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Batch and Fed-batch Production of Hyperthermostable α-L-Arabinofuranosidase of *Thermotoga maritima* in Recombinant *Escherichia coli* by Using Constitutive and Inducible Promoters

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Abstract A thermostable α-L-arabinofuranosidases (α-L-AFase) is an industrially important enzyme for recovery of L-arabinose from hemicellulose. The recombinant α-L-AFase from *Thermotoga maritima* was expressed in *Escherichia coli* by using a constitutive pHCE or an inducible pRSET vectors. In batch fermentation, the constitutive expression system resulted in slightly faster growth rate (0.78 vs. 0.74/hr) but lower enzyme activity (2,553 vs. 3,723 units/L) than those of the induction system. When fed-batch fermentation was performed, biomass and enzyme activity reached the highest levels of 36 g/L and 9,152 units/L, respectively. The fed batch cultures performed superior results than batch culture in terms of biomass yield (4.62-5.42 folds) and enzyme synthesis (3.39-4.00 folds). In addition, the fed-batch induction strategy at high cell density resulted in the best productivity in cell growth as well as enzyme activity rather than the induction method at low cell density or the constitutive expression.

Keywords: α-L-arabinofuranosidase, *Escherichia coli*, fed-batch, batch, fermentation

Introduction

The α-L-arabinofuranosidases (α-L-arabinofuranoside arabinofuranohydrolases, EC3.2.1.55, α-L-AFases) are the enzymes involved in the hydrolysis of α-L-arabinose linkages. These enzymes have been purified from several bacteria, fungi, and plants (1-4). The α-L-AFases catalyze the hydrolysis of terminal non-reducing α-L-1,2-, α-L-1,3-, and α-L-1,5-arabinofuranosyl residues from various oligosaccharides, glycoconjugates, and polysaccharides including various pectin, homo-hemicelluloses (branched arabinans, debranched arabinans), and heteropolysaccharides (arabinogalactans, arabinoxylans, arabinoxyloglucans, glucuronoarabinoxylans, etc) (5-8). The enzymes do not distinguish between the saccharide links to the arabinofuranosyl moiety and thus exhibit wide substrate specificity (3,9).

In recent times, α-L-AFases have received boosting attention due to not only their role in the degradation of lignocelluloses but also their positive effect on the activity of other enzymes acting on lignocelluloses. Accordingly, these enzymes are used in many biotechnological applications including clarification of fruit juices, wine industry, and digestion enhancement of animal feedstuffs and as a natural improver for bread (2,10). In addition to these, the use of α-L-AFase is focused on the production of L-arabinose, because the sugar has various functions as a food additive. L-Arabinose is known to selectively inhibit intestinal sucrose and reduces the glycemic response in plasma after sucrose ingestion in human. Naturally occurring L-arabinose is not metabolized in animals; thus it is a noncaloric sugar. L-Arabinose has 50% sweetness and quite similar sweet taste compared with sucrose. Based on these observations, when consumed with sucrose, L-arabinose can be used as a functional sugar in preventing diabetes or obesity with the inhibitory activity against sucrose digestion (3,11).

Thermostable enzymes offer potential benefits in the hydrolysis of lignocellulosic substrates; higher specific activity decreasing the amount of enzymes, enhanced stability allowing improved hydrolysis performance and increased flexibility with respect to process configurations, all leading to improvement of the overall economy of the process (for review, read 12, 13). A gene encoding hyperthermostable α-L-AFase from *Thermotoga maritima* was isolated and expressed in recombinant *Escherichia coli* (14). The purified recombinant α-L-AFase showed very high specific activity and thermostability (active at 100°C at pH 5.5-6.0). Significant amount of L-arabinose was released from oatspelts arabinoxylan after α-L-AFase treatment at 100°C, suggesting that L-arabinose could be efficiently produced from natural polysaccharides using this enzyme.

In the present investigation, to achieve overproduction of recombinant α-L-AFase, batch and fed-batch culture have been extensively employed by using high cell density culture of *E. coli*, which is often used for high yield and productivity of the desired product. *E. coli* expression system has no natural secretion mechanism and hence the concentration of the produced protein is proportional to cell concentration. Consequently, the processes optimized for

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high cell densities are beneficial (9,15,16). For this, a substrate limited fed-batch strategy was used by controling the feeding rate to maintain glucose below the critical level for over flow metabolism (17). Also, for higher α-L-AFase productivities, 2 different *E. coli* expression vectors (constitutive pHCE vs. inducible pRSET) were compared for fed-batch culture and, in the case of inducing system, induction times were evaluated at different culture period (low cell density vs. high cell density).

Materials and Methods

Bacterial strains and plasmids A gene (*abf*B) encoding α-L-arabinofuranosidase (α-L-AFase) was isolated from the hyperthermophilic microorganism, *Thermotoga maritima* (ATCC 43589D) (14). The *abf*B gene was 1,455 bp long and encoded 484 amino acid residues with a molecular weight of 55,265 Da. The *abf*B gene was introduced into the HCE promoter-based constitutive expression vector, pHCEIIB (BioLeaders Co., Daejeon, Korea) between the *Nde*I and *Pst*I restriction sites downstream of HCE promoter. The constructed vector, pHCETAF, was expressed in *E. coli* MC1061. The pHCEIIB vector (3.7 kb) originated from pUC19 contained HCE promoter and Shine-Dalgano sequence derived from upstream of D-amino acid aminotransferase (EC 2.6.1.21) of thermostable *Geobacillus toebii* with Amp^r gene and it was developed to express heterologous protein in *E. coli* without induction (18). At the same time, the *abf*B gene was also introduced in the T7 promoter-based inducible expression vector, pRSET-B (Novagen, WI, USA) between the *Nde*I and *Bgl*II restriction sites downstream of the T7 promoter. The constructed vector, pRBTAF, was expressed in *E. coli* BL21(DE3, pLysS) by induction with isopropyl β-D-1-thiogalactopyranoside (IPTG). The detailed construction process of the plasmid pRBTAF is described in our previous report (14).

Culture and media *E. coli* cells harboring plasmids (pRBTAF or pHCETAF) were routinely grown and maintained in Luria-Bertani (LB) medium (tryptone 10 g/L, yeast extract 5 g/L, NaCl 10 g/L) supplemented with 50 μg/mL of ampicillin. For long-term storage, 15% glycerol stock of culture was stored at 80°C. Medium used for batch fermentation contained the following components: KH_2PO_4 5 g/L, K_2HPO_4 3 g/L, $MgSO_4 \cdot 7H_2O$ 1.2 g/L, yeast extract 20 g/L, glucose 20 g/L, and 0.1 mM of ampicillin. The concentrated feed solution for fed-batch fermentation contained the following components per L: 247 g glucose, 211 g yeast extract, 1.5 g $(NH_4)_2SO_4$, 1 g $MgSO_4 \cdot 7H_2O$, and 0.1 mM ampicillin. The salts were sterilized separately in the vessel at 121°C for 15 min and ampicillin was added after sterile filtration.

Batch and fed-batch fermentation Bioreactor cultivations were performed in a 5-L fermentor (Kobiotech, Incheon, Korea) starting as a batch culture with an initial medium volume of 1.5 L. The agitation speed and flow rate of aeration were set at 900 rpm and 1.5 L/min, respectively. Culture temperature was at 37°C and pH was controlled at 6.8 by the addition of 28% ammonia water. Pure oxygen was supplied to accommodate the increasing rate of oxygen consumption accompanying cell growth. By this means, the dissolved oxygen concentration could be maintained above 20% of air saturation during the culture. Carbon substrate feeding was performed by the pH-stat strategy with a high limit of 6.82 and a low limit of 6.80. When glucose was depleted, pH was increased rapidly to 6.83 and then the feed solution was added until the high limit. Accordingly, the glucose concentration was kept under 1.5 g/L during the fed-batch culture to avoid accumulation of acetic acid. Induction was carried out by using IPTG. Fermentations and assay conditions were standardized to avoid any bias and for accurate and reproducible comparison.

Analytical methods The biomass concentration in the fermentor was measured by taking the optical density (OD) at 600 nm and the dry cell weight (DCW) was obtained from the correlation between OD_{600} and DCW determined by weighing dry cells in batch culture (19). α-L-AFase activity was assayed according to 3,5-dinitrosalicylic acid (DNS) method (20) by determining the amount of reducing sugar. One unit of enzyme activity was defined as the amount of enzyme releasing 1 μmol of arabinose equivalent per min under the assay conditions. For analysis of protein expression pattern, protein extracts prepared by sonication of *E. coli* after IPTG induction were analyzed by 8.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and stained with Coomassie blue. The specific growth rate (μ) was determined from the slope of a graph of the log of cell mass (DCW) against time (hr).

Results and Discussion

Batch culture and protein analysis Cell growth and enzyme productivities of recombinant *E. coli* harboring pHCETAF and pRBTAF were monitored during the batch fermentation using 5-L fermentor. As shown in Fig. 1, when *E. coli* carrying pHCETAF was inoculated, cells started to grow exponentially with specific growth rate of 0.78/hr and, after 7 hr, biomass and enzyme activity reached the highest level of 7.8 g DCW/L, 2,553 units/L, respectively. In case of *E. coli* carrying pRBTAF, inoculum was added in the medium with IPTG and cells grew exponentially with specific growth rate of 0.74/hr. After 11 hr, biomass and enzyme activity reached the highest levels of 8.32 g/L, and 3,723 units/L, respectively.

The constitutive expression system resulted in slightly faster growth rate (0.78 vs. 0.74/hr) but lower enzyme activity (2,553 vs. 3,723 units/L) than those of the induction system. This is explainable with the difference of promoter strengths between T7 and HCE. The T7 promoter might strongly induce the expression of α-L-AFase from the early phase of cell growth; caused a heavy load in the intracellular metabolic flux and it could suppress a vivid cell growth. This is supported by the results of enzyme expression levels during the fermentations. The constant expression level (300-400 units/g DCW) of α-L-AFase along with the whole culture period in the constitutive system reveals the expression pattern of primary metabolite during the batch culture. Meanwhile, the induction system, where IPTG was added with inoculum, shows a pattern of an initial high expression (600-800 units/g DCW) with a late low enzyme production (400 units/g DCW) in each cell. This result also provides a clue to design a reasonable

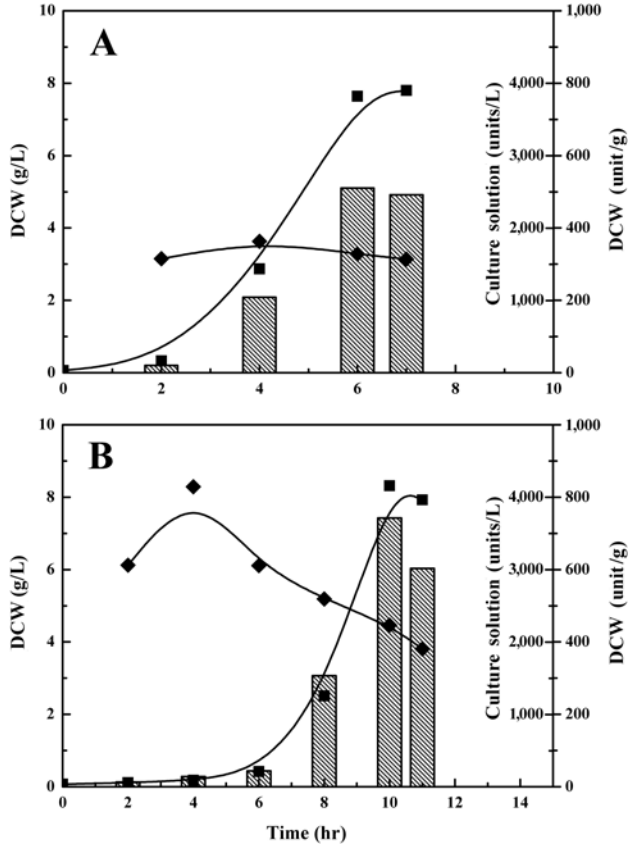

Fig. 1. Time profiles during batch fermentation of *E. coli* harboring pHCETAF (constitutive) or pRBTAF (inducible) plasmids. The recombinant *E. coli* was cultivated in 2 L of medium in a 5-L fermentor. A, Constitutive expression of α-L-AFase using pHCETAF system; B, induction expression of α-L-AFase using pRBTAF system. Cell growth (dry cell weight, DCW) (■), cellular enzyme activity (◆), and volumetric enzyme activity (▨).

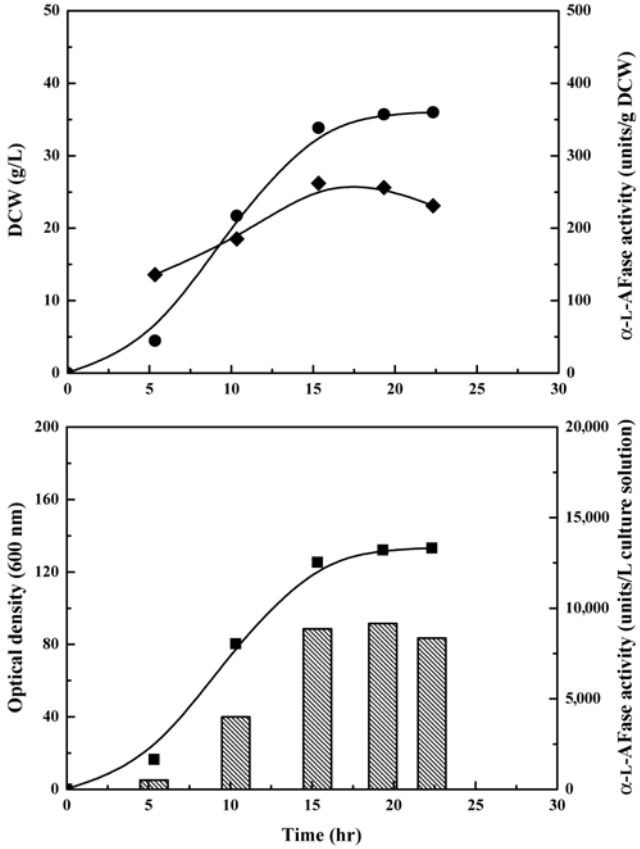

Fig. 2. Time profiles during fed-batch fermentation of *E. coli* harboring pHCETAF (constitutive) plasmid. The recombinant *E. coli* was cultivated in a 5-L fermentor by feeding glucose stock medium with pH-stat strategy. Dry cell weight (DCW, ●), optical cell density (■), cellular enzyme activity (◆), and volumetric enzyme activity (▨).

strategy to manage the fed-batch fermentation of α-L-AFase in terms of induction time to accumulate higher amount of proteins in the cell.

The SDS-PAGE analysis of α-L-AFase expression in *E. coli* (pRBTAF) proved the synthesis of enzymes in cells and efficient purification after heat treatment at 80°C for 20 min (data not shown). A large amount of enzyme was produced in the *E. coli* but some of them were aggregated as an insoluble form. The folding process for the active enzyme seemed to be accomplished insufficiently due to the strong promoter action or other environmental factors (21). As protein aggregation is favored as the intracellular concentration of nascent polypeptide chains is increased, a slow and lower level of protein synthesis is often found to result in a higher amount of soluble protein (22). Accordingly, the pH-stat strategy employed in fed-batch experiment, which maintains low glucose level and slow protein synthesis along the fermentation, is supposed to result in soluble over-expression of enzymes.

Fed-batch culture with constitutive expression Based on the above analysis on batch culture, fed-batch fermentation of recombinant *E. coli* harboring a constitutive promoter in pHCETAF was carried out in a complex medium using a pH-stat strategy. Just after inoculation, cells started to grow exponentially and, when glucose was exhausted in the fermentor, pH was rapidly increased and the concentrated feed medium was added by the pH rising over the set value of 6.83. During the fed-batch fermentation, cell growths and enzyme activities were monitored with the time course (Fig. 2). Just after inoculation, enzyme activity was detected in the cell lysates, and after 20 hr, biomass and enzyme activity reached the highest levels of 36 g DCW/L and 9,152 units/L, respectively. In the fed-batch phase, the specific growth rate (μ) descended to 0.20/hr due to the nutrient limitation in medium by glucose (<1.5 g/L) along with the whole culture period. By using fed-batch cultivation, the biomass and enzyme activity were increased over 4- and 3-folds, respectively, than those of batch culture.

Fed-batch culture by induction at low cell density By the same method with the above experiment, fed-batch culture of recombinant *E. coli* harboring pRBTAF plasmid was carried out. In order to compare the effect of induction time on the protein yields, 0.1 mM of IPTG was added in broth both at low and high cell densities. First, the low cell density expression was performed with 0.1 mM IPTG at 8.4 g DCW/L. Figure 3 shows the time course of biomass

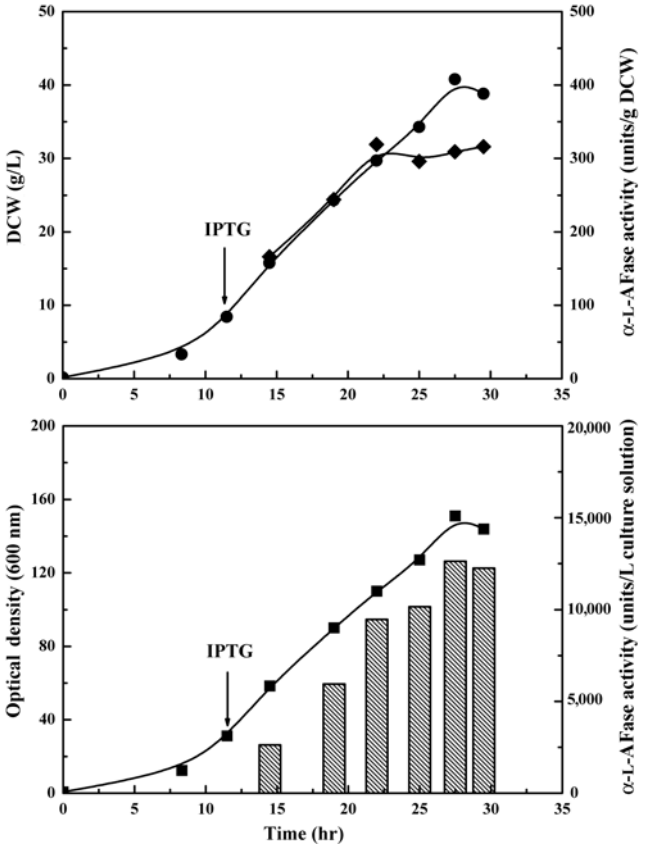

Fig. 3. Time profiles during fed-batch fermentation of *E. coli* harboring pRBTAF plasmid after induction at low cell density. The recombinant *E. coli* was cultivated in a 5-L fermentor by feeding glucose stock medium with pH-stat strategy and α-L-AFase expression was induced by 0.1 mM IPTG at 8.4 g DCW/L. Dry cell weight (DCW, ●), optical cell density (■), cellular enzyme activity (◆), and volumetric enzyme activity (▨).

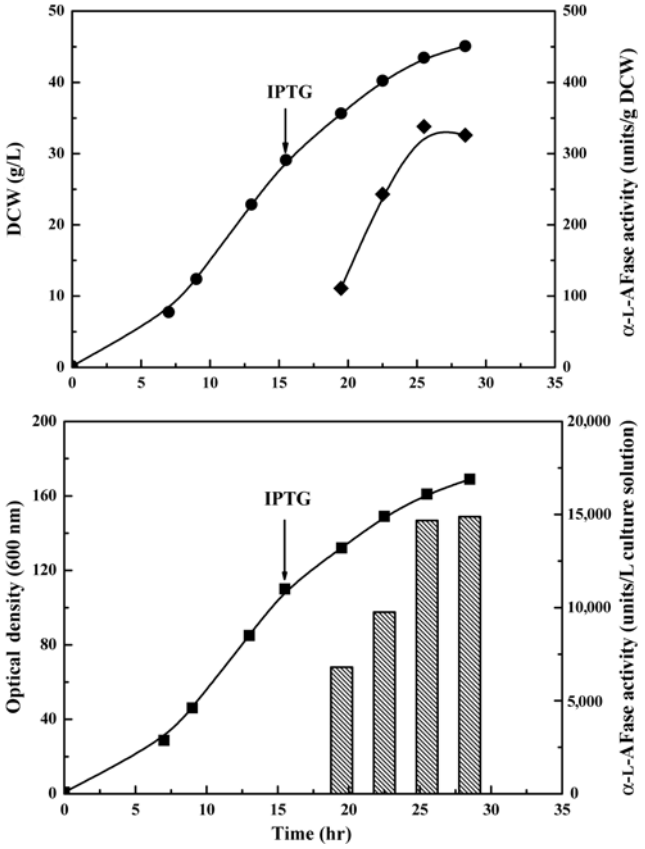

Fig. 4. Time profiles during fed-batch fermentation of *E. coli* harboring pRBTAF plasmid after induction at high cell density. The recombinant *E. coli* was cultivated in a 5-L fermentor by feeding glucose stock medium with pH-stat strategy and α-L-AFase expression was induced by 0.1 mM IPTG at 29.7 g DCW/L. Dry cell weight (DCW, ●), optical cell density (■), cellular enzyme activity (◆), and volumetric enzyme activity (▨).

and enzyme activity. As soon as IPTG was added in the medium, α-L-AFase activity was detected in the cell extract and the total activity gradually increased following the cell growth. After 25 hr of fed-batch fermentation, the biomass and enzyme activity reached the highest levels of 40.8 g DCW/L (150 OD_{600}) and 319 units/g DCW (12,630 units/L), respectively. In the fed-batch phase, the specific growth rate (μ) was 0.12/hr. Compared to yields of biomass and enzyme activity obtained at the batch culture, the fed-batch cultivation by induction at low cell density showed about 5-folds and over 3-fold increments, respectively.

Fed-batch culture by induction at high cell density Fed-batch culture of recombinant *E. coli* harboring pRBTAF plasmid was also carried out by induction at relatively high cell density (29.7 g DCW/L). As IPTG was added in the medium after 15 hr from inoculation, α-L-AFase activity was detected and the total activity was rapidly increased (Fig. 4). After 28 hr of fed-batch fermentation, the biomass and enzyme activity reached the highest levels of 45.1 g DCW/L (170 OD_{600}) and 338 units/g DCW (14,892 units/L), respectively. In the fed-batch phase, the specific growth rate (μ) was 0.10/hr. Compared to yields of biomass and enzyme activity obtained at the batch culture, the fed-batch cultivation by induction at high cell density showed over 5- and 4-fold increments, respectively.

The experimental results are summarized in Table 1 comparing the 2 expression systems (constitutive vs. inducible) as well as the 2 different fermentation methods (batch vs. fed-batch). The fed batch cultures performed superior results than batch culture in terms of biomass yield (4.62-5.42 folds) and enzyme synthesis (3.39-4.00 folds). In addition, the fed-batch induction strategy at high cell density resulted in the best productivity in cell growth as well as enzyme activity rather than those of induction method at low cell density or the constitutive expression method.

Gomes *et al.* (23) reported that they have produced thermostable α-L-AFase by cultivation of thermophilic eubacterium *Rhodothermus marinus* with 4.2 g/L of maximum biomass and 2,700 units/L of enzyme activity. Refer to those yield using wild type strain, the present fed-batch fermentation using recombinant *E. coli* significantly enhanced the enzyme productivity over 5-folds. Growing *E. coli* to high density is currently the method of choice for the production of recombinant proteins, mainly because of the high volumetric productivity associated with this method. After long-time efforts of industrial microbiologists, the

Table 1. Summary of α-L-AFase production under different culture conditions in 2 recombinant *E. coli* systems

		Constitutive *E. coli* (pHCETAF)		Inducible *E. coli* (pRBTAF)		
		Batch	Fed-batch	Batch	Fed-batch Induction at low cell density	Fed-batch Induction at high cell density
Cultivation time (hr)		7	23	11	30	29
Specific growth rate (/hr)		0.78	0.20	0.74	0.12	0.10
Maximal biomass	OD_{600}	30	130	32	150	170
	DCW g/L	7.80	36.00	8.32	40.80	45.10
	Folds	1.00	4.62	1.00	4.90	5.42
Maximal enzyme activity	units/g DCW	363	262	828	319	338
	units/L	2,553	9,152	3,723	12,630	14,892
	Folds	1.00	3.58	1.00	3.39	4.00
Productivity (units/L · hr)		365	398	338	421	514

recombinant *E. coli* has become a production machine for heterologous proteins. However, techniques to obtain the highest possible density of productive *E. coli* as well as high protein productivity should be developed for each target proteins (24,25) by following optimization of culture systems like this study.

While the constitutive expression system resulted in slightly lower yields in this experiment, the system has several merits when used in biotechnology, since the current vectors with induction promoters have some constraints to be overcome in real fermentation; cost and safety concerns with IPTG induction against *trp* or *lac* promoter (26,27), inclusion body formation and heat shock protease expression with thermal induction against phage λ promoter (28,29). The constitutive expression vector is still a useful tool in protein overproduction for both batch and fed-batch cultivation with the convenience and economical advantage. Now a day, development of a novel expression system for the production of food enzymes are widely investigated (30). The *E. coli* system, on the other hand, which has been broadly used for biotechnological production of pharmaceutical protein, still have a potential for the production of food enzymes like α-L-AFase under the condition of safety assessment.

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