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Constitutive overexpression of *Pseudoalteromonas carrageenovora* arylsulfatase in *E. coli* fed-batch culture

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Abstract−The arylsulfatase gene (*astA*) from *Pseudoalteromonas carrageenovora* genome was subcloned into pHCE-IA vector, in which the hyper constitutive expression (HCE) promoter from the D-amino acid aminotransferase (D-AAT) gene of *Geobacillus toebii* was employed. When the constructed pHCE-AST was introduced into *E. coli*, the transformant showed the hydrolyzing activity for 4-methylumbelliferyl-sulfate and *p*-nitrophenyl-sulfate. When the cell was cultured on fermentor containing MaxyBroth-HD medium with 1% glycerol, the enzyme activity reached 12.8 unit/mL. On MaxyBroth-HD medium with 2% glycerol, the cell showed 2.7-fold higher arylsulfatase expression than that with 1% glycerol. The fed-batch cultivation employing MaxyBroth-HD medium and additional feeding of glycerol gave about 143 unit/mL of arylsulfatase at 20 h, which corresponds to 4-fold higher enzyme activity than that of 2% glycerol batch culture. Most of arylsulfatase activity in fed-batch culture was produced in the extracellular medium, whereas the activity in the batch cultures was localized in the periplasmic cell space.

Key words: Arylsulfatase, Constitutive Expression, *Pseudoalteromonas carrageenovora*, *E. coli*

INTRODUCTION

Agar is a structural polysaccharide found in the cell walls of red algae such as *Gelidium* and *Gracilaria* and is generally considered to be a mixture of about 70% agarose and 30% agaropectin [1]. Agarose consists of a repeating unit of alternating 1,4 linked 3,6-anhydro-α-L-galactosyl residues and 1,3 linked α-D-galactosyl residues, which can be named as agarobiose [2-4]. The structure of agaropectin is like agarose but additionally contains sulfate groups and carboxyl groups [2,5]. At the present time, agarose and agaropectin in agar were isolated by organic solvents [6]. A weak point of these methods is that they are solvent- and labor-consuming. Therefore, this needs added-equipment of organic solvent recovery process and waste water treatment. Agarose production process using an enzymatic process was required.

Enzymatic hydrolysis of sulfate groups in agaropectin or agar simplifies the process of agarose preparation. To produce a value-added agarose from agar, the prerequisite is the supply of a large amount of arylsulfatase [7]. Incorporation of sulfate groups in agarose residues causes weakened gel strength due to avoidance of cross-linked structure during gelation [8]. Arylsulfatase (aryl-sulfate sulfohydrolase, EC3.1.6.1) is a class of glycosulfohydrolase involved with desulfation of sulfated polysaccharides, which catalyzes to hydrolyze arylsulfate ester bond, producing the aryl compounds and inorganic sulfate. Several studies have reported on various arylsulfatases isolated from bacteria such as *Klebsiella pneumoniae* [9], *Salmonella typhimurium* [10,11], *Serratia marcescens* [12], and *Pseudomonas aeruginosa* [13]. *P. carrageenovora* arylsulfatase is considerably smaller (36 kDa) than the other bacterial sulfatases, and does not display sequence similarity to any of the sulfatases [14]. It shows a broad optimum in its activity at pH 8.5. The enzyme appears to be located in the periplasm, and is probably involved in desulfation of sulfated polysaccharides [15]. The gene encoding arylsulfatase of *P. carrageenovora* was cloned and the nucleotide sequence was reported [15].

This report describes the cloning and overexpression of the arylsulfatase of *P. carrageenovora* in *E. coli*, and the use of the recombinant enzyme in the production of a desulfated agarose. Therefore, in this study, the culture conditions and process parameters like medium composition were systematically examined, and high cell-density fed-batch fermentation employing optimal glycerol feeding strategies was conducted. To our best knowledge, this is the first report about functional constitutive overexpression of *P. carrageenovora* arylsulfatase by *E. coli* fed-batch culture.

MATERIALS AND METHODS

1. Bacterial Strain and Plasmids

E. coli BL21 [(*DE3*), *F-*, *ompT*, *rB-*, *mB-*] and DH5α [*recA1 endA1 gyrA96 thi1 hsdR17 supE44 relA1 lacZ* ΔM15] were used as host strains. Genomic DNA of *P. carrageenovora* ATCC 43555 was used for the source of *astA* gene. For the construction of *E. coli* constitutive expression plasmid, the pHCE-IA vector (3,865 bp, BioLeaders Co., Korea), which is used for the high-level production of recombinant protein [16], was used for the subcloning and constitutive expression of *astA* gene.

2. Culture Condition

P. carrageenovora and *E. coli* were grown on the ZoBell's 2216e medium (cultivating heterotrophic marine bacteria, Difco, USA)

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and Luria-Bertani (1% Bacto-tryptone, 0.5% Bacto-yeast extract, 0.5% NaCl) media, respectively. For the selection of *E. coli* transformants, LBAM medium (LB, 30 μg/mL ampicillin, 5-100 mM 4-methylumbelliferyl-sulfate) was used. *E. coli* high expression media, MaxyBroth-FC and HD in which various essential ingredients for the heterologous protein expression in the recombinant *E. coli* cell, such like organic nitrogen sources and inorganic salts, etc., were contained, were purchased from Bioprogen Co. (Taejon, Korea).

3. Construction and Transformation of Arylsulfatase Expression Plasmids

The *astA* gene of 0.9 kb was amplified by PCR, in which genomic DNA (*P. carrageenovora*) was used as a template [15]. For genomic DNA extraction, genomic DNA isolation kits were used and purchased from Nucleogen Inc (Ansan, Korea). For the in-frame connection with MCS site of pHCE-IA, two PCR primers were designed to have proper restriction enzyme sites at the 5′ and 3′ ends, and synthesized as the following sequences: 5′-GTACTCATATGATCGAGGTCATT-3′ and 5′-TGCCGGTCGACTTAACGTATCTT-3′. After amplification, the *astA* gene was treated with *Bam*HI and *Hin*dIII and then subcloned into the pHCE-IA vector, resulting in pHCE-AST plasmid (4,852 bp).

4. Fermentation

Flask cultures were carried out in 250 mL flask containing Maxy Broth-FC medium supplemented with 50 mg/mL ampicillin in a shaken incubator at 37 °C and 200 rpm. The fermentor was run at 37 °C and 400-700 rpm. An automatic regulator maintained the pH, agitation, and aeration. Batch and fed-batch cultures were carried out in a 5 L jar fermentor (KoBiotech Co., Korea). Seed culture was prepared in a 500 mL flask containing 50 mL Maxybroth-FC medium at 37 °C and transferred into a fermentor containing 1 L Maxybroth-HD medium plus 1% and 2% glycerol. The culture of pH was controlled at 7.0, except for the periods of pH rise due to glycerol depletion, by addition of 5 N NaOH. Fed-batch cultivation employing MaxyBroth-HD medium and additional feeding of 2% glycerol was performed.

5. Analyses

The *E. coli* cell growth was monitored by measuring the optical density at 600 nm. The *E. coli* culture broth was centrifuged, and then the supernatant was used for the measurement of extracellular arylsulfatase activity. The soluble and insoluble fractions of *E. coli* were obtained by centrifugation (10,000 rpm, 10 min) of ultrasonic lysates. The assay of arylsulfatase activity was described in our previous reports [17-19]. 1.0 unit of arylsulfatase activity was defined as the enzyme amount producing 1 μM *p*-nitrophenol from *p*NPS per minute at 40 °C. The protein concentrations of pooled enzyme solution from each purification step were determined according to the method of Lowry (Protein detection kit, Sigma Co., St Louis, USA) with bovine serum albumin (BSA) as a standard.

RESULTS AND DISCUSSION

1. Construction of Arylsulfatase Expression Plasmids

The open reading frame (ORF) with its own signal sequence (25 amino acids, MQKISIIFNLFLSL GCLAFTFNGSA) of *astA* gene of *P. carrageenovora* was amplified and then connected with the downstream of constitutive HCE promoter, which was derived from the D-amino acid aminotransferase (D-AAT) gene of *Geobacillus toebii*, in pHCE-IA vector [16]. The nucleotide sequence in the junction region between HCE promoter and *astA* gene was analyzed and agreed well with the expected sequence (data not shown). The resulting plasmid was named as the pHCE-AST plasmid (4.8 kb). By the HCE promoter system, the total amount of human tumor necrosis factor-α was approximately two times higher than that produced by the pET system under the same culture conditions [16].

2. Overexpression of Arylsulfatase in *E. coli*

When the transformed *E. coli* cells were grown on LB agar plate containing MUFS (4-methylumbelliferyl sulfate), only *E. coli* BL21 (DE3) harboring pHCE-AST showed an intense fluorescence at 360 nm due to the liberated 4-methylumbelliferone (data not shown). This result indicated that the arylsulfatase gene was successfully expressed in *E. coli*.

The cell growth and arylsulfatase activity of *E. coli* BL21 (DE3)/pHCE-AST in the flask cultures with MaxyBroth-FC medium and MaxyBroth-FC medium containing 0.4% glucose or 0.4% glycerol are summarized in Table 1. When grown on MaxyBroth-FC medium containing 0.4% glycerol, 2-fold higher expression level of arylsulfatase, about 5.8 unit/mL, was obtained over the culture medium Maxybroth-FC. However, when glucose was used as carbon and energy source, the arylsulfatase activity was detected with very low level, 0.1 unit/mL. These results imply that the expression of arylsulfatase in *E. coli* BL21(DE3)/pHCE-AST was severely repressed by glucose and was accelerated by glycerol. In many cases, the use of glycerol resulted in even better production of recombinant proteins in *E. coli* than glucose does [20,21]. In the expression of green

Table 1. Comparison of cell growth and arylsulfatase production of *E. coli* BL21(DE3)/pHCE-AST in different culture modes and medium conditions

Culture mode	Culture condition	Culture time (h)	Cell growth (OD_{660})	Total arysulfatase activity (unit/mL)
Flask culture	FC[a]	30	9.7	3.1
	FC+0.4% glucose[a]	30	9.1	0.1
	FC+0.4% glycerol[a]	30	6.8	5.8
Batch culture	HD[b]	12	7.8	11.7
	HD+1% glycerol[b]	12	16.8	12.8
	HD+2% glycerol[b]	12	23.9	34.7
Fed-batch culture	HD+2% glycerol[b]	20	39.9	143.2

[a]MaxyBroth-FC (flask culture)

[b]MaxyBroth-HD (fermentor culture)

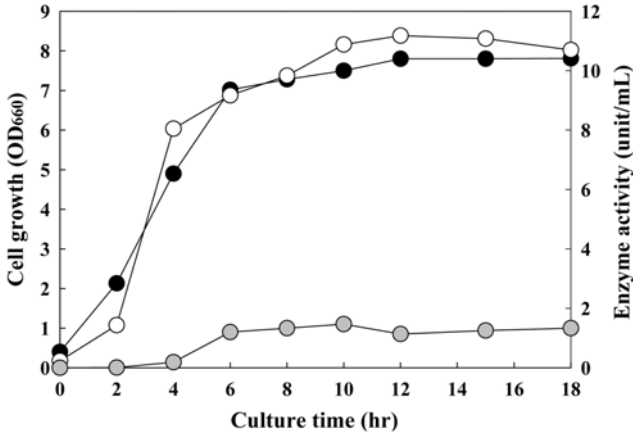

Fig. 1. Time profiles of cell growth and arylsulfatase activity in the fermentor batch culture of *E. coli* BL21(DE3)/pHCE-AST on MaxyBroth-HD medium. Black symbols (●), cell growth; white symbols (○), intracellular activity; gray symbols (●), extracellular activity.

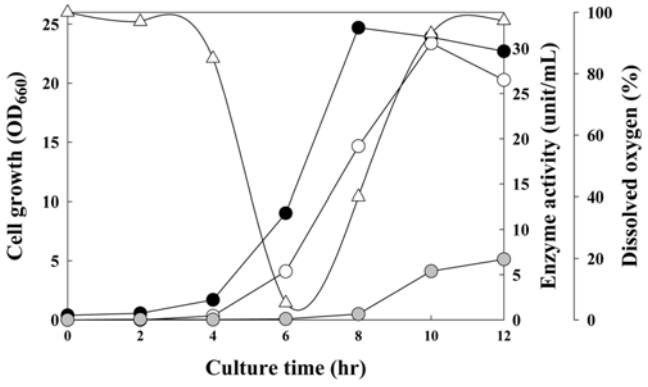

Fig. 3. Time profiles of cell growth and arylsulfatase activity in the fermentor batch culture of *E. coli* BL21(DE3)/pHCE-AST on MaxyBroth-HD+2% glycerol medium. Black symbols (●), cell growth; white symbols (○), intracellular activity; gray symbols (●), extracellular activity; triangle symbols (△), dissolved oxygen.

fluorescent protein gene in *E. coli* by using *Salmonella prpBCDE* promoter, the maximum cell concentration of 5.0 g/L and 6.3 g/L was obtained on glucose and glycerol, respectively [21]. Moreover, *E. coli* does not produce significant amounts of acetate while growing on glycerol. In this study, during the production of arylsulfatase on glycerol as the sole carbon source, the concentration of produced acetate was less than 1 g/L (data not shown).

3. Arylsulfatase Expression in the Batch Fermentation

The batch cultures of BL21/pHCE-AST were carried out on Maxybroth-HD media with different glycerol concentrations. Fig. 1 shows the time profiles of cell density (OD_{660}) and intracellular and extracellular activities of arylsulfatase with only Maxybroth-HD medium (no glycerol). When the cell growth reached a maximum at 18 h, the total arylsulfatase activity was about 11.9 unit/mL, which is higher than that in a LB broth of arylsulfatase (5.6 unit/mL). As shown in Fig. 2, on Maxybroth-HD medium containing 1% glycerol, the cell growth reached a maximum at 12 h, and the activity of 12.8 unit/mL was obtained. Moreover, on MaxyBroth-HD medium containing 2% glycerol, the maximal cell growth was achieved at 8 h and the expression level of arylsulfatase was increased by 2.7-fold (34.7 unit/mL) over 1% glycerol (Fig. 3). The arylsulfatase was produced with a growth-associated manner and found predominantly within the cell, especially in the periplasmic space, during the whole culture period.

To compare the effects of two different promoters on the arylsulfatase expression, the recombinant *E. coli* BL21(DE3) cells harboring pHCE-AST or pAST-A1 [17] were cultivated on Maxybroth-FC medium containing 0.4% glycerol. As shown in Table 1, on the Maxybroth-FC medium, the expression level of arylsulfatase in *E. coli* BL21(DE3)/pHCE-AST was about 3.1 unit/mL, whereas that of *E. coli* BL21(DE3)/pAST-A1 was about 2.7 unit/mL. On the Maxybroth-FC medium containing 0.4% glycerol, the constitutive expression system either in *E. coli* DH5α and *E. coli* BL21(DE3) gave

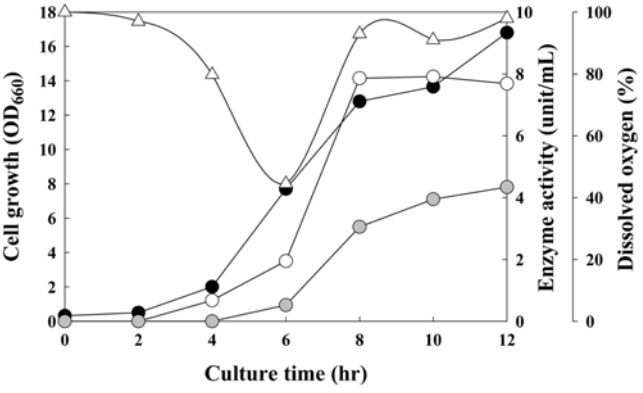

Fig. 2. Time profiles of cell growth and arylsulfatase activity in the fermentor batch culture of *E. coli* BL21(DE3)/pHCE-AST on MaxyBroth-HD+1% glycerol medium. Black symbols (●), cell growth; white symbols (○), intracellular activity; gray symbols (●), extracellular activity; triangle symbols (△), dissolved oxygen.

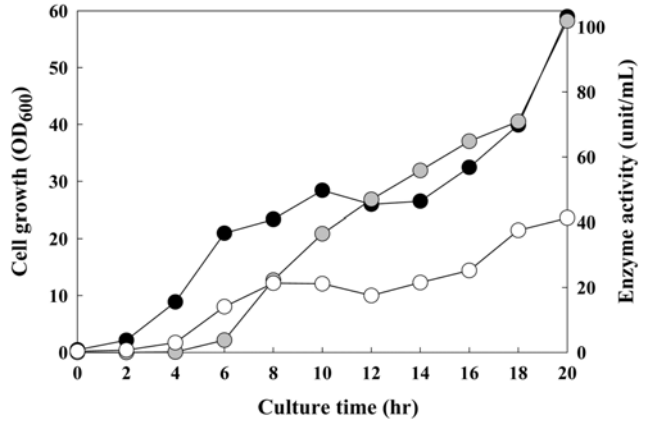

Fig. 4. Time profiles of cell growth and arylsulfatase activity in the fermentor fed-batch culture of *E. coli* BL21(DE3)/pHCE-AST on MaxyBroth-HD+2% glycerol medium. Black symbols (●), cell growth; white symbols (○), intracellular activity; gray symbols (●), extracellular activity; triangle symbols (△), dissolved oxygen.

5.8 unit/mL, which is 74% higher expression level of arylsulfatase than that of the inducible expression system (3.3 unit/mL). From these results, it was found that the constitutive HCE promoter system is superior to the pET T7 promoter system for the overexpression of arylsulfatase gene in *E. coli*.

4. Arylsulfatase Expression in the Fed-batch Fermentation

Fig. 4 shows the fed-batch cultivation profile of *E. coli* BL21 (DE3)/pHCE-AST on MaxyBroth-HD medium and additional feeding of glycerol. The enzyme activity of intracellular and extracellular arylsulfatase increased with increasing the cell density, and the final activity of extracellular arylsulfatase reached about 98 unit/mL. The total enzyme activity of 143.3 unit/mL was more than 4-fold higher than that of batch cultivation on 2% glycerol. Most of the arylsulfatase activity (68.4%) in the fed-batch culture was produced in the extracellular medium. This high level secretion at high cell density could suggest that the change in permeability of the outer membrane may result from a disturbance of cell envelope biogenesis caused by competition between the overproduced protein and envelope components for the sites of translocation across the cytoplasmic and outer membranes, or for common metabolic precursors [22-25]. Nesmeyanova's studies indicate that translocation across the outer membrane may have resulted in a change of alkaline phosphatase conformation due to transmembrane movement, or alteration of the enzyme environment, and probably depends on the mechanism of protein translocation. The maximum activity of alkaline phosphatase reached about 180 unit/mL. Up to 50-60% of the alkaline phosphatase produced is secreted into the medium where it is the prevalent protein. The intracellular β-glucosidase was employed as an indicator for the examination of cell integrity. In our experiment, this protein was not detected within the extracellular medium, which indicates that the arylsulfatase was not released as the result of cell lysis (data not shown).

The constitutive HCE promoter system has been successfully applied in the gram-scale production of human leptin (2.1 g/L) [26] and poly-γ-glutamic acid (3.7 g/L) [27]. When the recombinant *E. coli* cell employing the HCE promoter system is used, the recombinant fermentation process can be easily regulated using the continuous and controlled feeding of glycerol, so that the recombinant protein under the hyper-constitutive expression could be overproduced as a secreted form in *E. coli* high cell-density fed-batch culture.

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