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Mutational Selection and Characterization of a Lysine-Producing Strain of *Corynebacteriumglutamicum*

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Abstract: ***Bacterial strains of Corynebacteriumglutamicum isolated from various natural environments were initially selected for their capacity to accumulate glutamic acid and then subjected to EMS chemical mutagenesis. Strains were then selected for amino acid auxotrophies and their capacities to produce other amino acids (notably lysine) were examined. A strain initially isolated from bird droppings and having a double auxotrophy for threonine and methionine was thus selected and retained for characterization as to its capacity to produce significant amounts of lysine. This strain grew at rates approximately half that of the parent strain but accumulated lysine as a primary metabolite throughout growth. Final concentrations of approximately 50 g/L lysine could be produced on glucose medium and this fermentation limit was shown to be directly related to the end concentration of lysine. Glucose was shown to be the best carbon source though sucrose showed similar capacity albeit with slightly diminished rates of sugar consumption and lysine accumulation. Acetate gave only relatively poor fermentation performance. Bearing in mind that only a single generation of mutational selection was necessary to attain this performance, the strain shows interesting potential for further development.***

Keywords: *Corynebacteriumglutamicum*, lysine production strain, mutational selection, animal feed

1. Introduction

Food security remains the primary goal of Third World countries in which one of the major preoccupations is the qualitative and quantitative requirement for upgrading food supply in face of an expanding population. In light of the agricultural conversion efficiencies, it would appear logical to favourplant protein production rather than animal production, but it must be remembered that such vegetable matter has a lower nutritional quality, linked to the low levels of certain essential amino acids. Recent market estimations (1) indicate that the global demand for lysine was 1,697 Ktons in 2011 but is expected to reach 2,518 Ktons by 2018. This increase is driven by an increasing demand for meat in developing countries, with the animal feed market accounting for more than 90% of demand. In North Africa, the predominant food base is cereals and the capacity to use essential amino acid supplements either as a direct nutritional input or as a feed component for poultry is important, as has been used elsewhere to improve cattle production. Since these essential amino acids cannot be synthesized by the human body it is therefore necessary to find an efficient manner to produce such dietary compliments. One possibility that exists is to exploit bacterial capacity to produce specific amino acids via large scale fermentation of readily available carbon feedstocks (2). If this can be achieved using GRAS-rated microorganisms such as *Corynebacteriumglutamicum*, widely used industrially for glutamate and lysine production, the bacterial biomass can also be exploited for animal fodder use. However, this group of bacteria does not naturally produce amino acids other than glutamate and mutational studies are required to favor the large scale synthesis of other amino acids. If this has been achieved to high levels for lysine, other amino acids have also been shown to be realistic targets. Combined genetic mutational studies (3) and efficient screening techniques have been used hand in hand with optimized fermentation conditions developed over many years to achieve production levels significantly higher than 100 g/L. Many of these industrially developed strains are fully protected by patent literature but wild-type strains are abundant in the environment and can be good sources of potential future development to meet specific production constraints. The sequencing of the *C. glutamicum*genome (4) has provided a sound base for metabolic engineering (*5,6,7)* though consumer opinion still plays a role in determining whether such modern techniques are used or more classical mutational approaches employed.

The objective of this study was to examine the ease with which such coryneform bacteria could be isolated from different sources locally by screening for natural glutamic acid synthesizing strains, and then to further examine whether these strains could be the source of possible production strains for other more essential amino acids. This was achieved by a classical chemical mutagenesis step and screening for amino acid auxotrophies. A number of modified strains were achieved producing different cocktails of amino acids, notably for lysine, methionine, threonine and the branched chain amino acids (valine, leucine and isoleucine).

2. Materials and Methods

2.1 Isolation of Wild Type Strains and Screening Procedures

Four different biological samples were chosen as source material to screen for the presence of coryneform bacteria

able to produce glutamic acid naturally. These samples were bird droppings collected directly from the nest, soil samples, raw milk collected at source and spinach leaves obtained at a local market. In each case 20g of the sample material was suspended in 100ml of physiological saline solution (composition en0.9% NaCl) and twice vortexed to achieve good distribution, prior to diluting through a serial dilution into the same saline solution to 10^{-5} concentrations. These dilutions (0.1 ml) were plated onto TSA agar medium (see below) for bacterial isolation.

2.2 Growth media and culture conditions

Plating medium was TSA medium (tryptic casein (15 g/L) soybean papain (5g/L), NaCl (5 g/L) with pH adjusted to 7.3 and solidified with agar (15 g/L). This medium was supplemented with amphotericin B (anti-fungal) at concentrations of 50 µg/ml and nalidixic acid (antibacterial inhibiting gram negative bacteria) at 10 µg/ml to favour the selective recovery of Gram positive strains. The plates were incubated at 30°C for 48h before counting and cloning of the recovered bacteria. Growth was later examined on a number of alternative media: Glutamate production capacity was examined in two different liquid media(see table1) differing in the nitrogen source (M1 with $(NH_4)_2SO_4$ and M2 with urea) to check for urease activity. Growth was in shake flask cultures (120 rpm at 30°C) and samples were taken for chemical analysis of glutamate production after 72h.

2.3 Bacteriological Analysis

The selection of the bacteria isolated on the TSA medium was via the classical techniques described by Abe *et al* (8), based on morphological, physiological and biochemical characteristics. The capacity to produce amino acids, notably glutamic acid was investigated on various media (see above). Strains were also examined for their requirement for biotin since this is a characteristic trait of coryne form bacteria. The physiological and biochemical characteristics of isolated strains were examined using challenges for growth and fermentation on different carbohydrates and other carbon substrates, while respiratory capacity and growth response to temperature and pH profiles was also tested. The morphological characteristics were obtained via direct observation of colony form and colour and via microscopic examination of Gram staining and sporulation capacity.

2.4 Analytical Procedures

Glutamate synthesis was visualized from samples takenfrom spent growth media (see above) after biomass removal by centrifugation (10 min at 5000rpm at room temperature) and the capacity to produce glutamate was qualitatively assessed using TLC techniques after running standard amino acid samples on control plates at 5 g/L. Samples were spotted onto commercially available silica TLC plates and eluted with aqueous acetic acid.After drying the plates, amino acid spots were visualized using ninhydrin spray and compared to the control spot positions. This same technique was also used to identify the amino acids produced in mutated strains selected for amino acid auxotrophies (see below). Lysine concentrations were analyzed by the method ofChinard (9).

2.5 Mutational Procedures

After selecting the most promising strain based on coherence with existing literature characteristics for *C. glutamicum*this strain was mutated via exposure to EMS (ethyl methane sulphonate, 0.05 M) followed by washing and plating out to select strains able to naturally produce other amino acids. Colonies were recovered and replica plated onto selective media to test for amino acid auxotrophy (minimal media complimented with various amino acids: individual or in mixtures). Strains showing amino acid requirements were recovered and tested for their capacity to produce different amino acids in minimal media containing the relevant amino acids at 100 µg/L and the supernatant, after 72hours incubation was tested using the TLC analysis to check for each strain's capacity to produce amino acids.

3. Results

3.1 Selection of strains

A first selection was made based on morphological characters of colonies established by Abe *et al* (1967), in which 289 colonies were tested by differential gram staining and the characteristic form of the coryneform bacteria, notably their tendency to form Chinese letters (10). This preliminary microscope screening enabled 20 isolates to be preselected (see table 2).

The TLC analysis carried out on culture supernatants after fermentation showed that in the M1 medium, all the 20 strains produced a variety of amino acids though predominantly glutamic acid. However in M2 urea media, strains S1T, S2T, S3T, S1E, S2E, S1L, S3L and S6L did not grow or produce amino acids presumably because they do not possess urease activity. All strains were shown to have a requirement for biotin and grew as grey/black colonies on tellurite medium as would be expected for coryne form bacteria. None of the strains produced spores and all were aerobic or facultativelyanerobic. Growth was possible for a range of temperatures being optimal at 30-35°C and only poor at 20°C and not possible at 40°C. Neutral pH was best for growth with no growth observed at either pH4 or pH10.

Among the strains isolated, 14 lacked the decarboxylation enzymes capable of degrading amino acids confirming the theory of Kinoshita (10) that many amino acid producing bacteria have only relatively low capacity to degrade amino acids. Only those strains isolated from milk possessed these catabolic amino acid degrading enzymes. Further attribution was made based upon the classification procedures (11) involving growth responses on glucose, urea, sucrose and mannitol. Natural capacity to produce amino acids was best on mannitol negative strains. These strains showed variable capacity to produce small amounts of a number of amino acids (glutamic acid, branched chain amino acids and alanine) though no detectable production of lysine was observed.

Based on growth capacity and natural amino acid production profile a bird dropping strain was selected for further study. This strain, whose taxonomic identity was confirmed as belonging to the *C. glutamicum* taxa by RNA 16S sequence

analysis, was urease and sucrose positive but could not develop on mannitol. The amino acid production profile was interesting as it showed a capacity to naturally accumulate branched chain amino acids (isoleucine and valine) as well as glutamic acid and traces of alanine.

3.2 Mutation

EMS mutation was used to provoke random mutations within the genome and a screening process to detect strains in which additional amino acids were produced was examined. In the initial mutation challenge a large number of undefined mutations were obtained able to form colonies on complex agar medium. It was observed as seen by others, a wide variety of colony pigmentations varying from white to orange and pink. This carotenoid variability is indeed exploited industrially (12). Approximately 4% of these colonies were shown to be auxotrophic for one or more amino acids. When examined for their capacity to produce lysine (or other essential amino acids) 70 strains were seen to accumulate amino acids in mineral medium when growth was restored by amino acid supplementation. Strains with double auxotrophy for threonine and methionine specifically accumulated lysine. Four strains were further examined and shown to have essentially the same levels of lysine production and growth. One of these strains was therefore selected for more detailed physiological characterization.

3.3 Physiological Analysis of Lysine Accumulation

The growth characteristics of the selected mutant strain were compared with the parent strain for growth rate sugar consumption and amino acid production using a glucose minimal medium supplemented with threonine and methionine to overcome the nutritional auxotrophies.The exponential growth rate of the mutant strain was approximately 50% of that measured for the parent strain ($\mu = 0.23\ h^{-1}$ for the mutant strain and $0.52\ h^{-1}$ for the parent strain) while sugar consumption was similar in the mutant strain ($4.3\ mmol.g^{-1}.h^{-1}$ in the parent strain and $4.6\ mmol.g^{-1}.h^{-1}$ in the mutant strain).Substrate turnover was therefore slightly higher in the mutant strain possibly due to the presence of additional substrates (threonine and methionine) to compensate auxotrophies. The presence of these amino acids would effectively diminish oxaloacetate requirement for anabolic biomass synthesis by approximately 25% and $NADPH_2$ requirements by 13% for biomass synthesis and thereby favor lysine synthesis. While the parent strain produced only trace amounts of amino acids under normal exponential growth (predominantly glutamic acid and no lysine) the mutant strain produced $14\ g.l^{-1}$ of lysine after 7 hours cultivation on glucose (Figure 1) and minor quantities of other amino acids (alanine and valine). This strain did not produce any detectable amounts of glutamic acid. The accumulation of lysine in the culture medium was directly coupled to the growth of the bacteria with a primary metabolite production profile. When glucose was replaced with sucrose the mutant strain produced considerably less lysine despite a similar rate of growth, probably linked to the fact that sucrose consumption was slower than that observed for glucose. Similarly lysine production was weak on acetate ($6.5\ g.l^{-1}$) as would be expected from the poor energetic balance associated with acetate consumption (13). Thus, despite the abundant availability of acetate as a potential feedstock in North Africa, this feedstock is not economically favorable for widespread fermentation of lysine. Using ammonium acetate as N source enabled lysine to be produced at virtually the same final concentration as with inorganic ammonium salts but no gain in yield or rates of production were observed.

One of the potentially limiting factors of lysine production is the inhibition by accumulation in the medium. To examine the natural potential of the mutant strain to accumulate lysine a series of cultures were inoculated with various initial concentrations of lysine (10, 20, 30, 40 and 50 g/L). Glucose concentration was increased to enable the fermentation to proceed to higher levels of lysine accumulation. In all cases lysine production halted when a final concentration of approximately 50 g/L was attained (Table 3). Indeed in the fermentation initiated with this amount of initial lysine, no further production occurred and sugar consumption was extremely low. This suggests that this value of 50 g/L is the maximal threshold concentration which can be attained using this strain and to go beyond this value will require additional evolution of the strain. Further investigations should also examine whether this strain can use low-cost substrates such as agronomic process by-products (14,15).

4. Discussion

Screening coupled to mutational selection procedures has enabled a promising strain of *C. glutamicum*to be isolated which shows good potential for lysine production. Final concentrations are as yet relatively low compared to many developed industrial strains, but bearing in mind that this strain was selected after a single round of chemical mutation with no specific factors to favour amino acid overproduction, it can reasonably be anticipated that further selection would enable higher levels of lysine to be attained. Strains developed in Germany (16) show that amino acid export can be a major bottleneck for accumulating high concentrations of amino acids as intracellular accumulation of the amino acid ultimately counters much of the genetic selection to remove allosteric feedback control on the biosynthetic pathway. This is probably the next logical strategy to further develop this strain. Stoichiometric metabolic flux analysis can be examined to see how flux through central metabolism influences amino acid yields (17). When used here such an analysis shows that the lysine yields, based on experimentally determined rates of sugar consumption, lysine production and specific growth rate optimization, were close to the maximal levels which can be expected(figure 1 and 2). Indeed carbon recovery yields are close to 30% which is basically the yields predicted for the strain when growth rate and substrate consumption rates are used. Such calculations of stoichiometric flux analysis need always to fix certain variables: substrate uptake rate was assumed to be constant at all theoretical growth rates tested (value observed in that mutant strain) and the flux through pentose phosphate pathway was held constant at 66% of the available glucose-6P) which led to a requirement for alternative pathways of $NADPH_2$ generation to facilitate an optimized carbon flux which increased in proportion to the amount of lysine theoretically produced though not

necessary in the parent strain. This could be attained via transhydrogenase activity though *C. glutamicum* is frequently described as not possessing this activity, or by exchange mechanisms involving pyruvate/PEP and oxaloacetate/malate carboxylation exchange mechanisms in which malic enzyme could generate some $NADPH_2$ (18). The fact that the observed experimental yield is close to that predicted suggests that one of these mechanisms is indeed functional or that flux through the pentose phosphate pathway was considerably higher (effectively a flux of 100% of available glucose-6P would be necessary to meet the $NADPH_2$ demand which has never been observed in the multiple fluxomic studies using lysine-producing strains, suggesting a role for malic enzyme to attain the cofactor balance necessary for high lysine yields as observed here. If yields are acceptable they could be further improved by appropriate fed-batch fermentation strategies in which growth rate was further diminished assuming that sugar uptake can be maintained. However, further development is necessary to improve the final concentrations of lysine which are still relatively low compared to currently exploited industrial strains. They are however rather promising for a strain having undergone a single round of mutation. The strain is therefore a relatively efficient lysine producer whose biotechnological value could be further improved by additional mutational selection strategies to facilitate a non-OGM source of feed for improving poultry production. Future work with this strain will concentrate on mutational strategies to increase the final concentration of lysine which can be accumulated via screening for lysine tolerance, and when this has been achieved, an optimized fedbatch fermentation strategy to prolong the period of growth dissociated high productivity, thereby increasing the overall lysine yield. Such additional input should achieve production characteristics compatible with economic production of this essential ingredient for the animal feed market.

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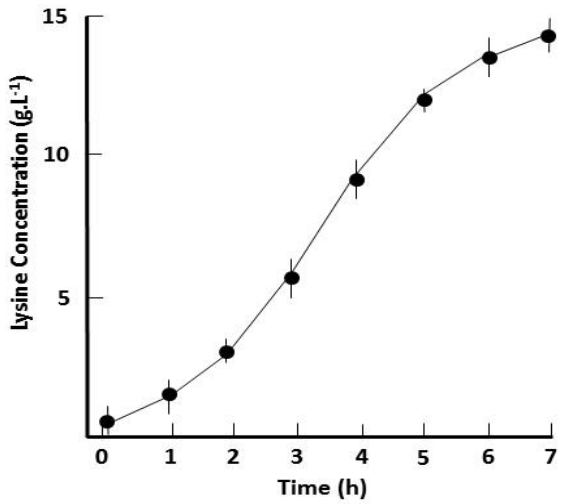

Figure 1: Lysine production profile of a mutant strain of *C. glutamicum* cultivated in shake-flask cultures.The parent strain did not produce any detectable amounts of lysine

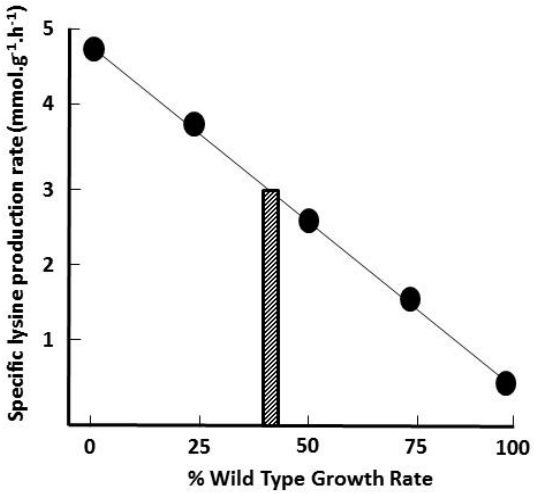

Figure 2: Estimated yields of lysine from stoichiometric flux balancing approach using known metabolite requirements for growth of *C. glutamicum* and the experimental rates of sugar consumption observed in the mutant strain. Flux through the pentose phosphate pathway was fixed at 65% of available glucose-6P and a malic enzyme shunt was allowed to meet the $NADPH_2$ requirement. The bar marker shows the experimental yields obtained with the mutant strain positioned at its observed rate of exponential growth relative to the parent strain.

Table 1: Composition of liquid medium (M1 and M2), andsolidminimaland complex medium usedforcolonyisolation. Concentrations are given as g/L.

	M1	*M2*	*Minimal medium*	*Complex medium*
Glucose	50	50	1.0	100
$(NH_4)_2SO_4$	20		1.0	40
Urea		8.0		
Peptone	2.0	2.0		
Meat Extract	2.0	2.0		
Yeast Extract				1.0
K_2HPO_4	1.0	1.0	7.0	3.0
KH_2PO_4			2.0	1.0
$MgSO_4,7H_2O$	0.5	0.5	0.1	0.4
$FeSO_4$			5.0	5.0
$MnSO_4$			5.0	5.0
$CaCO_3$	30			50
Na-Citrate			0.4	
Thiamin				2.10^{-4}
Biotin				3.10^{-4}
Agar			25	20
pH	7.2	7.2	7.5	7.5

Table 2: Distribution of isolates from natural source material

Source material	*Number of samples*	*Total number of colonies*	*Number of gram+ colonies*	*Isolated coryneform colonies*
Birddroppings(E)	1	344	92	4
Soil (T)	1	261	65	7
Milk (L)	1	154	87	6
Spinach(P)	1	128	45	3

Table 3: Consequences of lysine supplementation on the capacity to accumulate lysine.

Initial Lysine Concentration (g/L)	Additional Lysine Produced (g/L)
10	40.1
20	29.7
30	**19.3**
40	9.2
50	0