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OPTIMIZING THE CONCENTRATION OF BIOTIN FOR *L*-GLUTAMIC ACID PRODUCTION BY A LOCALLY ISOLATED CORYNEFORM STRAIN

B. Niaz[1*], M. I. Rajoka[4], K. A. Al-Ghanim[3], S. Yousaf[2], S. Mahboob*[3, 1] and S. Nadeem[2]

[1*]Govt. College University, Allama Iqbal Road, Faisalabad-38000, Pakistan
[2]Animal Sciences Division, NIAB, P.O. Box 128, Faisalabad-38000, Pakistan
[3]Department of Zoology, College of Science, King Saud University, Riyadh, 11455, Kingdom of Saudi Arabia.
[4]Department Bioinformatics and Biotechnology, Govt. College University, Allama Iqbal Road, Faisalabad-38000, Pakistan
Corresponding Author email: shahidmahboob60@hotmail.com

ABSTRACT

Various concentrations of biotin were tested in order to achieve fluent excretion of glutamic acid (glu) in a fermentation medium composed of a locally isolated *Corynebacterium glutamicum* strain. The behaviour of the strain was observed to vary with varying concentrations of biotin. Maximum values for specific growth rate (μ, h^{-1}), cell mass yield (Yx/s, g/g), substrate consumption rate (Q_S, g/l h) and cell mass specific productivity (q_X, g/g h) were 0.34, 0.24, 2.92 and 1.43, respectively, given 10 μg biotin/100 ml under working conditions of a shake flask with temperature 30 °C, in 100 g glucose/l medium (pH 7.0). The kinetic parameters calculated for glutamate production under the above conditions were 4.94 g/l h, 4.4 g/g cells, 0.66 g/g, and 1.5 g/g ·h for Q_P, $Y_{P/X}$, $Y_{P/S}$, and q_P, respectively, and were comparatively higher than the respective values reported for some *C. glutamicum* strains growing on glucose in batch culture studies. Conversely, at biotin concentrations of 1.0, 2.5, 5.0, 15 μg/ ml, significantly less improvement in *glu* production was noticed, but at 10 μg/ml a nearly 2.2-fold increase in Q_P was observed, though the only statistically significant difference was in the QS value.

Key words: Biotin; optimization; glutamic acid.

INTRODUCTION

Proteins are considered to be the mother source of amino acids, which are amongst the most fundamental constituents of life. The amino acids cover a wide range of activities. In addition to regulating the water balance of the human body, controlling growth and forming the structural basis of chromosomes, amino acids are also involved in coordinated motion, catalysis, transportation as well as the mechanical support and transport of nerve impulses (Nadeem *et al.*, 2013; Park and Lee, 2008). Our bodies use glutamic acid (*glu*) not only to manufacture proteins but also as the most effective neurotransmitter in the central nervous system. Moreover, in heart patients, *glu* plays a vital role in the protection of heart muscles (Niaz *et al.*, 2009). Georget *et al*. (2006) have very well illustrated the elementary role of *glu* in relation to the stability of androgen receptors and in the activities of androgen/ antiandrogen in human beings.

Due to the swift rise in the demand for its sodium salt, monosodium glutamate (MSG), as a flavour enhancer, *glu* has already captured the world market to a great extent. In 1996, the annual production of MSG was around one million tons (Ikeda, 2003; Kumagai, 2000), which due to a 6% annual increase (Hermann, 2003) is now more than two million tons (Zhang *et al*., 2012).

L-glutamic acid fermentation is a classic example of aerobic fermentation (Ishizaki *et al*., 1993). A large variety of microbes, wild as well mutants, and particularly various strains of *Brevibacterium* and *Corynebacterium*, now jointly termed as coryneforms, especially *C. glutamicum* (Khan *et al*., 2013; Nadeem *et al*., 2013; Shakoori *et al*., 2012; Ali *et al*., 2011; Kimura, 2003) have been successfully used for the production of *glu*, along with many other amino acids, for the last five decades (Sahm *et al*., 2000). The improvement of any wild type strain for better industrial-scale amino acid production has for long been a top priority for researchers (Mitsuhashi, 2014).

Manipulation of the metabolic and regulatory processes may be made in order to enhance the extracellular yield of *glu* (Nadeem *et al*., 2013). Glutamic acid is secreted from cells in response to various stress situations (Nadeem *et al*., 2011), and these can be re-created using various treatments such as biotin limitation, addition of penicillin (Amin and Al-Talhi, 2007; Nunheimer *et al.*, 1970), or explicit fatty acid derivate and ester surfactants Choi *et al.*, 2004; Hirasawa *et al.*, 2001; Takinami *et al.*, 1965; Shiio *et al*., 1962), fermentations employing oleic acid (Kitano *et al.*, 1972) or glycerol-auxotrophs (Kikuchi *et al.*, 1972; Nakao *et al.*, 1972).

Biotin, a water-soluble B-complex vitamin, plays a vital role in assisting the body to metabolize proteins and process glucose. It is also an essential constituent of enzymes that break down several substances, such as carbohydrates and fats present in the

body (Rathman *et al*., 2002). Also called vitamin H or B7, biotin is found in meagre amounts in several food items.

One of the distinctive features of *C. glutamicum* is that it needs biotin for growth. It is well accepted that in order to accumulate reasonable amounts of extracellular *glu*, biotin is required in minor quantities. Complete details of this mechanism are not yet known, however. It has been claimed that changes in the membrane permeability are necessary for frequent excretion of *glu* into the medium (Kinoshita, 1999). A lot of work is being done to understand how biotin works together with *C. glutamicum* for enhanced *glu* production (Ikeda *et al*., 2013; Schneider *et al.*, 2012; Peters-Wendisch *et al*., 2012). The present study was planned with the same objective, but specifically to explore the impact of varying biotin concentrations upon the membrane permeability of a Coryneform strain, *Corynebacterium glutamicum* NIAB SS-27.

MATERIALS AND METHODS

Microorganisms and inoculum: *Corynebacterium glutamicum* NIAB SS-27 (wild type), isolated from soil samples, was obtained from the NIAB (Nuclear Institute of Agriculture and Biology) culture collection. Initially it was preserved in agar slants from where the inoculum was prepared by shifting the cells into a 250 ml Erlenmeyer flask containing 50 ml glucose-yeast extract (GYE) seeding medium as (10% v/v): glucose, 0.5% and yeast extract, 0.3%.

Production medium: The *C. glutamicum* strain NIAB SS-27, was grown in a Glucose-trypticase fermentation medium with a composition of (g/100 ml): glucose, 10.0; trypticase, 0.75; KH_2PO_4, 0.07; K_2HPO_4, 0.4; $CaCO_3$, 2.0; $(NH_4)_2SO_4$, 3.0; $MgSO_4.7H_2O$, 0.03; thiamine HCl, 5 mg/l; biotin, 60 µg/l. Glucose, thiamine HCl and biotin were filter-sterilized individually through a membrane filter (0.45 μm), whereas the rest of the medium was autoclaved at 121 ºC and 15 psi for 15 min. Thereafter, all the ingredients were mixed aseptically and pH was adjusted at 7.0 with KOH.

Fermentation conditions: The culture grown overnight was inoculated in the fermentation medium as mentioned above, and incubated at 150 rpm and 30±1 ºC in a gyratory shaker. From this fermentation broth, a 3 ml fraction was removed after every 2 hr for the assessment of bacterial growth. After 96 h, the growth was terminated and processed after following Nadeem *et al*. (2013).

Effect of biotin: A sterilized solution of biotin in seven different concentrations (0.5, 1.0, 2.5, 5.0, 10, 25 and 50 µg/ml) was added aseptically to the fermentation medium (GYE). After inoculation, the culture was incubated for 96 h at 30±1ºC and 150 rpm. The whole process was regularly monitored for the impact of biotin upon extracellular accumulation of *glu*. A control with no biotin was also fermented sidewise under the same set of fermentation conditions in order to provide a comparison.

Glucose utilization: In order to work out the residual glucose in the fermentation broth, dinitrosalicylic acid (DNS) reagent (Costa-Ferreira and Duarte, 1992) was applied. The sample (1 ml fermented broth) with 2 ml water and 3 ml DNS reagent (3,5-dinitrosalicylic acid) taken in a test tube, was placed in a boiling water bath for 10-15 minutes. The tube was removed and placed at room temperature for five minutes. It was then given an ice bath and its O.D. was observed at 550 nm. A standard curve of glucose solution containing 200 µg to 1 mg concentration was plotted.

Growth curve: In order to co-relate the growth pattern and product formation of the strain under study, a growth curve was plotted (Costa-Ferreira and Duarte, 1992). The Coryneform strain, NIAB SS-27, was grown in the GYE medium, 50 ml per flask (Erlenmeyer, 250 ml), at 30 ºC and 150 rpm. The sample was monitored at regular intervals and the harvest pH observed. Empty, sterilized, centrifuged tubes along with their plugs were weighed (W-I). The broth was centrifuged in these tubes at 10,000 x g until a pellet was formed. The tubes, along with the fresh pellets, were weighed again (W-II), and placed at 70 ºC until the pellet was thoroughly dried (48-72 h). Thereafter, they were weighed in triplicate and the average weight calculated (W-III). Using these three weights (W-I, W-II, W-III) the dry cell weight percentage was calculated as: (W-III – W-I) / (W-II – W- I) x 100. Finally, a growth curve illustrating the growth pattern of NIAB SS-27 was plotted on the basis of its dry mass (DM).

Kinetic study: Kinetic parameters, namely maximum volumetric rate of glutamate formation (Q_P), product yield ($Y_{P/S}$), specific product yield ($Y_{P/X}$) and specific rate of glutamate formation were determined by applying equations 1-4.

$$dP/dt = Y_{P/X}.\ dX/dt \quad (1)$$

$$Y_{P/X} = dP/dX \quad (2)$$

$$Y_{P/S} = -dP/dS \quad (3)$$

$$q_P = 1/X.\ dP/dt \quad (4)$$

Similarly, the specific growth rate, cell yield and specific substrate consumption rate was determined by using the following equations:

$$dX/dt = \mu X \quad (5)$$

$$Y_{X/S} = -dX/dS \quad (6)$$

$$q_S = 1/X\ dX/dt.\ dX/dS \quad (7)$$

The volumetric rate of substrate consumption and glutamate formation were represented by the maximum respective slopes of the substrate and glutamate curves against the time of fermentation (h).

Statistical analysis: All experiments were replicated in three flasks and the data are presented as the mean of these three independent experiments. Tukey-Kramer one way ANNOVA (Instat 3 software) was used to determine the significant differences between the mean values at the 5 % level of confidence

RESULTS

Effect of biotin concentration on product formation: The best results were obtained at a concentration of 10 μg/ml, at which point the strain supported a volumetric productivity of 4.94 g *glu*/l.h. At 1.0, 2.5 and 5.0 μg/ml biotin supported Q_P of 2.81, 3.49 and 4.42 *glu*/l.h, respectively, in the fermentation broth, which was still significantly higher than that produced by the control (2.26 g/l.h) (Table I). At higher concentrations the strain supported lower volumetric rates compared to the rate at 10 μg biotin /ml, as shown in Table 1. This table also shows that 10 μg biotin/ml supported significantly higher values (P=0.001) of $Y_{P/X}$, $Y_{P/S}$ and q_P than those supported by all other concentrations of biotin (Table 1).

Table 1. Effect of biotin concentrations on glutamate production parameters.

Biotin concentration (µg/ml)	Q_P (g l^{-1} h^{-1})	$Y_{P/X}$ (g g^{-1} cells)	$Y_{P/S}$ (g.g^{-1} substrate)	q_p (g. g^{-1} h^{-1})
0	2.26±0.11 e	1.85±0.02e	0.26±0.02e	0.54±0.01e
1	2.81±0.13d	2.28±0.02d	0..37±0.02d	0.71±0.01d
2.5	3.49±0.16c	2.680±0.03c	0.52±0.03c	0.88±0.01c
5	4.24±0.24b	3.61±0.03b	0.60±0.03a	1.19±0.02b
10	4.94±0.22a	4.4±0.2a	0.66±0.03a	1.5±0.1a
15	3.4±0.22c	2.85±0.04c	0.59±0.02b	0.91±0.02c
25	2.1±0.21e	1.51±0.03f	0.46±0.02d	0.445±0.02e
p	0.001	0.001	0.001	0.001
Significance level	HS	HS	HS	HS

HS stands for highly significant

Effect of biotin concentration on glucose consumption: The glucose consumption rate of 2.92 g/l h for NIAB SS-27 with 10 μg biotin/ml in the medium was significantly higher than that of the control (no biotin) and, indeed, significantly higher than the values supported by all other concentrations. Biotin used at 5 and 15 μg/ml produced glutamate rates that were not significantly different from each other; while all other concentrations supported significantly different values of QS (Table 2), although the values of all other substrate consumption and cell formation kinetic parameters were non-significantly different across the biotin concentrations in the medium (Table 2), as calculated from Fig. 1-2. The specific growth rate, and specific substrate uptake rates and other substrate consumption parameters are shown in Table 2. During growth on glucose culture medium, enhanced production of *glu* may have occurred due to enhanced cell wall permeability at this concentration compared with all other concentrations.

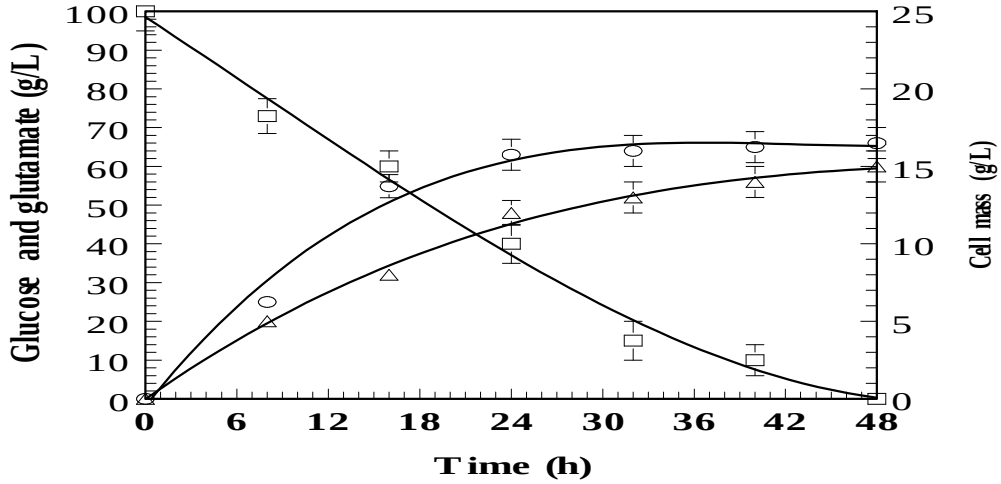

Fig. 1. Time course of production of glutamate (o), cell mass (Δ) from glucose (□) present in the fermentation medium in the presence of 10 µg biotin/ mL medium.

Table 2. Effect of biotin concentrations on substrate consumption parameters.

Biotin concentration (µg/ml)	Q_S ($g.l^{-1} h^{-1}$)	$Y_{X/S}$ ($g. g^{-1}$)	µ (h^{-1})	q_S ($g\ g^{-1} h^{-1}$)
0	1.59f	0.22	0.29	1.32
1	1.89d	0.22	0.31	1.41
2.5	2.21c	0.23	0.33	1.43
5	2.65b	0.23	0.33	1.43
10	2.92a	0.24	0.34	1.42
15	2.62b	0.24	0.32	1.38
25	1.65e	0.22	0.29	1.32
P	0.0001	0.7248	0.7248	0.8480

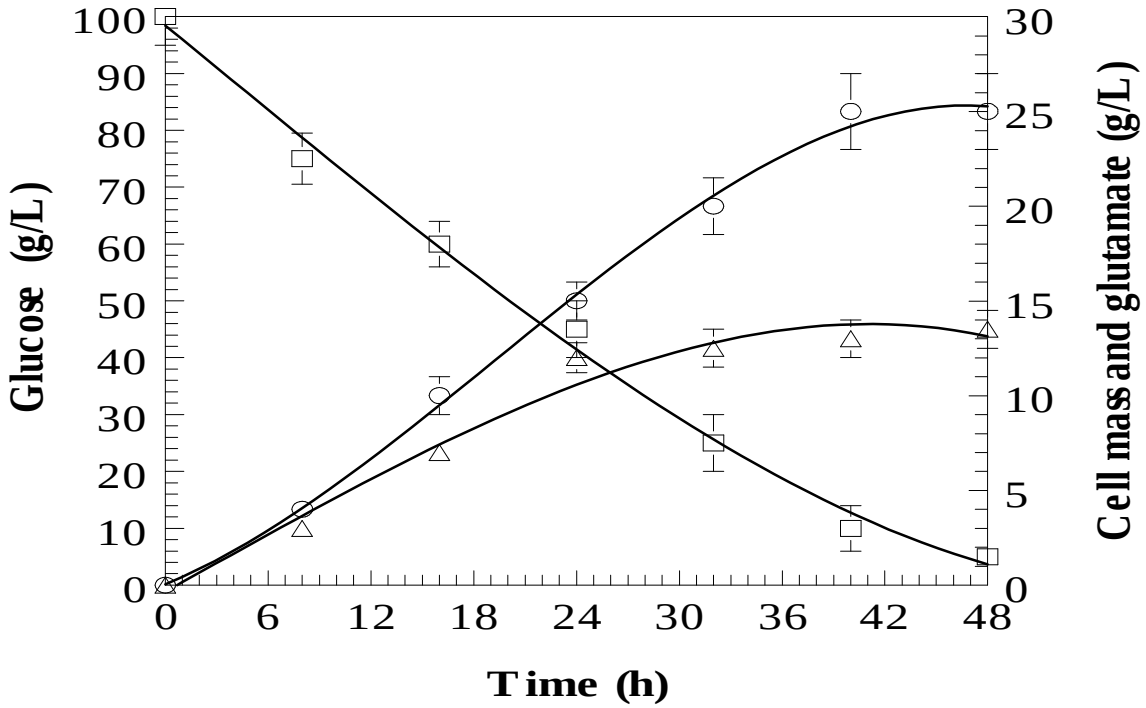

Fig. 2. Time course of production of glutamate (o), cell mass (Δ) from glucose (□) present in the fermentation medium in the absence of biotin.

The best kinetic parameters for glutamate production with 10 µg/ml biotin in the medium were 4.94 g/l h, 4.4 g/g cells, 0.66 g/g, and 1.5 g/g ·h for Q_P, $Y_{P/X}$, $Y_{P/S}$, and q_P, respectively, when glucose was used as a carbon source in the production medium (Table 1), and these differed significantly ($p \leq 0.001$) from the results for all other biotin concentrations. Minimum production occurred in the absence of biotin (values of 2.26±0.11, 1.85±0.02, 0.26±0.02, 0.54±0.01, respectively for the above kinetic parameters). Maximum values for specific growth rate (µ, h^{-1}), cell mass yield (Yx/s, g/g), substrate consumption rate (Q_S, g/l h) and cell mass specific productivity (q_X, g/g h) were 0.34, 0.24, 2.92 and 1.43, respectively, with 10 *µ*g biotin/ ml under working conditions of a shake flask with a temperature of 30 °C, in 100 g glucose/l medium (pH 7.0). There was a significantly ($p \leq 0.001$) higher variation in the specific growth rates, as well as larger variations in the synthesis of *glu* (Table 1*)*.

DISCUSSION

Our previous study made on this strain regarding the effect of various vitamins showed a 2.5-fold increase in *glu* production (Niaz *et al.,* 2009). Biotin was not included in that study, however; an omission that this study corrects.

One of the distinctive features of *C. glutamicum* is that it entails some fractions of biotin for its growth. When biotin is deficient, it may be because of the scarcity of two enzymes, pimelyl-CoA synthetase, and 7-keto-8-aminopelargonic acid synthetase (Kinoshita, 1985). It has been a proven that in order to excrete reasonable amounts

of *glu* from the cell into the medium, small quantities of biotin are required. Although, membrane permeability is the key factor for this excretion and a lot of studies have been made into this, the real secret behind this mechanism is yet to be properly unearthed (Khan *et al*., 2013; Schneider *et al.,* 2012; Vijayalakshmi and Sarvamangala, 2011).

The role of cell membrane permeability was realized some fifty years ago and since then the scientists have been working on it in depth (Kinoshita and Tanaka, 1972; Philips and Somerson, 1963; Shiio *et al.,* 1962). In 1987, Nakayama claimed that one of the specific conditions that allow bacteria to excrete reasonable amounts of *glu* is the nutritional constraint for biotin (Nakayama, 1987). It has been widely reported that in order to gear-up the flow of *glu*, the bacteria should be grown in biotin-limited media (Patil *et al.*, 2009; Aoki *et al.,* 2005; Shiio and Ujigawa, 1980), since under such an environment the permeability barrier for the excretion of *glu* is quite relaxed, facilitating the excretion of *glu* into the medium Kinoshita and Tanaka, 1972; Demain and Birnbaum, 1968).

The most suitable concentration of biotin to maximise the accumulation of *glu* in the medium has been a field of particular interest for scientists. In the present study, attempts were made to find out the most suitable biotin concentration for the NIAB SS-27 strain. Seven different concentrations were applied and showed significantly different concentrations of *glu.* The *glu* production rate for the strain NIAB SS-27 was better than the control (without biotin). It reflects that the strain not only has better metabolic potential and also has better yield conversion rate. Maximum values of Q_P, $Y_{P/X}$, $Y_{P/S}$, and q_P are higher than those reported for other *C. glutamicum* strains (Amin and Al-Talhi, 2007; Choi *et al*,. 2004; Jyothi *et al*. 2005; Aoki *et al.* 2005; Ikoda *et al.* 2013). Kinoshita (1999) reported obvious changes in the external layer of the cell membrane at lower concentrations of biotin.[28] He further explained these changes through electron micrographs depicting the swelling, elongation and branching out of cells, giving a disordered look to the membrane. He strongly correlated these changes with the *glu* accumulation in the cells. In addition, he showed that excessive biotin in the medium is also not desirable as in such a condition the *glu* from within the cells cannot permeate out through the membrane. He also proved that there was no pleomorphic change at higher biotin concentrations (Kinoshita, 1999). Different studies have reported quite different outcomes in terms of the optimum level of biotin for *glu* production. What is consistent, however, is that recommended levels of biotin are always on the low side; usually in the range from 0.001 μg/ml[46] to 5.0 μg/ml (Kinoshita, 1999). In the present study, however, the optimum level is indicated to be as high as 10 μg/ml. The variation in the desired level of biotin is dependent upon many factors, with the membrane structure and constitution being the major ones. Thus, it can be considered that the requisite biotin concentration for *glu* production may vary from one strain to another.[3]

Glucose is considered to be one of the most potent sources of carbon for the majority of the bacterial strains, supporting the growth and production of metabolites, particularly the amino acids (Sen *et al.,* 1992). The selection of a carbon source, however, largely depends upon the mode and choice of the respective organism. It has been reported that increased sugar metabolism results in enhanced fermentative production (Aoki *et al.,* 2005). Table II depicts that the glucose consumption rate within the initial 24 h was higher in the NIAB SS-27 strain compared to the control (no biotin). Substrate consumption kinetic parameters are considered to be a factor that might control product synthesis through different biotin concentrations. We found that the faster the organism consumed glucose in the presence of biotin Rajoka *et al*., 2006).

Generally, in *C. glutamicum*, glycolysis is operated at the pyruvate kinase level, which is considered to be the fundamental enzyme of the glycolytic pathway as dictated by cell energy level. The activity of this enzyme is controlled by ATP or AMP.[1] Thus, it can be speculated that pyruvate kinase of NIAB SS-27 was activated because of the reduced intracellular ATP concentration. The photo transferase system that converts phosphoenolpyruvate to pyruvate is tied to glucose uptake and seems to be highly active in this particular strain, potentially explaining the efficient glucose consumption. So, quite possibly, in the strain under discussion, phosphoenolpyruvate is converted into pyruvic acid hence increasing the intracellular concentration of Acetyl-CoA. Since the ratio of oxaloacetate/ Acetyl-CoA the major factor governing *glu* production, changes to this ratio may be instrumental in gearing up NIAB SS-27 for *glu* production. All of these changes could possibly play a vital role in the enhanced *glu* production/ excretion, but further work needs to be done to develop a better understanding of these processes.

Tanaka *et al*. (1960) contributed a lot in this context. They claimed that minor quantities of biotin when added in the fermentation media supported the excessive accumulation of *glu* in the broth. Kikuchi and his group,[48] meanwhile, were of the view that the decrease in the phospholipid content of the cell membrane relaxed the permeability barrier facilitating the frequent release of *glu* into the fermentation medium. Conditions in which biotin content was higher than 0.5 ng/mg of the cells produced sufficient amounts of oleic acid to increase the phospholipid content, ultimately reducing the excretion of endogenous *glu* (Patil *et al.,* 2009). In fact, biotin, oleic acid and phospholipids are closely linked with each other as far as *glu* excretion is

concerned. The meagre amounts of biotin in the medium restrict the biosynthesis of oleic acid, which leads to a decrease in the phospholipid concentration, which is supposed to be involved in regulating the membrane permeability for *glu*. Kinoshita *et al* (1972). investigated the relationship between *glu* production and α-ketoglutarate dehydrogenase activity, which is one-tenth of that of glutamate dehydrogenase, and which has quite an impact on *glu* production (Shio and Ujigawa, 1980). This difference of activity is one of the main reasons for the overproduction of *glu* (Kinoshita, 1985).

There is a school of thought that recommends "sub-optimal" rather than optimal concentration of biotin for the maximum *glu* excretion from the cell (Kinoshita, 1999, 1985). The logic behind this idea is that higher biotin concentrations provoke optimal cell growth leading to high lactate production, whereas *glu* is best released at sub-optimal growth levels (Kinoshita, 1999, 1985). The results of the present study are also in line with these findings.

Conclusion: The excretory process of *glu* from inside the cell to the fermentation medium is multi-factorial. Although the chemical and physical constitution of the cell membrane plays a vital role in this context, biotin is the dominant factor regulating the permeability barrier across the membrane, especially when added in minor quantities. The suitability graph of biotin varies from strain to strain. For the fluent excretion of *glu*, however, it never exceeds a sub-optimal level. The crux is that the amount of *glu* in the medium is directly related not to the biosynthesis but to its release from the cell facilitated by biotin.

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