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Fed-batch production of L-phenylalanine from glycerol and ammonia with recombinant *Escherichia coli*

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ABSTRACT

Glycerol was used as carbon source for L-phenylalanine production with recombinant *Escherichia coli*. In contrast to glucose, no consumption of the precursor phosphoenolpyruvate (PEP) is necessary for glycerol uptake. Additional lactic acid feeding was necessary for growth because the genes encoding the PEP consuming pyruvate kinase isoenzymes have been deleted. Thus a fed-batch process was developed with feeding of lactic acid and glycerol for biomass formation followed by feeding of glycerol and ammonia for L-phenylalanine production. Unfortunately, plasmid instability was observed in the first process. Plasmid stability could be successfully assured by replacing an ampicillin resistance gene by a kanamycin resistance gene cassette. The resulting maximum L-phenylalanine concentration of 13.4 g L^{-1} was improved by 26% and biomass specific productivity (22 $mg_{L\text{-}phe}$ g_{CDW}^{-1} h^{-1}) was raised by 69%. The final L-phenylalanine concentration of 13.4 g L^{-1} was thus improved by a factor of 2.4 compared to earlier reports.

1. Introduction

L-Phenylalanine is one of the commercially most important aromatic amino acids [1–4]. The main relevance for the industrial synthesis of L-phenylalanine is based on its role as building block for the artificial sweetener aspartame [2]. Furthermore, L-phenylalanine is used as additive in the food and feed industry [2] as well as for pharmaceutically active compounds like HIV protease inhibitor, anti-inflammatory drugs [4], and catecholamines [5].

High L-phenylalanine concentrations of 38 g L^{-1} [6], 46 g L^{-1} [7] and 50 g L^{-1} [8] can be achieved with metabolically designed *Escherichia coli* (*E. coli*) strains with glucose as carbon source. A rational approach for metabolic engineering of *E. coli* for enhanced L-phenylalanine production was applied [6; reviewed in 2]. First, an *E. coli* strain was used (F4, a derivative of wild type strain W3110) which had been engineered to carry a precise chromosomal deletion of the gene cluster *pheA-aroF-tyrA*. Deletion of *tyrA* was necessary to avoid the formation of L-tyrosine. Then, the gene *pheA** encoding a feedback resistant enzyme PheA (chorismatemutase/prephenatedehydratase) and the wild type genes, *aroF* (tyrosine-sensitive DAHP synthase), *aroB* (dehydroquinate synthase), and *aroL* (shikimate kinase) were combined to an artificial operon under the control of a *lacI*/P_{tac} expression system (pF81) which was equipped with an ampicillin resistance gene (*bla*) (in the following specified as $pF81_{amp}$). The combination of these genes led to the minimization of intermediates of the aromatic biosynthesis pathway in *E. coli* (e.g. 3-deoxy-D-arabino-heptulosonate-7-phosphate, shikimate and 3-dehydroshikimate). L-Phenylalanine concentrations of up to 38 g L^{-1} were thus achieved in a fed-batch process using glucose as carbon source [6].

In recent years, alternative carbon sources like glycerol have been studied for microbial L-phenylalanine production [1,5,9–11]. Glycerol is an inevitable by-product of biodiesel production [12]. For each kilogram of biodiesel approximately 75–100 g of crude glycerol are produced [12,13]. Through the exponential growth of biodiesel production from vegetable oils and animal fats in the last decade [12,13] the market price of crude glycerol decreased considerably [12]. With a price of US $ 0.13–0.24 per kilogram [14], glycerol has become an attractive alternative carbon source compared to sugars like glucose.

Glycerol as carbon source also provides application-oriented advantages. For glycerol the degree of reduction per carbon (κ) is significantly higher ($C_3H_8O_3$: $\kappa = 4.67$) than that of glucose ($C_6H_{12}O_6$: $\kappa = 4$) [15]. Glycerol uptake in *E. coli* occurs either by passive diffusion or through protein-assisted facilitated diffusion [16,17]. Compared to the uptake of glucose by the phosphotransferase system (PTS), no phosphoenolpyruvate (PEP) is necessary for glycerol uptake. Therefore pure stoichiometric carbon balancing without consideration of energy metabolism reveals a carbon recovery of 90% for glycerol, whereas PTS using glucose conversion

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can only achieve 45% [11]. For energetic balancing one negative aspect of glycerol metabolism is its first phosphorylation step to glycerol-3-phosphate and as a consequence the necessary ATP regeneration. This point is partly compensated by ubiquinol generation during DHAP synthesis. Comparable to ATP loss in glycerol metabolism, ATP loss is obtained on glucose during fructose-1,6-bisphosphate synthesis, which is produced heading for PEP generation. As two molecules of PEP are necessary for the aromatic biosynthesis pathways, PEP-saving could be an interesting feature and glycerol could be an interesting carbon source for microbial PEP-consuming reactions [18].

Reports on microbial L-phenylalanine production from glycerol as carbon source have mainly highlighted single enzyme variations like alteration of phenylalanine dehydrogenase in combination with amino acid exporter and glycerol transport facilitator [10]. Thereby results focus on comparing different recombinant *E. coli*. The product concentrations are rather low if simple batch processes are applied: 0.4 g L^{-1} within 240 h [10] up to 5.6 g L^{-1} within 70 h [5].

This work deals with the design of a controlled fed-batch process for L-phenylalanine-production with glycerol as carbon source making use of genetically engineered *E. coli* [2]. An *E. coli* strain with enhanced expression of the shikimate pathway key enzymes and deletion of the pyruvate kinase genes was used to provide more phosphoenolpyruvate for the aromatic biosynthesis route. This attempt is similar to earlier approaches improving PEP availability if glucose is the carbon source for aromatics [18,21–23]. As glycerol catabolism via the lower glycolytic trunk is reduced by the loss of pyruvate kinase activity, these strains require a source of pyruvate to resume good growth in minimal media with glycerol (growth on glucose is less affected as the PTS provides pyruvate from PEP; [18,21–23]).

2. Material and methods

2.1. Microorganisms and plasmids

The bacterial strains used in this study were *E. coli* DH5α and *E. coli* FUS4.11$_{kan}$ (*E. coli* W3110 Δ*pheA-tyrA-aroF*, Δ*lacIZYA*::P_{tac}-*aroFBL*, Δ*pykA*::FRT, *pykF*::FRT-Kan-FRT [11]). *E. coli* FUS4.11$_{kan}$ was constructed from strain *E. coli* F4 [6,19] by the introduction of an artificial chromosomal P_{tac}-*aroF-aroB-aroL* operon, resulting in strain *E. coli* FUS4 [11]. Chromosomal deletion of the genes encoding the two pyruvate kinase isoenzymes by a recombineering method [20], yielding in a strain with *pykA* gene deleted and *pykF* disrupted by a kanamycin resistance cassette (strain FUS4.11$_{kan}$) [11].

The kanamycin-resistance cassette of strain *E. coli* FUS4.11$_{kan}$ was eliminated using the plasmid pCP20 as previously described [24] to yield strain FUS4.11.

The exchange of the ampicillin resistance gene (*bla*) of pF81$_{amp}$ [6] by a kanamycin resistance gene (*kan*) was conducted using the λ-Red recombineering technique as described by Sharan et al. [25]. In brief, the *kan* gene was PCR amplified from plasmid pJF-crtZ-FRT-kan-FRT [26] using the following primers:

5′ TTCTAAATACATTCAAATATGTATCCGCTCATGAGACAATAACCCTGATAGCTTGCAGTGGGCTTACATGG 3′ and
5′ TGAAGTTTTAAATCAATCTAAAGTATATATGAGTAAACTTGGTCTGACAGGAACTCCAGCATGAGATCCCCG 3′.

The PCR product was electroporated into λ-Red expressing *E. coli* DH5α cells carrying plasmid pF81$_{amp}$. The transformants were selected on LB-kanamycin-agar plates. Plasmid DNA of these kanamycin-resistant clones was then isolated and retransformed into strain DH5α with selection for kanamycin resistance. The resulting plasmid, pF81$_{kan}$, was verified by restriction analysis and DNA sequencing. Maps of plasmids pF81$_{amp}$ and pF81$_{kan}$ are shown in the supplementary material (Fig. S1).

Fig. 1 represents a schematic pathway illustration of metabolically engineered *E. coli* FUS4.11$_{kan}$ pF81$_{amp}$ and *E. coli* FUS4.11 pF81$_{kan}$. To underline differing genetic features during growth and production phase, metabolic pathways are illustrated before (Fig. 1A) and after induction of protein expression (Fig. 1B).

Transformation of *E. coli* strains by plasmids pF81$_{amp}$ or pF81$_{kan}$ was performed by heat-shock transformation of chemical competent cells. The resulting recombinant strains *E. coli* FUS4.11$_{kan}$ pF81$_{amp}$ and *E. coli* FUS4.11 pF81$_{kan}$ were stored on defined medium agar plates at 4 °C before inoculation of the preculture.

2.2. Growth medium and preculture

A defined minimal medium [27] was modified and used for all cultivations, which contained (g L^{-1}): 3.00 KH_2PO_4, 12.00 K_2HPO_4, 5.00 $(NH_4)_2SO_4$, 0.10 NaCl, 0.30 $MgSO_4{\cdot}7H_2O$, 0.015 $CaCl_2{\cdot}2H_2O$, 0.1125 $FeSO_4{\cdot}7H_2O$, 1.50 sodium citrate, 0.0075 thiamin, 0.075 L-phenylalanine, 0.075 L-tyrosine and antibiotics (0.10 ampicillin or 0.05 kanamycin). For precultures the pH was adjusted to pH 7.0 before sterilization of 1.04-times concentrated salt components (121 °C, 20 min) and addition of filtrated (0.2 μm) stock solutions of the temperature sensitive components ($MgSO_4{\cdot}7H_2O$: 300.00 g L^{-1}, $CaCl_2{\cdot}2H_2O$: 15.00 g L^{-1}, $FeSO_4{\cdot}7H_2O$: 22.50 g L^{-1}, sodium citrate: 300.00 g L^{-1}, thiamin: 7.50 g L^{-1}, L-phenylalanine: 10.00 g L^{-1}, L-tyrosine: 15.00 g L^{-1} (titrated with 10 M potassium hydroxide for solubility) and ampicillin or kanamycin: 50.00 g L^{-1}).

A trace-element solution adapted from literature [28] was used (1 mL L^{-1}) in the fed-batch processes containing (g L^{-1}): 11.20 $MnSO_4{\cdot}H_2O$, 10.00 $AlCl_3{\cdot}6H_2O$, 7.33 $CoCl_2{\cdot}6H_2O$, 2.00 $ZnSO_4{\cdot}7H_2O$, 2.00 $Na_2MoO_4{\cdot}2H_2O$, 1.00 $CuCl_2{\cdot}2H_2O$ and 0.50 H_3BO_3.

Precultures were obtained in two steps: First two single *E. coli* colonies were picked from the agar plate to inoculate 20 mL of defined minimal medium with 4.5 g L^{-1} glycerol and 2.5 g L^{-1} lactic acid in two 100 mL shake flask. After incubation in an orbital shaker for 24 h at 37 °C and 100 rpm the whole culture was used to inoculate ten 500 mL shake flasks. Each shake-flask contains 100 mL of defined medium with 4.5 g L^{-1} glycerol and 2.5 g L^{-1} lactic acid. Flasks were incubated for 20 h at 37 °C and 200 rpm. The complete preculture was used to inoculate the stirred tank reactor used for fed-batch fermentations.

2.3. Bioreactor and feeding solutions

Fed-batch cultivations were performed in a 42 L stainless steel stirred tank reactor with four equidistant baffles and three six-bladed Rushton impellers (Techfors, Infors HT, Bottmingen, Switzerland). The vessel was filled with a concentrated solution of the salts of the medium before *in situ* sterilization for 20 min at 121 °C. After cooling down (37 °C), microfiltrated stock solutions of the temperature sensitive medium components and separately sterilized glycerol (1000 g L^{-1}) and lactic acid (600 g L^{-1}) were pumped aseptically into the reactor to achieve initial substrate concentrations of 2.4 g L^{-1} glycerol and 1.6 g L^{-1} lactic acid at an initial volume of 15 L. Oxygen supply was realized by air-gassing (up to 1.75 vvm). Dissolved oxygen concentration (probe: 322756800, Mettler Toledo GmbH, Giessen, Germany), was kept above 40% air saturation by controlling stirrer speed and gassing rate. The pH (probe: 405-DPAS-SC-K8S/120, Mettler Toledo GmbH, Giessen, Germany) was controlled to pH 7.0 by addition of either H_3PO_4 (42%) or NH_4OH (25%).

Four different feed solutions were used successively for fed-batch operation. Feed solutions contained carbon sources (glycerol