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ORIGINAL PAPER

Production of L-phenylalanine from glycerol by a recombinant *Escherichia coli*

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Abstract The production of L-phenylalanine is conventionally carried out by fermentations that use glucose or sucrose as the carbon source. This work reports on the use of glycerol as an inexpensive and abundant sole carbon source for producing L-phenylalanine using the genetically modified bacterium *Escherichia coli* BL21(DE3). Fermentations were carried out at 37°C, pH 7.4, using a defined medium in a stirred tank bioreactor at various intensities of impeller agitation speeds (300–500 rpm corresponding to 0.97–1.62 m s^{-1} impeller tip speed) and aeration rates (2–8 L min^{-1}, or 1–4 vvm). This highly aerobic fermentation required a good supply of oxygen, but intense agitation (impeller tip speed ~1.62 m s^{-1}) reduced the biomass and L-phenylalanine productivity, possibly because of shear sensitivity of the recombinant bacterium. Production of L-phenylalanine was apparently strongly associated with growth. Under the best operating conditions (1.30 m s^{-1} impeller tip speed, 4 vvm aeration rate), the yield of L-phenylalanine on glycerol was 0.58 g g^{-1}, or more than twice the best yield attainable on sucrose (0.25 g g^{-1}). In the best case, the peak concentration of L-phenylalanine was 5.6 g L^{-1}, or comparable to values attained in batch fermentations that use glucose or sucrose. The use of glycerol for the commercial production of L-phenylalanine with *E. coli* BL21(DE3) has the potential to substantially reduce the cost of production compared to sucrose- and glucose-based fermentations.

Keywords *Escherichia coli* BL21(DE3) · Glycerol · L-Phenylalanine · Fermentation

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Introduction

This work reports on the production of the essential amino acid L-phenylalanine ($HO_2CCH(NH_2)CH_2C_6H_5$) by a recombinant *Escherichia coli* using glycerol as the inexpensive and readily available sole carbon source. The production of L-phenylalanine by various recombinant *E. coli* strains using glucose or sucrose as carbon sources has been discussed extensively in the literature [2, 8, 9, 14, 19, 26, 31, 32], but reports on the use of glycerol for this fermentation are nonexistent.

As a consequence of the increase in the production of biodiesel from triacylglycerol oils, there has been a massive increase in the availability of glycerol, or 1,2,3-propanetriol, a by-product of biodiesel manufacture [6, 10, 33]. There are only a few uses of glycerol and so its price continues to plummet. In the foreseeable future, glycerol is

likely to become an inexpensive carbon source for many industrial fermentations. The availability of glycerol is expected to continue to increase as the production of renewable biodiesel increases from its current low levels. Glycerol can be used as a carbon source by some industrial microorganisms. Compared to sugars such as glucose, glycerol has a higher degree of reduction [22, 34] and therefore yields more energy for each unit of carbon present within its structure. The use of glycerol as a fermentation substrate has the potential to improve the production economics of both biodiesel and other biotechnology-derived products [10]. A wide range of industrial chemicals can be produced by the fermentation of glycerol, as discussed by da Silva et al. [10]. Glycerol itself can be produced using microbial processes [30] and chemical synthesis, but these routes have been almost entirely superseded by low-cost glycerol from biodiesel processing.

L-Phenylalanine is used as a nutritional supplement and as a precursor for the production of various catecholamines and the artificial sweetener aspartame. L-Phenylalanine is mostly produced commercially by fermentation involving recombinant *E. coli* [20] and sucrose. The yield of this amino acid on sucrose is about 0.20–0.25 g g^{-1}, and the final concentration in the fermentation broth can be nearly 50 g L^{-1} [20]. Attaining such a high concentration inevitably requires a fed-batch fermentation process. Mixed carbon sources involving predominantly but not exclusively glycerol have been used to produce L-phenylalanine in highly aerobic fed-batch cultures of *Corynebacterium glutamicum* [24]. A final product concentration of nearly 23 g L^{-1} and a productivity of 0.32 g L^{-1} h^{-1} were reported in an extensively optimized fed-batch fermentation [24]. Optimization of the oxygen supply was claimed to alleviate the feedback inhibition of the fermentation by the product [24].

Use of glycerol as a fermentation substrate has particular advantages in anaerobic fermentations [11, 22, 33], and *E. coli* is capable of anaerobically metabolizing glycerol to reduced compounds [34]; however, amino acid fermentations are generally highly aerobic and quite sensitive to changes in the supply of dissolved oxygen [1, 12, 15, 24]. The concentration of dissolved oxygen is known to affect the expression of respiratory enzymes such as dehydrogenases and oxidases in some microorganisms [28], and these changes in turn influence the production of metabolites such as organic acids [16], biopolymers [29] and polysaccharides [17]. The supply of dissolved oxygen to a fermentation is strongly affected by the aeration rate and agitation speed of the impeller [4, 7]. In view of the importance of the oxygen supply, this work focused on the influence of the aeration and agitation conditions on the production of L-phenylalanine from glycerol using the novel genetically modified *Escherichia coli* BL21(DE3), an overproducer of L-phenylalanine [25].

Materials and methods

Microorganism

Escherichia coli BL21(DE3) [genotype: F^- *ompT* $hsdS_B$ (r_B^- m_B^-) *gal dcm* (DE3)] was the host strain (Invitrogen Corporation, Carlsbad, CA, USA) used to express the phenylalanine dehydrogenase gene of *Acinetobacter lwoffii*. The phenylalanine dehydrogenase gene was closed using pET-17b (Novagen; Merck KGaA, Darmstadt, Germany) as an expression vector. The plasmid contained an ampicillin resistance gene as the marker gene. This recombinant strain had been constructed at the Department of Biochemistry, Faculty of Science, Chulalongkorn University, Thailand [25], and was previously shown to overproduce L-phenylalanine on various sugars [23]. The expression of phenylalanine dehydrogenase by this strain was not affected by IPTG (isopropyl-β-D-thiogalactopyranoside) relative to the control [23, 25].

Growth medium and culture conditions

E. coli BL21(DE3) was maintained on Luria–Bertani (LB) agar slants containing 50×10^{-3} g L^{-1} of ampicillin. The pH of the medium was adjusted to 7.4 prior to autoclaving (121°C, 15 min). Slants were incubated at 37°C for 24 h and then stored at 4°C. Subculturing was carried out once every four weeks.

A defined medium was used in all fermentations and it contained (g L^{-1}): 10 glycerol, 50 $(NH_4)_2SO_4$, 0.81 $MgCl_2$, 2.43 KH_2PO_4, 2.43 K_2HPO_4, 0.085 yeast extract, 0.0085 thiamine–HCl, 0.002 $FeSO_4$, 0.002 $MnSO_4$, 0.05 $CaCl_2$, and 0.01 $ZnSO_4$. The $MgCl_2$ solution was sterilized separately. Prior to inoculation, the pH of the sterilized (121°C, 15 min) and cooled medium was adjusted to 7.4.

The seed culture was grown in 250 mL Erlenmeyer flasks containing the above-specified medium (50 mL) supplemented with 50×10^{-3} g L^{-1} of ampicillin. The flasks were incubated at 37°C on an orbital shaker at a rotation speed of 200 rpm for 16–18 h to attain an optical density of 0.6–0.8 measured at 600 nm. Inoculum volume was 5% (vol/vol) of the working volume (2 L) of the fermenter.

Bioreactor and fermentations

All experiments were carried out in a 3.3 L (2 L working volume) glass stirred tank reactor (BioFio III; New Brunswick Scientific, Edison, NJ, USA). The bioreactor vessel had a diameter of 13.8 cm and was fully baffled with four equidistant baffles. Agitation was provided by a six-bladed Rushton turbine impeller, 6.2 cm in diameter. Agitation speed and air flow rate were controlled at specified values.

The dissolved oxygen concentration was measured using a polarographic electrode (InPro 6800 Series O_2 Sensors, Mettler-Toledo, Switzerland).

Batch fermentations were carried out at 37°C. The pH was controlled at 7.4 by the automatic addition of 3 M NaOH. Foam was controlled by automatic addition of the sterile silicone antifoaming agent 1614 (catalog no. AF606211; Dow Corning Corporation, Midland, MI, USA). The effects of impeller agitation speed and aeration rate on L-phenylalanine and biomass production were investigated at the aeration rate values of 2.0, 4.0, 6.0 and 8.0 L min^{-1} and the agitation speeds of 200, 300, 400 and 500 rpm. The latter corresponded to impeller tip speed values of 0.65, 0.97, 1.30 and 1.62 m s^{-1}, respectively. All experiments were carried out in triplicate.

Analytical methods

The biomass concentration in the broth was calculated from the optical density measured at 600 nm (OD_{600}) and a calibration curve relating the dry cell weight (DCW) to OD_{600} (1 unit of OD_{600} was equivalent to 1.72 g DCW L^{-1}). A culture broth sample was centrifuged at 10,000*g* for 10 min. The supernatant was then filtered through a syringe filter (0.2 µm pore size). L-Phenylalanine, glycerol and ammonium concentrations were measured in the filtered supernatant, as explained below.

L-Phenylalanine in the culture supernatant was derivatized as follows: 50 µL of 1.5 M $NaHCO_3$ (pH 9.0) was added to a 110 µL aliquot of the supernatant. A 100 µL solution of dabsyl chloride (2 mg mL^{-1} in acetone) was then added. The mixture was vortexed and then heated at 70°C for 10 min. The solution was then dried under vacuum and the solids were resuspended in 200 µL of 70% ethanol. The resulting solution was centrifuged for 2 min at 14,000*g*, filtered through a syringe filter (0.2 µm pore size) [27] and analyzed by HPLC (SUPELCO, LC-DABS column, 15 cm × 4.6 mm ID, 3 µm particles) at room temperature. The mobile phase consisted of a 70:30 v/v mixture of a phase A (25 mM potassium dihydrogen phosphate, pH 6.8) and a phase B (acetonitrile and 2-propanol, 75:25 v/v). The mobile phase flow rate was 1.0 mL min^{-1}. The detection wavelength was 436 nm.

The concentration of glycerol in the culture supernatant was measured by a colorimetric method using periodate and acetylacetone reagents [21]. The spectrophotometric absorbance of the samples was measured at 410 nm. The absorbance was converted to a glycerol concentration using a calibration curve (1 unit of OD_{410} was equivalent to a glycerol concentration of 0.047 g L^{-1}).

The ammonium ion concentration was measured as specified by Jeffery et al. [18]. Briefly, a known excess of a standard solution of sodium hydroxide was added to a known volume of the culture supernatant. The ammonia in the resulting solution was boiled off. The excess of sodium hydroxide in the remaining solution was determined by titration with a standard HCl solution using methyl red as the indicator.

Results and discussion

A typical profile of L-phenylalanine fermentation is shown in Fig. 1. Biomass growth and production of the amino acid occurred on glycerol as the sole carbon source. The final biomass yield on glycerol ($Y_{X/S}$) was 0.56 g g^{-1}. The maximum value of the product yield on the carbon source ($Y_{P/S}$) was 0.48 g g^{-1} at 36 h. Because of rapid consumption of the dissolved oxygen during exponential growth and the inability of the oxygen supply to meet demand, the dissolved oxygen concentration declined to nearly zero, despite continued aeration at a relatively high 4 L min^{-1} (2 vvm) and agitation at an impeller speed of 300 rpm (Fig. 1). The dissolved oxygen concentration gradually recovered to ~100% of the air saturation value after 24 h due to a declining rate of oxygen consumption as a consequence of slowed growth resulting from a depletion of glycerol. L-Phenylalanine was produced only during rapid biomass growth (Fig. 1).

Biomass production and glycerol consumption profiles of the fermentations carried out at an aeration rate of 4 L min^{-1} and different impeller agitation speeds (200–500 rpm) are shown in Fig. 2. Both the biomass growth rate and the substrate consumption rate were influenced by the oxygen supply, which improved progressively with increased speed of agitation. The specific growth rates calculated using the data in Fig. 2 are shown in Fig. 3. Increasing the agitation speed to 400 rpm increased the specific

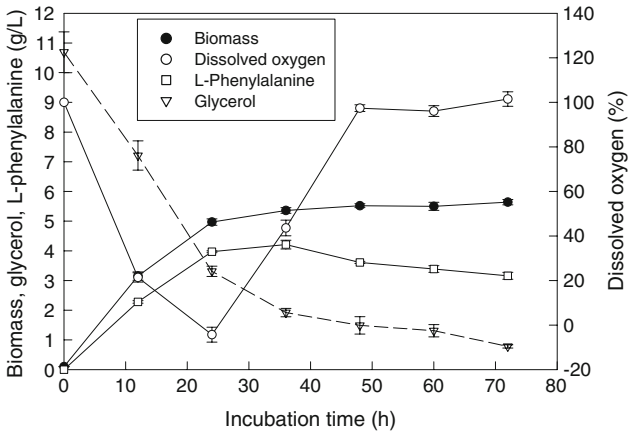

Fig. 1 A typical fermentation profile at an aeration rate of 4 L min^{-1} (2 vvm) and an impeller speed of 300 rpm (tip speed = 0.97 m s^{-1}). *Error bars* are based on three replicate fermentations

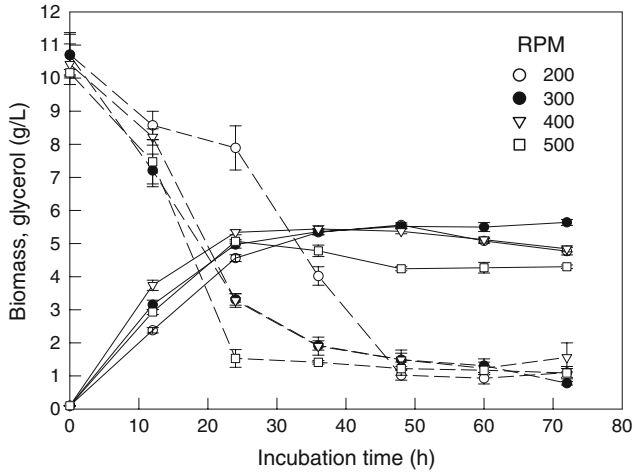

Fig. 2 Biomass growth (*solid lines*) and glycerol consumption (*dashed lines*) profiles during fermentation at an aeration rate of 4 L min^{-1} (2 vvm) and at various impeller speeds (rpm)

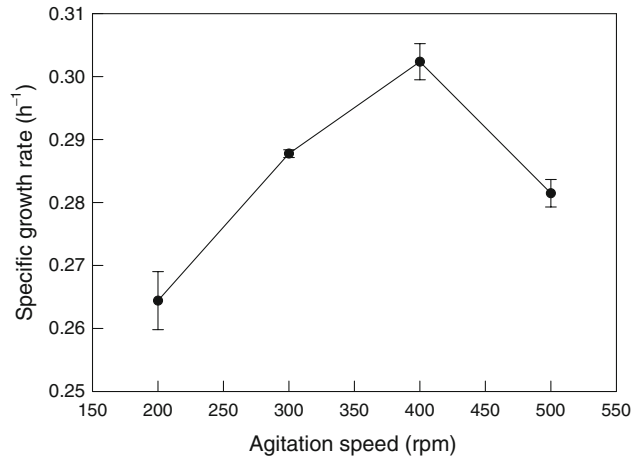

Fig. 3 Specific growth rate of the biomass at various impeller agitation speeds. All data were obtained at a fixed aeration rate value of 4 L min^{-1}

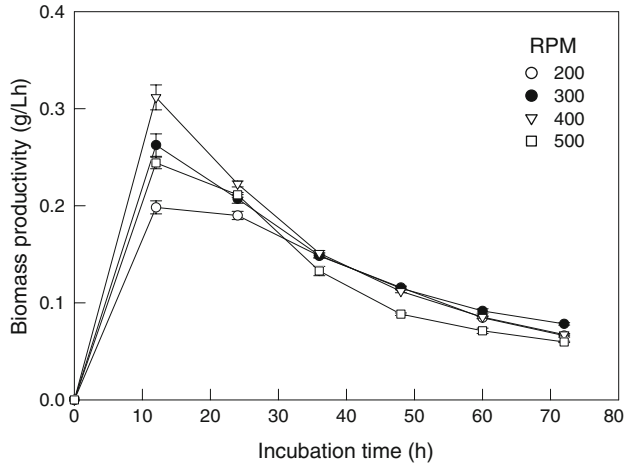

Fig. 4 Biomass productivity at various impeller agitation speeds (rpm) and a constant aeration rate of 4 L min^{-1}

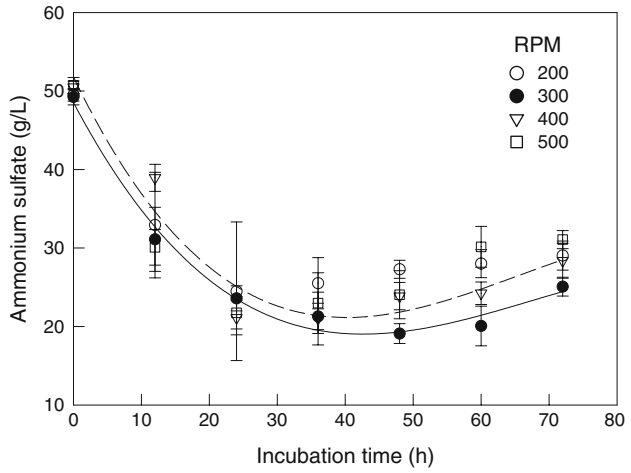

Fig. 5 Nitrogen (as ammonium sulfate) concentration profiles during fermentations at various impeller agitation rates (rpm). Lines are drawn only for the sets of data obtained at 300 rpm (*solid line*) and 400 rpm (*dashed line*). All data are for a constant aeration rate of 4 L min^{-1}. *Error bars* are based on three replicate fermentations

growth rate of the biomass (Fig. 3) because of improved oxygen supply, but growth rate declined with a further increase in agitation speed. This decline was attributed to a possible shear sensitivity of the recombinant *E. coli*. Many recombinant bacteria are known to be sensitive to excessive turbulence and high shear rates compared to wild-type strains [3, 5, 13]. The maximum observed specific growth rate was 0.302 h^{-1} (Fig. 3). Clearly, at an aeration rate of 4 L min^{-1}, an impeller agitation speed of 400 rpm (tip speed of 1.30 m s^{-1}) was best for obtaining rapid growth (Fig. 3). This impeller agitation speed also produced the highest biomass productivity (Fig. 4). The decline in biomass productivity after ~12 h (Fig. 4) was simply a consequence of a declining concentration of glycerol (Fig. 2), an inevitable characteristic of a batch fermentation. In all fermentations, nitrogen was in excess and the lowest measured nitrogen level, expressed as the ammonium sulfate concentration, never fell below 18 g L^{-1} (Fig. 5).

The measured oxygen concentration profiles (Fig. 6) confirmed that an increase in impeller agitation speed improved oxygen supply and reduced the length of time during which the fermentation was depleted in oxygen (Fig. 6). For agitation speeds of 200–400 rpm, during the first 20 h of rapid exponential growth, the agitation speed was insufficient to prevent oxygen depletion (Fig. 6), but increasing the agitation speed to >200 rpm reduced the length of the oxygen-depleted period (Fig. 6). At an agitation intensity of 500 rpm, the oxygen concentration never declined to less than 40% of the air saturation value (Fig. 6).

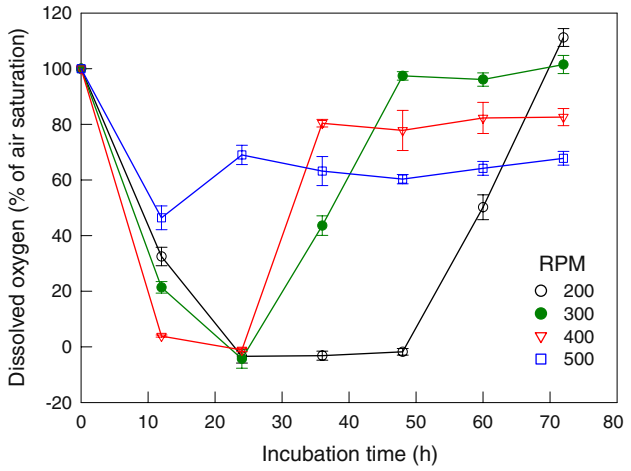

Fig. 6 Dissolved oxygen concentration profiles at an aeration rate of 4 L min^{-1} (2 vvm) and at various impeller speeds (rpm). *Error bars* are based on three replicate fermentations

Production of L-phenylalanine was evidently strongly associated with growth (Fig. 1), and so the conditions that produced the most rapid growth and biomass productivity also gave the highest final concentration and productivity of the amino acid (Fig. 7). At an agitation speed of 400 rpm, for example, the maximum concentration of the product was nearly 5 g L^{-1}, or nearly threefold greater than the value attained at the higher agitation speed of 500 rpm (Fig. 7a). Similarly, at 400 rpm, the maximum productivity of the amino acid was nearly 0.20 g L^{-1} h^{-1}, or nearly fourfold greater than the peak productivity at the agitation speed of 500 rpm (Fig. 7b). Although the dissolved oxygen concentration never declined to less than 40% of the air saturation value at an agitation speed of 500 rpm (Fig. 6), the presence of sufficient oxygen throughout the fermentation did not improve productivity relative to the values obtained at an agitation speed of 400 rpm. This fermentation clearly required a high rate of aeration but was adversely affected by excessively high turbulence. The high oxygen demand of this fermentation is partly linked to the stoichiometry of product formation: the synthesis of each mole of L-phenylalanine requires at least one mole of oxygen.

A high sensitivity to impeller agitation has been observed in other fermentations involving recombinant *E. coli*. For example, in a highly aerobic fermentation, the use of the low-shear method of bioreactor pressurization was found to be preferable for enhancing oxygen transfer in a recombinant *E. coli* culture compared to the use of increased impeller agitation speed [3].

In view of the high productivity and final concentration of biomass and L-phenylalanine at an agitation speed of 400 rpm, further experiments were conducted at this speed. In different experiments, the aeration rate was fixed at 2, 4, 6 and 8 L min^{-1}, corresponding to 1, 2, 3 and 4 vvm, respectively.

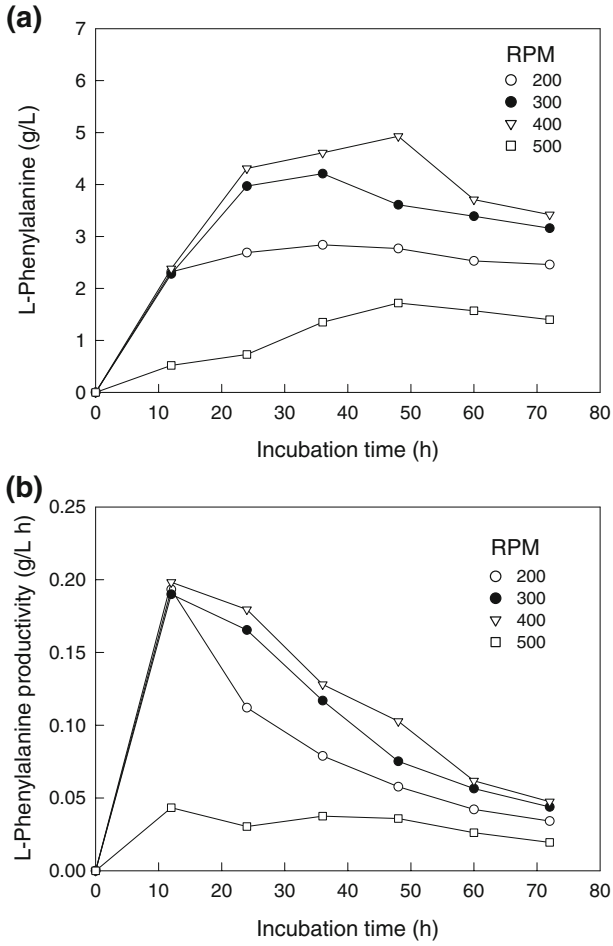

Fig. 7 L-Phenylalanine production profile (**a**) and productivity (**b**) at various impeller agitation speeds (rpm) and a constant aeration rate of 4 L min^{-1}

The effects of increasing the aeration rate (2–8 L min^{-1}) on the biomass growth and productivity profiles are shown in Fig. 8 for a constant agitation speed of 400 rpm. The highest values of the growth rate and biomass productivity occurred at the highest aeration rate of 8 L min^{-1} (Fig. 8). In contrast, at a low aeration value of 2 L min^{-1}, the biomass growth rate, the final concentration and the productivity were all distinctly low (Fig. 8). This further attested to the highly aerobic nature of this fermentation.

The L-phenylalanine concentration and productivity profiles for the fermentation depicted in Fig. 8 are shown in Fig. 9. L-Phenylalanine concentration and productivity were clearly quite low at the low aeration rate of 2 L min^{-1} (Fig. 9). Both the concentration and productivity of amino acid increased substantially with an increase in the aeration rate to 4 L min^{-1} (Fig. 9). A further increase in aeration rate did not significantly influence the final product concentration and productivity (Fig. 9). These results suggest that

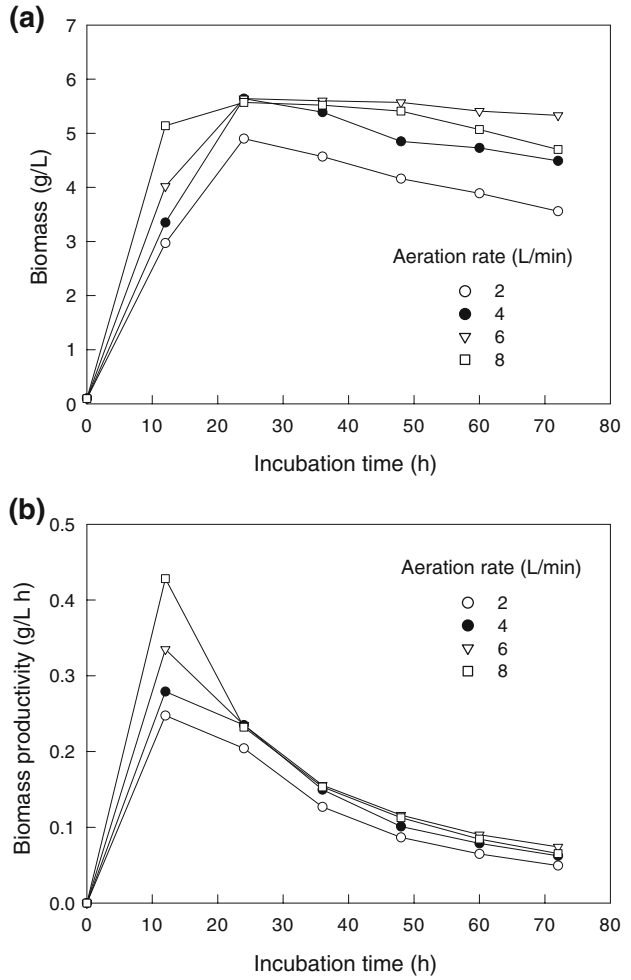

Fig. 8 Biomass growth profile (**a**) and productivity (**b**) at various aeration rates and at a constant agitation speed of 400 rpm (tip speed = 1.30 m s^{-1})

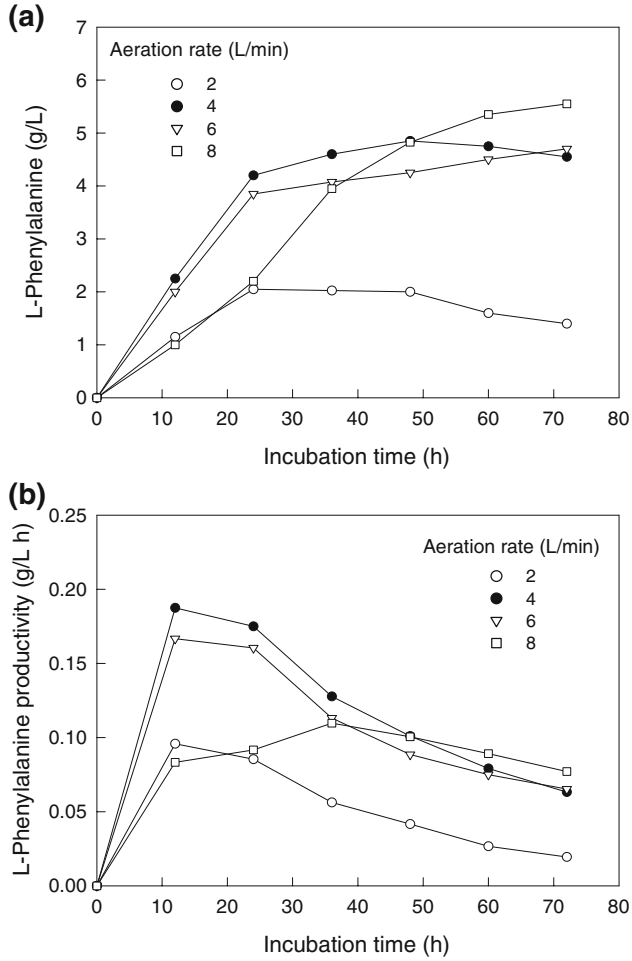

Fig. 9 L-Phenylalanine production profile (**a**) and productivity (**b**) at various aeration rates and at a constant impeller speed of 400 rpm (tip speed = 1.30 m s^{-1})

judicial selection of the aeration rate and impeller agitation speed is necessary to attain optimal L-phenylalanine production in this fermentation.

For recombinant *E. coli* strains grown on glucose, the L-phenylalanine yield and productivity of course depend on the strain being used, whether batch, fed-batch, or continuous fermentation is employed, and the inhibition of the fermentation by the product, among other factors [19]. Under the best conditions (400 rpm, 8 L min^{-1} air flow), the yield of product on substrate ($Y_{P/S}$) was an impressively high 0.58 g g^{-1} in our case. A combination of high yield and inexpensive substrate is required to minimize the cost of producing L-phenylalanine. In comparison with the yield obtained in this work, the highest reported yield for the commercial production of L-phenylalanine on sucrose is about 0.25 g g^{-1} [20], or less than 45% of the yield we obtained. In fed-batch fermentations with various recombinant *E. coli* strains, the highest reported product yield on glucose appears to be ~0.17 g g^{-1} [19], or only 29% of our yield. Generally low yields have been reported for L-phenylalanine on glucose in recombinant *E. coli* fermentations. For example, for two recombinant *E. coli* strains, the yields on glucose were 0.031 and 0.057 g g^{-1}, respectively [31]. A high product yield on glycerol is a reflection of a high expression level of L-phenylalanine dehydrogenase in the strain used and the fact that the three-carbon molecule of glycerol is easier to metabolize by glycolysis than a six-carbon sugar is. Furthermore, compared to glucose and sucrose, glycerol provides more energy per carbon in its structure.

In industrial processes, the final concentration of L-phenylalanine approaches 50 g L^{-1} [20], but only in fed-batch fermentations. In fed-batch fermentation involving recombinant *E. coli* growing on glucose, a final product concentration of 46 g L^{-1} has been reported [19]. Batch fermentations invariably attain a low final product concentration because only a small concentration of substrate can be added to the medium at the start to prevent reduced water activity and substrate inhibition of the fermentation.

For a given microbial strain and fermentation medium, the use of the fed-batch strategy can increase the final product concentration by tenfold or more compared to the batch fermentation. For example, a batch fermentation of *C. glutamicum* that started with 100 g L^{-1} molasses achieved a maximum L-phenylalanine concentration of only 9.3 g L^{-1}, but this could be increased to nearly 23 g L^{-1} in a highly optimized fed-batch operation [24]. In our fermentations, the initial concentration of glycerol was 10 g L^{-1}, and this led to a final product concentration of 5.6 g L^{-1}, quite comparable to the best values reported in the batch fermentations involving *C. glutamicum* [24]. In view of the exceptionally high product yield on glycerol, a fed-batch fermentation with our recombinant *E. coli* has the potential to raise the final product concentration to around 50 g L^{-1} through the cumulative feeding of approximately 100 g of glycerol per liter.

Concluding remarks

The genetically engineered *Escherichia coli* BL21(DE3) was successfully grown on glycerol in order to produce L-phenylalanine in a highly aerobic fermentation. The recombinant bacterium appeared to be shear sensitive, and this imposed an upper limit of around 1.3 m s^{-1} on the tip speed of the Rushton turbine impeller. In an optimally aerated and agitated batch fermentation, an exceptionally high L-phenylalanine yield of 0.58 g g^{-1} was attained on glycerol. The final concentration of the product was comparable to values that have been reported in other highly productive batch fermentations that use glucose or sucrose instead of glycerol. In conclusion, using the new recombinant *E. coli* BL21(DE3) and glycerol as the carbon source, a production process for L-phenylalanine can be developed that is likely to be competitive with the current fed-batch fermentation processes that are based on sucrose and glucose.

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