor-at-a-time method

3.1.1. Effect of carbon source

To choose a suitable carbon source for urease production by *A. niger* PTCC5011, nine carbon sources including glucose, fructose, inositol, manitol, galactose, lactose, sucrose, starch and molasses were provided at concentrations of 20 and 40 g l^{-1} in the basal medium. Among the carbon sources evaluated, the highest urease production was about 0.56 and 0.68 units ml^{-1} in glucose and sucrose media at concentration of 40 g l^{-1}, respectively.

Subsequently, the effects of glucose and sucrose at concentrations of 10–100 g l^{-1} in basal medium were studied on urease production. The highest urease production was about 0.65 and 0.73 units ml^{-1} at concentration of 50 g l^{-1} glucose and sucrose in production medium, respectively.

The pH value decline was seen with increasing the concentrations of sucrose and glucose from 1 to 10% in basal medium. It seems with increasing the glucose and sucrose concentrations, more acidic end products accumulate in the medium. The enzyme activity decreases linearly in the presence of equal to or more than 5% of these two sugars and the subsequent decrease of pH below 3.00.

Table 1
Assignment of the experimental factors and their levels, and main effects of medium components on urease activity for nutritional improvement by taguchi method (first stage)

Factors	Symbols	Level 1 (g dl^{-1})	Enzyme activity (units ml^{-1})	Level 2 (g dl^{-1})	Enzyme activity (units ml^{-1})	Level 3 (g dl^{-1})	Enzyme activity (units ml^{-1})
$CaCl_2$	A	0.03	0.847	0.06	0.684	–	0.00
Sucrose	B	2.5	0.951	5.0	0.744	10	0.602
Urea	C	0.0425	0.837	0.085	0.924	0.17	0.536
Yeast extract	D	0.17	0.717	0.34	0.832	0.68	0.747
$MgSO_4{\cdot}7H_2O$	E	0.025	0.728	0.05	0.822	0.1	0.746
KH_2PO_4	F	0.665	0.865	1.33	0.855	2.66	0.576
K_2HPO_4	G	0.0175	0.623	0.035	0.931	0.070	0.742
$NiSO_4{\cdot}6H_2O$	H	0.0016	0.861	0.0032	0.902	0.0064	0.534

3.1.2. Effect of nitrogen source

To investigate the effect of nitrogen sources on urease production, conidia were inoculated in to the media containing sucrose at concentration of 50 g l^{-1} and various nitrogen sources including sodium nitrate, ammonium sulfate, ammonium carbonate, ammonium dihydrogen phosphate, ammonium acetate, ammonium chloride, soy bean flour, corn steep powder, peptone and yeast extract, where each nitrogen source was added to the basal medium at an amount to reach to the N/C ratio of 1/20.

Amongst the 10 nitrogen sources examined, yeast extract led to highest urease production, i.e. 1.40 units ml^{-1}.

3.2. Orthogonal array designs

3.2.1. Experimental design for the first orthogonal array (OAD1)

Factor and level assignments including main effects of each factor on urease activity are given in Table 1. Each value for level 2 was based on the optimized medium determined by the one-factor-at-a-time method. The values of levels 1 and 3 were half and twice of the level 2, respectively.

Based on L_{18} orthogonal array design, we carried out only 18 experiments in triplicate. In full-factorial experimental designs, to reach the same results as those of the orthogonal array method with this variety of factors, at least 2189 experiments are necessary. The experimental conditions for each run are listed in Table 2, including the enzyme activities in the last column. The ANOVA for the experimental results obtained by OAD1 and optimal levels of each factor for obtaining higher urease are given in the upper part of Table 3. The order of factor effects (contribution percent) on urease production was found to be urea > $NiSO_4$ > sucrose > KH_2PO_4 > K_2HPO_4 > $CaCl_2$ > y-yeast extract > $MgSO_4$. The results show that the effects of urea and $NiSO_4$ were more significant than those of the other nutrients. These findings are concordant with the fact that nickel is known to be a cofactor for urease activity [20] and urea may act as an inducer. To confirm the data, an experiment was carried out using optimal concentrations of nutrients according to upper part of the last column of Table 3. The measured urease activity obtained was 1.93 units ml^{-1}.

Table 2
The L_{18} orthogonal array applied for urease production by *A. niger* PTCC5011 (first stage)

Run	A	B	C	D	E	F	G	H	Activity (units ml^{-1})
1	1	1	1	1	1	1	1	1	1.08 ± 0.04
2	1	1	2	2	2	2	2	2	1.60 ± 0.06
3	1	1	3	3	3	3	3	3	0.33 ± 0.01
4	1	2	1	1	2	2	3	3	0.71 ± 0.02
5	1	2	2	2	3	3	1	1	0.76 ± 0.03
6	1	2	3	3	1	1	2	2	0.91 ± 0.06
7	1	3	1	2	1	3	2	3	0.55 ± 0.04
8	1	3	2	3	2	1	3	1	1.07 ± 0.07
9	1	3	3	1	3	2	1	2	0.49 ± 0.02
10	2	1	1	3	3	2	2	1	1.24 ± 0.14
11	2	1	2	1	1	3	3	2	0.85 ± 0.02
12	2	1	3	2	2	1	1	3	0.48 ± 0.10
13	2	2	1	2	3	1	3	2	1.02 ± 0.07
14	2	2	2	3	1	2	1	3	0.51 ± 0.06
15	2	2	3	1	2	3	2	1	0.54 ± 0.03
16	2	3	1	3	2	3	1	2	0.40 ± 0.10
17	2	3	2	1	3	1	2	3	0.62 ± 0.06
18	2	3	3	2	1	2	3	1	0.45 ± 0.06
Control	2	2	2	2	2	2	2	2	1.33 ± 0.03

Fermentations were carried out for 2 days at 30 °C with initial pH 5.5. Values are mean ± S.D. of triple determinations.

3.2.2. Experimental design for the second orthogonal array (OAD2)

The results obtained in OAD1 allowed further adjusting the levels of previous medium components for urease production.

Table 3
Results of ANOVA and optimal factor levels by Taguchi method (first and second stages)

Factors	DOF	Sum of squares	Variance	*F*-ratio	Pure sum	Contribution percent	Optimum level
(OAD1)							
$CaCl_2$	1	0.362	10.362	56.135	0.356	5.294	1
Sucrose	2	1.109	0.554	85.907	1.096	16.307	1
Urea	2	1.497	0.748	115.875	1.484	22.063	2
Yeast extract	2	0.127	0.063	9.875	0.114	1.701	2
$MgSO_4$	2	0.089	0.044	6.916	0.076	1.136	2
KH_2PO_4	2	0.966	0.483	74.795	0.953	14.173	1
K_2HPO_4	2	0.868	0.434	67.251	0.855	12.724	2
$NiSO_4$	2	1.459	0.729	112.999	1.446	21.510	2
Error	38	0.244	0.006			5.092	
(OAD2)							
Tween 80	1	0.001	0.001	0.110	0.000	0.000	2
Yeast extract	1	8.752	8.752	662.851	8.739	61.138	1
$NiSO_4$	2	0.063	0.031	2.409	0.037	0.260	2
Sucrose	2	2.379	1.189	90.094	2.352	16.460	2
Urea	2	0.167	0.083	6.361	0.141	0.990	2
KH_2PO_4	2	1.067	0.533	40.440	1.041	7.286	2
K_2HPO_4	2	1.183	0.591	44.831	1.157	8.097	2
$CaCl_2$	2	0.162	0.081	6.157	0.136	0.952	1
Error	39	0.514	0.013			4.817	

Table 4
Assignment of the experimental factors and their levels, and main effects of medium components on urease activity for nutritional improvement by taguchi method (second stage)

Factors	Symbols	Level 1 (g dl^{-1})	Enzyme activity (units ml^{-1})	Level 2 (g dl^{-1})	Enzyme activity (units ml^{-1})	Level 3 (g dl^{-1})	Enzyme activity (units ml^{-1})
Tween 80	A	Yes (0.07)	0.884	No	0.895	No	–
Yeast extract	B	Yes (0.34)	1.174	No	0.32	No	–
$NiSO_4{\cdot}6H_2O$	C	0.002	0.842	0.003	0.919	0.004	0.908
Sucrose	D	1.5	0.852	2	1.163	2.5	0.653
Urea	E	0.06	0.896	0.085	0.954	0.1	0.818
KH_2PO_4	F	0.45	0.759	0.55	1.085	0.665	0.824
K_2HPO_4	G	0.025	0.69	0.035	1.044	0.045	0.934
$CaCl_2$	H	0.004	0.935	0.003	0.921	0.03	0.812

Also Tween 80 was added as a surfactant to study its effects on the permeability of mycelia and accelerate their growth and production rate by facilitating mass transfer phenomena through the cell wall [14]. An L_{18} ($3^7\&1^2$) orthogonal array experimental design was performed. Table 4 shows the factor and levels assignments including main effects of medium components on urease production. For formulating this experiment, the following strategy was employed: (1) for each factor, if the optimum level is L1, then take L1 as L3 and new values less than it for L2 and L1, arbitrarily specified. (2) For each factor, if the optimum level is L2, then L2 is not modified and a shorter interval between L1, L2 and L2, L3 is arbitrarily specified. (3) For each factor, if the optimum level is L3, then take L3 as L1 and new values more than it for L2 and L3, arbitrarily specified. Yeast extract was an exception, determining just the consequence of its presence or absence in urease production. Magnesium sulfate was not further studied due to its negligible effect on urease activity. The experimental conditions for each run are listed in Table 5, including the enzyme activities in the last column.

Table 5
The L_{18} orthogonal array applied for urease production by *A. niger* PTCC5011 (second stage)

Run	A	B	C	D	E	F	G	H	Activity (units ml^{-1})
1	1	1	1	1	1	1	1	1	0.83 ± 0.06
2	1	1	2	2	2	2	2	2	1.86 ± 0.04
3	1	1	3	3	3	3	3	3	0.81 ± 0.07
4	1	2	1	1	2	2	3	3	0.50 ± 0.01
5	1	2	2	2	3	3	1	1	0.37 ± 0.13
6	1	2	3	3	1	1	2	2	0.20 ± 0.03
7	1	1	1	2	1	3	2	3	1.34 ± 0.05
8	1	1	2	3	2	1	3	1	0.91 ± 0.15
9	1	1	3	1	3	2	1	2	1.03 ± 0.07
10	2	1	1	3	3	2	2	1	1.15 ± 0.06
11	2	1	2	1	1	3	3	2	1.12 ± 0.20
12	2	1	3	2	2	1	1	3	1.06 ± 0.05
13	2	2	1	2	3	1	3	2	0.38 ± 0.03
14	2	2	2	3	1	2	1	3	0.00 ± 0.00
15	2	2	3	1	2	3	2	1	0.46 ± 0.06
16	2	1	1	3	2	3	1	2	0.84 ± 0.06
17	2	1	2	1	3	1	2	3	1.16 ± 0.10
18	2	1	3	2	1	2	3	1	1.16 ± 0.10
Control	2	2	2	2	2	2	2	2	1.40 ± 0.08

Fermentations were carried out for 2 days at 30 °C with initial pH 5.5. Values are mean ± S.D. of triple determinations.

The ANOVA for the experimental results obtained by OAD2 and optimal conditions are given in the lower part of Table 3. The order of factor effects (contribution percent) on urease activity was found to be yeast extract > sucrose > K_2HPO_4 > KH_2PO_4 > urea > $CaCl_2$ > $NiSO_4$. The results show that the effects of yeast extract and sucrose were more significant than those of the other nutrients. The enzyme activity and mycelial growth decreased significantly in trials without yeast extract, so it should not be omitted. The effect of Tween 80 on urease activity was not significant. To confirm these data, an experiment was carried out according to lower part of the last column of Table 3. The measured activity was 2.44 units ml^{-1}, implying that the optimal conditions obtained after OAD2 was the best.

Thus, to obtain higher urease activity, the medium composition after optimization was determined to be 2% sucrose, 0.085% urea, 0.34% yeast extract, 0.003% $NiSO_4{\cdot}6H_2O$, 0.05% $MgSO_4{\cdot}7H_2O$, 0.004% $CaCl_2$, 0.55% KH_2PO_4 and 0.035% K_2HPO_4.

Fig. 1 shows the time course comparison of urease production by *A. niger* PTCC5011 in basal and optimized media by one-factor-at-a-time and taguchi methods. In the basal medium, maximum urease production was obtained after 84 h of incubation. In our study, the improved medium obtained after one-factor-at-a-time showed maximum urease activity at 48 h of incubation, hence the fermentations were terminated after 48 h in next stages of optimization. The enzyme activity in

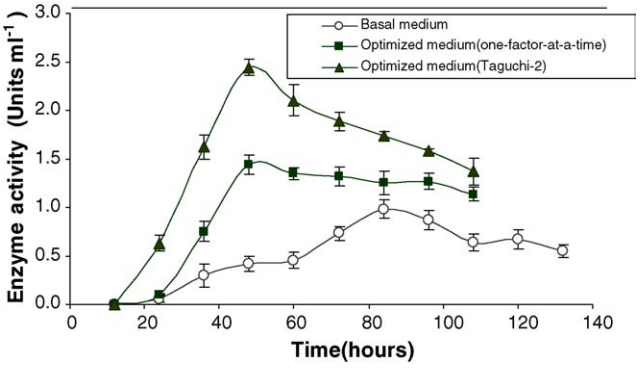

Fig. 1. Time course of urease production by *A. niger* PTCC5011 on three growth media. Experimental data are mean ± S.D. of triple determinations. (○) basal medium, (■) one-factor-at-a-time and (▲) optimum medium based on second taguchi medium.

optimized medium after one-factor-at-a-time and second stage of taguchi was found to be 1.42 and 2.44 units ml^{-1}, about 1.41 and 2.74 times more than the basal medium, respectively. So, the productivity increased from 0.01 to 0.05 units h^{-1}. The decrease of the enzyme activity after 48 h may be explained by the detrimental effects of acidic pH or some by-products such as proteases.

4. Conclusion

To the best of our knowledge, there is not enough information concerning optimum nutritional requirements for urease production by *A. niger*.

Using the one-factor-at-a-time and orthogonal array methods, it was possible to optimize nutritional components of medium to achieve higher urease activity by *A. niger* PTCC5011. Two optimization techniques used in this work can be widely practical to other processes for optimizing growth and production conditions. The optimized medium obtained in this study is a chemically defined medium and in comparison to complex media, has some advantages for further studies such as purification of urease.

Acknowledgments

This research was supported by Iranian Research Organization for Science and Technology (IROST), grant #1012180055. We would like to thank Dr. N. Ghaemi and Dr. F. Mohseni for their helpful assistance.

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