EFFECT OF SURFACE ACTIVE AGENTS, CHELATING AGENTS AND ANTIBIOTICS ON L-METHIONINE FERMENTATION BY A MULTIPLE ANALOGUE RESISTANT MUTANT *CORYNEBACTERIUM GLUTAMICUM* X300

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Keywords: surface active agents; antibiotics; chelating agents; analogue resistant; mutant; *Corynebacterium glutamicum* X300; L-methionine; fermentation

In this present study, it was intended to examine the effects of different surface active agents (Tween 80, oleic acid, linoleic acid, palmatic acid, stearic acid , sodium laurylsulfate, laurylmethylgluceth-10-hydroxypropyldiammonium chloride, dimethyldicatadecylammonium chloride, tetramethyl ammonium hydroxide, Centimonium chloride and Bromidox), antibiotics(Penicillin G, Erythromycin, Chloramphenicol , Streptomycin, Tetracycline-HCl and Gentamycin) and chelating agents (EDTA, NTA, DTPA, Catechol, Protocatechuate and Citrate) on L-methionine fermentation by a multiple analogue resistant mutant *Corynebacterium glutamicum* X300. All these agents showed stimulatory effects on the fermentation process.

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Introduction

L-methionine is an essential amino acid, required in human nutrition. Deficiency of L-methionine leads to developments such as rheumatic fever, muscular paralysis, hair loss, depression, toxemia, impaired growth etc.[1] Deficiency can be overcome by dietary supplementation. As per recommendation of Complementary Medicines Evaluation Committee (CMEC), L-methionine can be used therapeutically without any substance specific restriction. Because of inexpensive nature of fermentative production of other amino acids, efforts have been made to produce L-methionine by fermentation.[2-4]

Corynebacterium glutamicum is regarded as a model organism for L-amino acid production because of its simplified metabolic nature.[5,6] Thus, new strains can easily be produced from this parent strain by changing metabolic flux in order to produce L-methionine. The microbial production of L-methionine was performed in Japan in the 1970s.[7] In our previous study, we have already developed a high yielding multiple analogue resistant mutant of *Corynebacterium glutamicum* by protoplast fusion.[8] In this present investigation, we were intended to examine the effect of different surface active agents, antibiotics and chelating agents on L-methionine fermentation by the mutant *Corynebacterium glutamicum* X300.

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 from its parent strain *Corynebacterium glutamicum* (basically a L-glutamic acid producing bacterium which does not accumulate L-methionine) which was isolated from North Bengal soil was subjected for mutational study.

Chemical and Physical mutagensis

To develop a high L-methionine yielding strain, the above mentioned regulatory strain was subjected to mutational treatments using ethyl methanesulfonate (EMS) and UV irradiations as chemical and physical mutagens, respectively, as follows:

Exposure to EMS

1 mL cell suspension (containing $3x10^8$ cells) was added to 9 mL EMS solution of different concentrations (221.8 mmol mL^{-1}, 186.3 mmol mL^{-1}, 76.9 mmol mL^{-1} respectively) and was incubated (10, 20, 30, 40 and 60 minutes respectively). From each sample, 1 ml cell suspension was then plated on CD agar medium and kept at 30 °C for 48 h.[8]

Treatment with UV irradiation

Strains (namely, *Corynebacterium glutamicum* X164 which is high L-methionine yielding and *Corynebacterium glutamicum* X124 which is a multiple analogue resistant strain) were selected for protoplast fusion. 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; $MgSO_4.7H_2O$, 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 µg mL^{-1} in 250 ml Erlenmeyer conical flask at 30 °C: 2 ml cell suspension (containing $3x10^8$ cells mL^{-1}) was taken in a petridish (5 cm diameter) and expose it to UV irradiation, using Hanovia germicidal lamp (15 W) 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.[8]

Development of multiple L-methionine resistant strain

Multiple L-methionine analogue-resistant strain was develop by adding different L-methionine analogue (20-100 mg mL) to the growth medium (namely: α-methyl methionine , DL-methionine, D-methionine sulphate and DL-norleucine.[8]

Protoplast preparation, fusion and regeneration

Two superior for 24 hours. Then the cell suspensions were centrifuged separately at 10,000 rpm for 10 minutes. The pellets were collected and transferred aseptically to a protoplasting medium composed of: sucrose, 0.5 M, maleate buffer (pH 6.5), 0.02 M; $MgCl_2.H_2O$, 20 mM and lysozyme, 100 µg 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 °C for 10 minutes and then it was diluted 10 fold with protoplast medium buffer (pH 6.5). The suspension was then centrifuged for 5 minutes at 25,000 rpm at 5 °C using a cold centrifuge apparatus (EPLX3761).

The pellet was collected and plated for colony formation for 48 h at 30 °C. The colonies were transferred to agar (2%) slants containing the same growth medium[8].

Viable counting of protoplast (Reversion of protoplast)

Protoplasts were diluted with 10 ml of dilution fluid and plated into Petri dish (diameter 5cm) containing agar medium allowed to grow at 30 °C 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, 60µg[8].

Optimum cultural conditions

Volume of medium, 25 ml; initial pH, 7.0; shaker's speed, 150 rpm; age of inoculum, 48 hours; optimum cell density, $4.0X10^8$ cells mL^{-1}; temperature 28 °C and period of incubation, 72 h.[9]

Composition of synthetic medium(per liter)

Glucose, 100 g; $(NH_4)_2SO_4$, 8.0 g (in terms of nitrogen); K_2HPO_4, 2.2 g; $MgSO_4.7\ H_2O$, 1.5 g; $FeSO_4.7H_2O$, 0.03 g; KH_2PO_4, 2.0 g; $ZnSO_4.7H_2O$, 1.6 mg; $CaCO_3$, 1.5 g; $Na_2MoO_4.2H_2O$, 5.0 mg; $MnSO_4.4H_2O$, 2.5 mg; biotin, 80 mg and thiamine-HCl, 70 µg.[10]

Addition of surface active agents

Varying concentrations of surface active agents (0.05-0.50 µl mL^{-1}) namely Tween 80, oleic acid, linoleic acid, palmitic acid, stearic acid, benzalkonium chloride, laurylmethylgluceth-10 hydroxypropyldiammonium chloride, dimethyl dicatadecyl ammonium chloride, tetramethyl-ammonium hydroxide, centrimonium chloride and bromidox were added separately to the synthetic medium in order to examine their effect on L-methionine fermentation by this mutant.[11]

Addition of antibiotics

Varying concentrations of antibiotics (5.0-20.0 µl mL^{-1}) of different antibiotics namely penicillin G, erythromycin, Chloramphenicol, Streptomycin Tetracycline-HCl and Gentamycin.[12]

Addition of chelating agents

Varying concentrations of chelating agents (10^{-3}-10^{-15} M) namely EDTA, NTA, DTPA, catechol, protocatechate and citrate.[13]

Analysis of L-methionine

Descending paper chromatography was employed for detection of L-methionine in culture broth and was run for 18 h on Whatman No.1 Chromatographic 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.[8] All the chemicals used in this study were analytical grade (AR) grade and obtained from E mark Borosil glass goods and triple distilled water used throughout the study.

Estimation of Dry Cell Weight (DCW)

The cell paste was obtained from the fermentation broth by centrifugation and dried in a dried at 100 °C until constant cell weight was obtained.[9]

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.

Results

The effect of different surface active agents, antibiotics and chelating agents on L-methionine production by *Corynebacterium glutamicum* X300 has been presented in Table 1-3 as follows:

Table 1. Effect of surface active agents on L-methionine fermentation by *Corynebacterium glutamicum* X300

Surface active agents	Concentration, μg mL^{-1}	L-methionine, mg mL^{-1}	Dry Cell Weight, mg mL^{-1}
Tween80	0.0(control)	52.1±1.668	28.5±0.891
	0.05	52.8±1.202	*29.1±0.661
	0.10	*53.1±1.668	*29.3±0.983
	0.20	*53.6±0.913	**29.5±0.591
	0.50	*53.6±1.813	**29.5±0.993
Oleic acid	0.0(control)	52.1±1.831	28.5±0.662
	0.05	52.4±1.887	28.7±0.713
	0.10	*52.9±0.973	*29.0±0.683
	0.20	*52.9±1.854	*29.0±0.991
	0.50	*52.9±1.785	*29.0±0.772
Linoleic acid	0.0(control)	52.1±1.882	28.5±0.913
	0.05	52.4±1.683	28.7±0.683
	0.10	52.6±1.786	28.8±0.613
	0.20	52.6±1.991	28.8±0.551
	0.50	52.6±1.661	28.8±0.691
Palmatic acid	0.0(control)	52.1±1.661	28.5±0.771
	0.05	52.3±1.872	28.6±0.642
	0.10	52.7±1.601	28.8±0.773
	0.20	53.0±1.553	*29.0±0.613
	0.50	53.0±1.713	*29.0±0.661
Stearic acid	0.0(control)	52.1±1.662	28.5±0.672
	0.05	52.4±1.681	28.7±0.413
	0.10	52.7±1.881	28.9±0.662
	0.20	*53.1±1.682	*29.1±0.713
	0.50	*53.1±1.913	*29.1±0.463
Sodium Lauryl Sulfate	0.0(control)	52.1±1.992	28.5±0.441
	0.05	52.3±1.462	28.6±0.771
	0.10	52.6±1.562	28.8±0.683
	0.20	52.9±1.452	*29.0±0.421
	0.50	52.9±1.897	*29.0±0.613
Benzalkonium Chloride	0.0(control)	52.1±1.772	28.5±0.662
	0.05	52.3±1.813	28.6±0.591
	0.10	52.6±1.662	28.8±0.551
	0.20	52.6±1.811	28.8±0.613
	0.50	52.6±1.683	28.8±0.662
Laurylmethylgluceth-10 hydroxypropyldiammonium chloride	0.0(control)	52.1±1.662	28.5±0.491
	0.05	52.3±1.661	28.6±0.683
	0.10	52.5±1.261	28.8±0.613
	0.20	52.5±1.661	28.8±0.442
	0.50	52.5±1.713	28.8±0.681
Dimethyldicatadecyl-ammonium chloride	0.0(control)	52.1±1.662	28.5±0.436
	0.05	52.4±1.613	28.6±0.441
	0.10	52.7±1.661	*29.0±0.613
	0.20	52.7±1.662	*29.0±0.771
	0.50	52.7±1.715	*29.0±0.913
Tetramethylammonium hydroxide	0.0(control)	52.1±1.009	28.5±0.683
	0.05	52.3±1.613	28.6±-0.721
	0.10	52.7±1.662	28.8±0.991
	0.20	52.7±1.683	28.8±0.683
	0.50	52.7±1.653	28.8±0.772

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Continuation of Table 1.

Centimonium chloride	0.0(control)	52.1±1.009	28.5±0.961
	0.05	52.3±0.986	28.6±0.777
	0.10	52.7±1.592	28.6±0.683
	0.20	53.1±1.821	28.9±0.661
	0.50	*53.1±1.452	28.9±0.513
Bromidox	0.0(control)	52.1±1.771	28.5±0.881
	0.05	52.3±1.562	28.6±0.771
	0.10	52.6±1.843	28.7±0.683
	0.20	53.0±1.661	*29.0±0.642
	0.50	53.0±1.832	*29.0±0.691

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

Table 2. Effect of antibiotics on L- methionine fermentation by *Corynebacterium glutamicum* X300

Antibiotics	**Concentration, µg mL^{-1}**	**L-methionine, mg mL^{-1}**	**Dry Cell Weight, mg mL^{-1}**
Penicillin G	0.0(control)	52.1±1.683	28.5±0.991
	5.0	*53.2±1.771	28.1±0.891
	10.0	*53.9±1.913	*27.4±0.662
	15.0	**54.1±1.665	**27.0±0.814
	20.0	**54.1±1.567	**27.0±0.682
Erythromycin	0.0(control)	52.1±1.723	28.5±0.881
	5.0	52.6±1.835	*28.0±0.714
	10.0	53.0±1.623	**27.3±0.824
	15.0	*53.4±1.991	**27.0±0.591
	20.0	*53.4±1.613	**27.0±0.991
Chloramphenicol	0.0(control)	52.1±1.771	28.5±0.791
	5.0	52.4±1.825	*28.0±0.771
	10.0	52.9±1.992	**27.3±0.682
	15.0	*53.1±1.623	**27.1±0.814
	20.0	*53.1±1.719	**27.1±0.714
Streptomycin	0.0(control)	52.1±1.881	28.5±0.881
	5.0	52.4±1.913	28.1±0.692
	10.0	52.9±1.823	**27.3±0.661
	15.0	*53.1±1.913	**27.0±0.771
	20.0	*53.1±1.881	**27.0±0.651
Tetracycline-HCl	0.0(control)	52.1±1.852	28.5±0.881
	5.0	52.6±1.881	28.1±0.791
	10.0	52.8±1.826	**27.9±0.791
	15.0	52.8±1.001	**27.9±0.581
	20.0	52.8±1.825	**27.9±0.991
Gentamycin	0.0(control)	52.1±1.961	28.5±0.691
	5.0	52.3±1.991	28.3±0.661
	10.0	52.5±1.851	*28.0±0.813
	15.0	52.9±1.710	*27.8±0.792
	20.0	52.9±1.601	*27.8±0.681

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

Table 3. Effect of chelating agents on L- methionine fermentation by *Corynebacterium glutamicum* X300

Chelating agent(s)	Concentration, µg mL^{-1}	L-methionine, mg mL^{-1}	Dry Cell Weight, mg mL^{-1}
EDTA	0.0(control)	52.1±1.915	28.5±0.661
	10^{-3}	52.6±1.993	28.7±0.913
	10^{-5}	53.0±1.682	*29.0±0.665
	10^{-10}	52.8±1.702	28.8±0.862
	10^{-15}	52.6±1.882	28.7±0.913
NTA	0.0(control)	52.1±1.602	28.5±0.771
	10^{-3}	52.4±1.883	28.6±0.684
	10^{-5}	52.3±1.991	28.6±0.661
	10^{-10}	52.0±1.003	28.4±0.813
	10^{-15}	51.7±1.875	28.1±0.813
DTPA	0.0(control)	52.1±1.992	28.5±0.661
	10^{-3}	52.6±1.915	28.8±0.613
	10^{-5}	52.9±1.683	28.9±0.882
	10^{-10}	*53.2±1.660	29.2±0.571
	10^{-15}	*53.2±1.503	29.2±0.882
Catechol	0.0(control)	52.1±1.991	28.5±0.613
	10^{-3}	52.4±1.962	28.6±0.661
	10^{-5}	52.9±1.889	28.8±0.711
	10^{-10}	52.6±1.993	28.6±0.945
	10^{-15}	52.3±1.691	28.4±0.661
Protocatechate	0.0(control)	52.1±1.713	28.5±0.683
	10^{-3}	52.6±1.002	28.8±0.702
	10^{-5}	*53.1±1.721	*29.1±0.817
	10^{-10}	52.8±1.005	28.9±0.864
	10^{-15}	52.6±1.691	28.8±0.883
Citrate	0.0(control)	52.1±1.188	28.5±0.613
	10^{-3}	52.4±1.682	28.6±0.771
	10^{-5}	52.7±1.773	28.8±0.956
	10^{-10}	*53.1±1.882	*29.1±0.887
	10^{-15}	*52.8±1.991	28.8±0.854

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

Thus, maximum production of L-methionine can be obtained with Tween 80,0.10 µg mL^{-1}; oleic acid, 0.10 µg mL^{-1}; linoleic acid, 0.10 µg mL^{-1}; palmitic acid, 0.20 µg mL^{-1}; stearic acid, 0.20 µg mL^{-1}; gluceth-10-hydroxypropyl diammonium chloride, 0.10 µg mL^{-1}; dimethyl dicatadecyl-ammonium chloride, 0.10 µg mL^{-1}; tetramethylammonium hydroxide, 0.10 µg mL^{-1}; Centimonium chloride, 0.10 µg mL^{-1}; Bromidox,0.20 µg mL^{-1}; Penicillin G, 15.0 µg mL^{-1}; Erythromycin, 15.0 µg mL^{-1}; Chloramphenicol, 15.0 µg mL^{-1}; Streptomycin,15.0 µg mL^{-1}; Tetracycline-HCl, 10.0 µg mL^{-1}; Gentamycin, 15.0 µg mL^{-1}; EDTA,10^{-5} M; NTA, 10^{-4} M; DTPA, 10^{-10} M; catechol, 10^{-5} M; protocatechate, 10^{-5} M and citrate, 10^{-10} M.

Discussion

It has extensively been reported that small quantities of antibiotics can increase L-amino acid yield by different strains of bacteria[14-17]. Zaki *et al* . (1982) reported that erythromycin and tetracycline-HCl increase L-lysine accumulation by *Micrococcus glutamicus* when added to the fermentation broth[14]. Various surface active agents alter the permeability in microorganism and thereby can influence the accumulation of L-amino acids in fermentation broth [18-20]. Komicek *et al*. (1991) claimed that L-lysine accumulation by *Corynebacterium glutamicum* increased by Tween 80 due to alteration of cellular structure[20]. Takinami *et al*. (1963) reported the stimulatory effect of saturated fatty acids on L-glutamic acid fermentation[21]. Different chelating agents like EDTA, DTPA, NTA etc acting as 'metal buffer' under crucial condition and thus, may increase the secondary metabolite production by microorganisms[22].

Conclusion

From this present study it can tentatively be concluded that all these surface active agents, antibiotics and chelating agents examined showed positive impacts on L-methionine fermentation by this mutant. Thus, the production can be increased by using those agents in appropriate concentration.

References

[1]Rose, W. C., *Physiol. Rev*., **1938**, *18*, 109-136.

[2]Pham, C. B., Galvez, C. F. and Padolina, W. G., *ASEAN Food J*., **1992**, *7*, 34-37.

[3]Umerie, S. C., Ekwealor, I. A. and Nawabo, I. O., *Bioresour. Technol*., **2000**, *75*, 249-252.

[4]Odunfa, S. A., Adeniran, S. A., Teniola, O. D. and Nordstorm, J., *Int. J. Food Microbiol*., **2001**, *63*, 159-163.

[5]Lee, H.S. and Hwang, B.J., *Appl. Microbiol. Biotechnol*., **2003**, *62*, 459-467.

[6]Gomes, J. and Kumar, D., *Biotechnol. Adv*., **2005**, *23*, 41-61.

[7]Kase, H. and Nakayama, K., *Agric. Biol. Chem*., **1974**, *38*, 2021-2030.

[8]Ganguly, S., Satapathy, K. B. and Banik, A. K., *Res. J. Pharm. Dose Forms Technol.*, **2014**, *6*, 303-310.

[9]Ganguly, S., and Satapathy, K. B., *J. Bioprocess Technol.,* **2013**, Photon **98**, 303-307.

[10]Ganguly, S. and Satapathy, K. B., *Int. J. Multidiscip. Educ. Res.*, **2013**, *2*, 252-261.

[11]Ganguly,S. and Banik, A. K., *Int. J. Univers. Pharm. Life Sci*., **2011**, *1*, 94-97.

[12]Ganguly, S. and Banik, A.K., *J. Pure Appl. Microbiol*., **2012**, 6, 271-279.

[13]Ghosh, P., Ganguly, S. and Banik, A.K., *Int. J. Ind. Pharm. Biosci.,* **2012**, *1*, 1-7.

[14]Zaki, D., Galal, O., Hazino-Wahba, S. A., Morsi, K. I. and Wakell, E. A., *Nutr. Rep. Int*., **1982**, *26*, 537-546.

[15]Sen, S. K. and Chatterjee, S. P.,, *Folia Microbiol*., **1983**, *28*, 292-300.

[16]Sen, S. K. and Chatterjee, S. P., *Acta Biotechnol*., **1985**, *5*, 215-218.

[17]Israildes, C. J., Weir, A. N. and Bull, A. T., *Appl. Microbiol. Biotechnol*., **1989**, *32*, 134-136.

[18]Oshima, K., Tanaka, K. and Kinoshita, S., *J. Gen. Appl. Microbiol*., **1964**, *10*, 333-342.

[19]Smekal, F., Bulant, V., Kindlova, E ., *Kvasny Prum*, **1982**, *28*, 39-40.

[20]Komicek, J., Smekal, F. and Koniskova-Rodochora, M., *Folia Microbiol*., **1991**, *36*, 587-589.

[21]Takinami, K., Okada, H. and Tsunoda, T., *Agric. Biol. Chem*.,**1963**, *27*, 858-863.

[22]Chaudhury, A. Q. and Pirt, S. J., *J. Gen. Microbiol*.,**1966**, *43*, 71-81.

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