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PRODUCTION OF L-METHIONINE AS A FUNCTION OF UTILIZATION OF CARBON AND NITROGEN BY A MULTIPLE ANALOG-RESISTANT MUTANT *Corynebacterium glutamicum* X300

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ABSTRACT
An experimental study was conducted to examine the pattern of changes in the cell mass, glucose utilization and changes in the nitrogen contents in the broth during L-methionine fermentation by a multiple analog-resistant mutant *Corynebacterium glutamicum* X300. Utilization of glucose increased gradually along with the gradual increase of dry cell mass. L-methionine content increased upto 72h, then decreased gradually due to excessive cellular population which might adversely affect L-methionine production. Amino nitrogen level increased upto 72h as it is an indicator of L-methionine production. Cellular nitrogen and ammonical nitrogen increased gradually through out the study with sharp decline in residual glucose level.

KEY WORDS: Experimental, *Corynebacterium glutamicum* X300, L-methionine, glucose.

INTRODUCTION

Carbon and nitrogen contents in the fermentation broth have profound influence on bacterial growthand product formation. Microorganisms changes their consortium of enzymes in response to the growth environment.

Ganguly and Banik (2011) have conducted an extensive study on some biochemical changes in the fermentation broth with the advancement of L-glutamic acid fermentation by a mutant *Micrococcus glutamicus* AB100.[1] Ghosh *et al*. (2012) have conducted similar pattern of study for citric acid fermentation by a mutant *Aspergillus niger* AB1801 using a synthetic medium.[2]

In our present study, we were intended to examine the pattern of changes in the glucose andnitrogen content in the fermentation broth during the production of L-methionine by a multiple analog-resistant mutant *Corynebacterium glutamicum* X300 with the advancement of fermentation period.

MATERIALS AND METHODS

Selection of microorganism

A regulatory mutant *Corynebacterium glutamicum* X1 (accumulated only 0.6 mg. ml^{-1} L-methionine) developed in our laboratory by induced mutation from its parent strain *Corynebacterium glutamicum* K (basically a L-glutamic acid producing bacterium which does not accumulate L-methionine) which was kindly given by the Japanese Company *Kyowa Hakko Kogyo Ltd*.[3]

Chemical and Physical mutagenesis

To develop a high L-methionine yielding strain, the above mentioned regulatory strain was subjected to mutational treatments using Ethyl Methane Sulfonate (EMS) and UV irradiations as Chemical and Physical mutagens respectively as follows.

2.1. Exposure to EMS

1.0 ml cell suspension (containing $3.0x10^8$ cells) was added to 9.0 ml EMS solution of different concentrations (221.8 mmol. ml^{-1}, 186.3 mmol. ml^{-1} and 76.9 mmol. ml^{-1} respectively) and was incubated (10, 20, 30, 40 and 60 min. respectively). From each sample, 1.0 ml cell suspension was then plated on CD agar medium and kept at 30^0C for 48 h.[4]

2.2. Treatment with UV irradiation

2.0 ml cell suspension (containing 3.0 $x10^8$ cells.ml^{-1}) was taken in a petridish (5.0 cm diameter) and expose it to UV irradiation, using Hanovia germicidal lamp (15 Watt) from a distance of 12 cm for different periods of time (1-9 min.). The UV treated cells were plated in similar ways as mentioned above.[4]

3. Development of multiple L-methionine resistant strain

Multiple L-methionine analog-resistant strain was develop by adding different L-methionine analogs (20-100 mg.ml^{-1}) to the growth medium (namely: α-methyl

methionine, DL-ethionine, D-methionine sulphate and DL-norleucine).[5]

4. Physical conditions for growth

The fermentation was carried out using medium volume, 30 ml; initial pH 7.0; shaker speed, 200 rpm; age of inoculum, 48 h; cell density, 3.0 x 10^8 cells. ml^{-1} at 30^0C.[6]

5. Protoplast preparation, fusion, and regeneration

Two superior strains (namely, *Corynebacterium glutamicum* X164 which is high L-methionine yielding and *Corynebacterium glutamicum* X124 which is a multiple analog-resistant strain) were selected for protoplasting. The cells were harvested in 100 ml growth medium composed of: glucose, 20 $g.L^{-1}$; peptone, 10 $g.L^{-1}$; yeast extract; 10 $g.L^{-1}$; NaCl, 2.5 $g.L^{-1}$; $MgSO_4.7H_2O$, 0.25 $g.L^{-1}$; $MnSO_4.4H_2O$, 0.1 $g.L^{-1}$; K_2HPO_4, 1.0 $g.L^{-1}$, KH_2PO_4, 1.0$g.L^{-1}$ and biotin, 2.0 $\mu g.ml^{-1}$ in 250 ml Erlenmeyer conical flask at 30^0C for 24 h. Then the cell suspensions were centrifuged separately at 10,000 rpm for 10 min. The pellets were collected and transferred aseptically to a protoplasting medium composed of: sucrose, 0.5 M, malate buffer (pH 6.5), 0.02 M; $MgCl_2.H_2O$, 20 mM and lysozyme, 100 $\mu g.ml^{-1}$. After protoplast fusion (observed under phase contrast microscope), protoplast were fused in a medium containing the same composition similar to the protoplasting medium along with polyethane glycol (30%), dimethyl sulfide (15%) and $CaCl_2$, 10 mM. The suspension was shaking at 50 rpm on a rotary shaker with incubator at 30^0C for 10 min. and then it was diluted 10 fold with protoplast medium buffer (pH 6.5). The suspension was then centrifuged for 5 min. at 25,000 rpm at 5^oC using a cold centrifuge apparatus (EPLX3761). The pellet was collected and plated for colony formation for 48 h at 30^0C. The colonies were transferred to agar (2.0%) slants containing the same growth medium.[7]

Viable counting of protoplast (Reversion of protoplast)

Protoplasts were diluted with 10 ml of dilution fluid and plated into petridish (diameter 5.0 cm) containing agar medium allowed to grow at 30^0C for 48 h and subjected for subsequent fermentation trials.[8]

Composition of basal salt medium for L-methionine production

L methionine production was carried out using the following basal salt medium (per litre): glucose, 60 g; $(NH_4)_2SO_4$, 1.5 g; K_2HPO_4, 1.4 g; $MgSO_4 \cdot 7H_2O$, 0.9 g; $FeSO_4 \cdot 7H_2O$, 0.01 g; biotin, 60 μg^9.

Optimum cultural conditions

Volume of medium, 25 ml; initial pH, 7; shaker's speed, 150 rpm; age of inoculum, 48 h; optimum cell density, 4.0X10^8 cells. ml^{-1}; temperature 28^0C and period of incubation, 72 h.[10]

Composition of maintenance Medium

glucose, 20 $g.L^{-1}$; $(NH_4)_2SO_4$; 1.6 $g.L^{-1}$; NaCl, 2.5$g.L^{-1}$; $MgSO_4$. $7H_2O$, 0.25 $g.L^{-1}$; $MnSO_4.4H_2O$, 0.1 $g.L^{-1}$; K_2HPO_4, 1.0$g.L^{-1}$; KH_2PO_4, 1.0 $g.L^{-1}$; biotin, 2.0$\mu g.ml^{-1}$; thiamine-HCl, 2.0 $\mu g.ml^{-1}$ and agar, 2.0% as a solidifying agent.[11]

22. Analysis of L-methionine

Descending paper chomatography was employed for detection of L-methionine in culture broth and was run for 18 h on Whatman No.1 Chomatographic paper. Solvent system used include n-butanol: acetic acid: water (2:1:1). The spot was visualized by spraying with a solution of 0.2% ninhydrin in acetone and quantitative estimation of L-methionine in the suspension was done using colorimetric method.[3]

24. Estimation of Dry Cell Weight (DCW)

The cell paste was obtained from the fermentation broth by centrifugation and dried in a dried at 100^0C until constant cell weight as obtained.[3]

25. Estimation of residual sugar

Residual sugar was determined by DNS method as proposed by Miller (1959).[12]

26. Estimation of total nitrogen

Total nitrogen was measured by the micro-Kjeldahl method of Allen (1931).[13]

27. Estimation of ammonical nitrogen

Ammonical nitrogen was estimated by Conway method of Allen (1931).[13]

Statistical analysis

All the data were expressed as mean ± SEM. Data were analyzed using One Way ANOVA followed by Dunett's post hoc multiple comparison test using a soft-ware Prism 4.0.

All the chemicals used in this study were of Analytical Reagent (AR) Grade and were obtained from Mark. Borosil glass goods and triple distilled water were used though out the study.

RESULTS AND DISCUSSION

Quqntitative uptake rates of nutrients provide useful information for monitoring all growth and metabolism. These changes in the cell mass are proportional to glucose utilization. Hence the knowledge of glucose concentration can provide a basis for estimation of cell growth. The utilization of glucose in response to cellular growth and L-methionine production by this mutant has been depicted in the table 1.

Table 1: Rate of production of L-methionine, cellular growth in relation to glucose consumption by *Corynebacterium glutamicum* X300.

Time (h)	Residual sugar ($mg.ml^{-1}$)	Dry cell weight ($mg.ml^{-1}$)	L-methionine ($mg.ml^{-1}$)
0.0 (control)	100 ±0.913	-	-
18	91.6±0.613	**6.1±0.661	**10.4±0.966
24	*84.3±0.866	**9.3±0.616	**15.6±0.692
36	**62.8±0.993	13.6±0.993	24.3±0.991
48	**54.2±0.681	18.8±0.871	34.7±0.832
60	**41.6±0.772	23.3±0.683	44.6±0.691
72	**32.2±0.691	28.5±0.992	53.4±0.881
84	**23.8±0.863	30.1±0.714	51.8±0.683

Values were expressed as mean± SEM (Values were expressed as mean± SEM, where n=6; *p<0.05 and **p<0.01 when compared to control).

Utilization of glucose increased gradually along with gradual increase of dry cell weight. L-methionine content increased upto 72h, then decreased gradually, probably because of excessive cellular population which may adversely affect L-methionine production by the mutant.

The pattern of changes of nitrogen content has been depicted I n table 2.

Table 2: Rate of utilization of nitrogen during L-methionine fermentation by the mutant *Corynebacterium* glutamicum X300

Time (h)	Amino nitrogen ($mg.ml^{-1}$)	Cell nitrogen ($mg.ml^{-1}$)	Ammonical nitrogen($mg.ml^{-1}$)	Residual nitrogen ($mg.ml^{-1}$)
0.0(Control)	-	-	-	8.0±0.991
18	0.36±0.661	0.07±0.653	0.03±0.692	7.54±0.741
24	*1.13±0.723	0.28±0.771	0.19±0.771	*6.40±0.681
36	**2.16±0.917	*0.51±0.791	0.32±0.601	**5.01±0.832
48	**2.93±0.662	*0.64±0.691	*0.68±0.701	**3.75±0.691
60	**4.16±0.601	*0.93±0.992	*0.81±0.692	**2.10±0.882
72	**5.02±0.661	**1.45±0.691	*0.91±0.881	**0.62±0.881
84	**4.81±0.693	*1.11±0.772	**1.55±0.991	**0.51±0.591

Values were expressed as mean± SEM (Values were expressed as mean± SEM, where n=6; *p<0.05 and **p<0.01 when compared to control)).

Amino nitrogen level increased upto 72h as it is an indicator of L-methionine production. Cellular nitrogen and ammonical nitrogen increased gradually throughout the fermentation period. Residual nitrogen level decreased sharply along with the advancement of the fermentation period.

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