The commonness and difference among the Lactobacillus feruloyl esterases expressed in Escherichia coli

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Research

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Abstract

Background: Construction of recombinant *Escherichia coli* strains carrying feruloyl esterase genes for secretory expression offers an attractive way to facilitate enzyme purification and one-step produce ferulic acid from agricultural waste. It was found that the *Lactobacillus crispatus* feruloyl esterase could be secreted into extracellular environment of *E. coli*. Whether other *Lactobacillus* feruloyl esterases share the same secretory characteristic is worth investigation.

Results: A total of ten feruloyl esterases derived from nine *Lactobacillus* species were used to analyze their commonness and compare their difference when heterologously expressed in *E. coli* BL21 (DE3). Extracellular activity determination showed all these *Lactobacillus* feruloyl esterases could be secreted out of *E. coli* cells. However, protein analysis indicated that they could be classified as three types. The first type presented a low secretion level, including feruloyl esterases derived from *Lb. acidophilus* and *Lb. johnsonii*. The second type showed a high secretion level, including feruloyl esterases derived from *Lb. amylovorus*, *Lb. crispatus*, *Lb. gasseri* and *Lb. helveticus*. The third type also behaved a high secretion level but easy degradation, including feruloyl esterases derived from *Lb. farciminis*, *Lb. fermentum* and *Lb. reuteri*. Moreover, these recombinant *E. coli* strains could directly release ferulic acid from de-starched wheat bran.

Conclusions: Recombinant *E. coli* strains expressing feruloyl esterase of *Lb. amylovorus*, *Lb. crispatus* and *Lb. helveticus* displayed high secretion level and stable extracellular activity. Furthermore, the highest yield of ferulic acid was 140 μg on the basis of 0.1 g de-starched wheat bran after 72 h cultivation of *E. coli* expressing *Lb. amylovorus* feruloyl esterase. These results provided a solid basis for the production of feruloyl esterase and ferulic acid.

Background

Feruloyl esterase, belonging to the hemicellulase family, is a type of hydrolase capable of degrading the ester bond between the ferulic acid and lignin in the cell wall of plants. It acts in conjunction with other cellulases and hemicellulases to synergistically open the crosslinked network structures of cell walls [1, 2]. This feature endows its applications in many areas, such as feed additives and pulp and paper. Due to the structural complexity of the natural macromolecular substances, a large part of the feed cannot be used after being ingested by the animals, leading to the low utilization rate. The feruloyl esterase is able to destroy the crosslinking between cellulose, hemicellulose and lignin, thus making the feed becoming sparse. The feruloyl esterase-treated feed is easy to be digested and absorbed by livestock [3]. In the pulp and paper making industry, the usage of feruloyl esterase avoids the environment pollution and energy consumption which is caused by the traditional chemical methods. The composition of the paper are mainly cellulose and hemicellulose. Therefore, the lignin component in the plant cell walls needs to be removed. This process is mainly attributed to feruloyl esterase [4]. Furthermore, feruloyl esterase has the reaction ability of esterification or transesterification, indicating that the ester bond between phenolic acid

and sugar can be synthetized by biological enzymatic method. The produced esters have the promising potential for application as antibacterial, antiviral and anti-inflammatory drugs [5].

Nowadays, feruloyl esterases have been found in a variety of microorganisms including bacteria and fungi [6, 7]. They showed different coding sequences, protein structures, physicochemical properties and catalytic activities. For characterization of a feruloyl esterase, the routine experimental procedure is to clone the coding gene, heterologously express the enzyme in recombinant strain, and then purify the feruloyl esterase. *Escherichia coli* expression systems is commonly used for prokaryotic feruloyl esterase expression [8]. This system is the most well-researched classical high-efficiency heterologous expression system for prokaryotic genes. *E. coli* has been used as a cell factory to produce a considerable number of enzymes and medical proteins due to its clear genetic background, simple and easy operation, and high protein yield [9]. However, because of its internal and external bilayer membrane structure, the secretory expression of the protein becomes a problem in *E. coli*. The feruloyl esterase produced by engineering *E. coli* is usually located in the cytoplasm. A complicated purification process is needed to obtain the desired product for further study [10]. Nevertheless, there had been very few reports concerning about that the recombinant proteins were detected extracellularly in the expression of heterologous proteins in *E. coli*, such as β-xylosidase, cellulase and cutinase [11, 12, 13]. In our previous study, we fortunately found that the *Lactobacillus crispatus* feruloyl esterase also could be secreted into extracellular environment of *E. coli* [14]. Considering that the feruloyl esterase coding genes widely exist in different *Lactobacillus* species, whether these feruloyl esterases share the same secretory characteristic is worth investigation.

Another benefit of extracellular secretory feruloyl esterase is the direct use for ferulic acid production. Ferulic acid, also known as 4-hydroxy 3-methoxycinnamic acid, is crosslinked with other components of cell wall of plants. Feruloyl esterase hydrolyzes ester bonds to release ferulic acid [15]. Studies have shown that ferulic acid has many important biological effects. As an antioxidant, it can remove various free radicals, thus functioning as an anti-aging regulating agent. Furthermore, ferulic acid has obvious effects in reducing inflammation, promoting wound healing and anti-tumor [16, 17]. Therefore, a variety of functional foods can be developed by using ferulic acid. Moreover, ferulic acid is the raw material to produce vanillin by microbial fermentation and metabolism, which is used as a spice in the food industry and cosmetics industry [18]. At present, feruloyl esterase alone or combination with other enzymes such as xylanase is applied to extract ferulic acid from the crop by-products including wheat bran and rice bran. The used feruloyl esterase is usually obtained from the expression in a heterologous host and subsequent purification [19]. Several researches were conducted by using the natural extracellular feruloyl esterase of microorganisms to produce ferulic acid, but the expression level of the enzyme is generally low [20, 21]. The utilization of high-efficiency expression systems to mass produce feruloyl esterase and secrete it extracellularly is a very attractive method for the production of ferulic acid.

In the present study, a total of ten feruloyl esterases derived from nine *Lactobacillus* species were used to analyze their commonness and compare their difference when heterologously expressed in *E. coli*. These feruloyl esterase coding genes were cloned and expressed in *E. coli* BL21 (DE3), respectively. The changes of extracellular feruloyl esterase activities were measured, and the profiles of cytoplasmic and

extracellular protein bands were visualized. Moreover, these recombinant *E. coli* strains were directly used for ferulic acid production in a medium containing de-starched wheat bran.

Results

Expression of *Lactobacillus* feruloyl esterases in *E. coli*

The feruloyl esterase coding genes widely exist in the genome of a variety of lactic acid bacteria. In the present study, nine *Lactobacillus* species containing *Lb. acidophilus*, *Lb. amylovorus*, *Lb. crispatus*, *Lb. farciminis*, *Lb. fermentum*, *Lb. gasseri*, *Lb. helveticus*, *Lb. johnsonii*, and *Lb. reuteri* were chosen to comparatively investigate their feruloyl esterases. Especially, *Lb. johnsonii* could produce two feruloyl esterases. Primer sets were designed to amplify the feruloyl esterase coding genes, respectively. To reduce the redundant amino acid sequences derived from the expression vector, an in vitro homologous recombination method that relied on homologous sequences was used to ligate these genes into pET-22b vector. The recombinant plasmids for feruloyl esterases production were presented in Table 1. A plasmid pET22b-FaeLcr constructed previously was also used at present study as for positive control and comparative analysis [14]. The ten enzymes shared sequence similarity in the range of 44% to 87%, indicating they had a certain degree of homology (data not shown). Furthermore, bioinformatic analysis by SignalP and TatP revealed that all these feruloyl esterases did not contain any predictable signal peptide sequences.

These pET-22b-deritative plasmids containing feruloyl esterase coding genes were further transformed into *E. coli* BL21 (DE3) for enzymes expression. The produced proteins were named as FaeLac (from *Lb. acidophilus*), FaeLam (from *Lb. amylovorus*), FaeLcr (from *Lb. crispatus*), FaeLfa (from *Lb. farciminis*), FaeLfe (from *Lb. fermentum*), FaeLga (from *Lb. gasseri*), FaeLhe (from *Lb. helveticus*), FaeLjo1 (from *Lb. johnsonii*), FaeLjo2 (from *Lb. johnsonii*) and FaeLre (from *Lb. reuteri*), respectively. The clear transparent circles appeared in the LB medium supplemented with substrate ethyl ferulate and inducer IPTG, when these transformants were inoculated and incubated at 37 °C for 18 h (Fig. S1). The results indicated that these feruloyl esterases were correctly and functionally expressed in the recombinant *E. coli* strains. Furthermore, the feruloyl esterases might also be secreted into extracellular environment like our reported FaeLcr previously [14].

The secretory characteristic of *Lactobacillus* feruloyl esterases in *E. coli*

To investigate whether these feruloyl esterases could be secreted out of the recombinant *E. coli* cells, an Oxford cup-based experiment was carried out. These *E. coli* strains were cultivated to an OD_{600} of 0.6, and then induced by 0.5 mM IPTG to produce feruloyl esterases for 12 h at 37 °C. The cell-free culture supernatants were collected and added into the Oxford cup to test the extracellular feruloyl esterase activity. As shown in Fig. 1, the hydrolysis rings were formed by each of the detected samples after 6 h incubation. While the *E. coli* including the pET-22b vector without any insert showed no clear area (data not shown). These results suggested that all these *Lactobacillus* feruloyl esterases could be secreted into

the extracellular environment of *E. coli*. Furthermore, the difference in the size of the transparent circles indicated the different extracellular feruloyl esterase activities of these *E. coli* strains.

The extracellular feruloyl esterase activity changes of these recombinant *E. coli* strains were determined by using ρNPF as substrate. These strains were cultivated at 37 °C, and the activities of the cell-free supernatants were measured at time intervals of 4, 8, 12, 24, 36, 48, 60 and 72 h after induction. As shown in Fig. 2, the extracellular feruloyl esterase activities of the *E. coli* strains exhibited different trends. The extracellular activities of *E. coli* expressing FaeLam and FaeLhe fast increased along with the fermentation, and slightly decreased at the end of the fermentation. The extracellular activities of *E. coli* expressing FaeLfa, FaeLfe and FaeLre rapidly increased and then drastically decreased. While the extracellular activities of *E. coli* expressing FaeLac, FaeLga, FaeLjo1 and FaeLjo2 slowly increased and decreased. Furthermore, these strains reached the maximum extracellular enzyme activity at different time points. The maximal activity of extracellular FaeLre was detected at 8 h. The maximal activities of extracellular FaeLfe, FaeLjo1 and FaeLjo2 were observed at 12 h. The maximal activities of extracellular FaeLac, FaeLam, FaeLfa, FaeLga and FaeLhe were detected at 24 h. Moreover, the maximal activities of these extracellular feruloyl esterases were determined as presented in Table S2.

Extracellular protein of recombinant *E. coli* strains

The extracellular components of these recombinant *E. coli* BL21(DE3) cultured in LB medium were also collected at time intervals of 4, 8, 12, 24, 36, 48, 60 and 72 h after induction, and then analyzed by using SDS-PAGE. As shown in Fig. 3, the secretory protein bands were observed intuitively. All the feruloyl esterases could be secreted out of the *E. coli* cells but with different content. The extracellular feruloyl esterase bands and extracellular activities had a certain degree of consistency. The extracellular FaeLam and FaeLhe gradually accumulated in the culture medium along with the fermentation (Fig. 3B and Fig. 3F). The extracellular FaeLfa, FaeLfe and FaeLre first increased and then decreased (Fig. 3C, Fig. 3D and Fig. 3I). While the extracellular FaeLac, FaeLjo1 and FaeLjo2 only showed less dramatic changes (Fig. 3A, Fig. 3G and Fig. 3H). These results were in accordance with the extracellular feruloyl esterase activity changes, indicating that the activities were affected by the secretion and degradation level of feruloyl esterases. However, the FaeLga progressively increased without obvious reduction in extracellular environment, while the activity displayed on a downward trend after 24 h fermentation (Fig. 3E). This might be due to the instability of the FaeLga. The extracellular protein concentrations of these recombinant *E. coli* were measured when the enzymatic activity reached maximum, respectively (Table S2). It should be noted that the secreted feruloyl esterases only accounts for a portion (small or large) of the extracellular proteins.

Comparison of cytoplasmic and extracellular activities

The extracellularly secretory proteins of these *E. coli* strains were first expressed intracellularly and then transported through the protein transport system. In order to fully understand the profile of feruloyl esterases, whole cell proteins and cytoplasmic proteins of recombinant *E. coli* strains were also analyzed by SDS-PAGE. The sampling time was also set at the maximum extracellular activity for each feruloyl

esterase. As shown in Fig. 4, it could be clearly observed that these recombinant proteins were expressed in *E. coli* in quantities. Furthermore, the secreted feruloyl esterases showed the same molecular weight with the un-transported feruloyl esterases (data not shown), indicating that these proteins were not cleaved or modified during the transport process. However, these *E. coli* showed difference in whole cell and cytoplasmic component. As for FaeLac, FaeLam, FaeLcr, FaeLfa, FaeLga and FaeLre, the content of intracellular soluble feruloyl esterases was only slightly lower than that of whole cell. While the majority of FaeLfe, FaeLhe, FaeLjo1 and FaeLjo2 disappeared in the cytoplasmic components. The feruloyl esterase activities in the cytoplasmic components were also determined using ρNPF as substrate. Fig. 5 showed the results of the ratio of extracellular enzyme activity to total (extracellular plus cytoplasmic) enzyme activity when the extracellular activity reached maximum. Except for *E. coli* expressing FaeLac, the extracellular activities account for more than 50% of the total activities. The highest ratio was detected in the *E. coli* expressing FaeLjo1. This was probably due to the little intracellular soluble feruloyl esterase.

Ferulic acid production form agricultural waste

Ferulic acid is widely found in agricultural waste, such as wheat bran and rice bran. Previous experiments were commonly carried out by using purified feruloyl esterase to release ferulic acid from the plant cell walls. In the present study, one-step production of ferulic acid was performed by utilizing the recombinant *E. coli* secreting feruloyl esterase and the de-starched wheat bran as substrate. Therefore, 5 mL of LB medium containing 0.1 g of de-starched wheat bran was prepared to cultivate these *E. coli* strains and produce ferulic acid. As shown in Fig. 6, all these recombinant strains could hydrolyze the de-starched wheat bran to release ferulic acid, suggesting that the feruloyl esterases also could be secreted in this medium. However, the hydrolytic abilities of those strains were different. The *E.coli* expressing FaeLam displayed the highest hydrolytic activity, while lowest amount of ferulic acid was obtained by *E. coli* expressing FaeLjo1. In conclusion, these recombinant strains could be directly used for ferulic acid production from agriculture waste, and the hydrolytic ability was in a strain-specific manner. The highest yield of ferulic acid was 140 μg on the basis of 0.1 g de-starched wheat bran after 72 h cultivation of *E. coli* expressing FaeLam.

Discussion

Lactic acid bacteria are a group of Gram-positive bacteria that can ferment soluble carbohydrates to produce lactic acid. Many species of different genera belong to lactic acid bacteria, in which *Lactobacillus* is an important member [22]. *Lactobacillus* strains are widely distributed in human intestines, dairy products, and fermented plant foods [23]. In recent years, the application value of *Lactobacillus* has been recognized along with the increase of related researches. Considering the generally recognized as safe status of lactic acid bacteria and the application of feruloyl esterase in the food, cosmetics and pharmaceutical industries, the feruloyl esterases produced by *Lactobacillus* strains have received increasing attention. Various feruloyl esterases-producing *Lactobacillus* have been isolated from gut or fermented plant products, including those investigated in the present study [8, 14, 24, 25, 26].

Their feruloyl esterases have been heterologously expressed in *E. coli* BL21(DE3), and then purified and characterized previously. However, a new understanding of these feruloyl esterases from another perspective was obtained in this study. Results have shown that these feruloyl esterases could be secreted into the extracellular environment of *E. coli*, but differ in terms of secretion levels and extracellular accumulation. In addition, these recombinant strains could be used to release ferulic acid directly from agricultural waste. These results provided a solid basis for the production of feruloyl esterase and ferulic acid.

Although all these feruloyl esterases could be secreted out of the *E. coli* cells, they can be classified into three types according to the concentration and variation of extracellular feruloyl esterases. The first type presented a low secretion level. The second type showed a high secretion level. The third type also behaved a high secretion level, but easy degradation. By analysis of whole cell and cytoplasmic proteins, it was found that a part of recombinant feruloyl esterases existed as inclusion bodies. The formation of inclusion bodies might affect their secretion levels, like FaeLjo1 and FaeLjo2. In order to increase extracellular enzyme activity, these feruloyl esterases should be induced at lower temperatures or with less inducer to weaken the protein expression. The second type feruloyl esterases were ideal candidates for mass production. Furthermore, these feruloyl esterases showed diverse biochemical characteristics [8, 14, 25, 26]. Therefore, a high expression platform was created to produce feruloyl esterases suitable for different applications. Especially, the fermentation broths containing feruloyl esterase could be directly used as additive in pulp and paper or feed industry. In addition, the third type feruloyl esterases were highly secreted in the early stage, and then rapidly degraded in the later stage. This might be due to the extracellular protease produced by *E. coli* [27]. Construction of protease-resistant mutants via predicting the action site of protease could provide a method to prevent the feruloyl esterase degradation.

Ferulic acid accounts for up to 3% of the dry weight of cells in plant [28]. Since ferulic acid usually binds to macromolecules such as hemicellulose or lignin in the plant cell walls, only the secreted feruloyl esterase by microorganisms can approach these substrates to release ferulic acid. Therefore, the purified enzymes were often used in previous studies. Four *Lactobacillus* feruloyl esterases were heterologously expressed in *E. coli*, and purified to produce ferulic acid from corn stover [8]. The feruloyl esterase PcFAE1 of *Penicillium chrysogenum* 31B was overexpressed in *Pichia pastoris* KM71H and then purified to release ferulic acid from natural substrates [19]. In the light of the secreted expression of these feruloyl esterases, the direct use of the recombinant *E. coli* strain would facilitate the ferulic acid production, because of the saving of enzyme purification process. FaeLam showed the excellent performance with the maximum releasing amount of 140 μg ferulic acid from 0.1 g de-starched wheat bran. It should be noted that the hydrolysis experiments were performed at a constant temperature of 37 °C in order to accommodate the growth of *E. coli*. However, most of the feruloyl esterases reached maximum secretion activity within 24 h. In the future, variable temperature fermentation by changing to the optimum temperature of enzymatic activity after 24 h can be tried to increase the yield or shorten the fermentation time.

One of the questions raised by this study is whether these *Lactobacillus* themselves are capable of secreting feruloyl esterase. *Lactobacillus* strains as probiotics have been used in fermented food to improve human health [29]. Those capable of producing feruloyl esterase show greater potential for application. Because the feruloyl esterase can release ferulic acid bound to macromolecules such as hemicellulose and lignin, when the plant-based food is mixed with *Lactobacillus* strains. The produced ferulic acid enhances the probiotic effects. The above idea is based on the secretory expression of feruloyl esterase in *Lactobacillus*. However, the localization of feruloyl esterase in *Lactobacillus* is controversial. Lai *et al.* [24] and Esteban-Torres *et al.* [30] supported *Lactobacillus* feruloyl esterase as intracellular enzyme. Because no signal peptide sequence was predicted in these feruloyl esterases, and the extracellular components of *Lb. plantarum* and *Lb. johnsonii* cultures were not capable of degrading the model substrate. On the contrary, there were reports that the extracellular feruloyl esterase activity was detected and the ferulic acid was released when natural substrates such as barley were fermented by *Lactobacillus* strains [31]. In the present study, we showed that all these *Lactobacillus* feruloyl esterases without predictable signal peptide sequences could be secreted into the extracellular environment of *E. coli*. This motivated us to explore the secretion of feruloyl esterase in their natural host in the future.

The other question is how these feruloyl esterases are transported by *E. coli*. *E. coli* has been used as a cell factory to produce a variety of enzymes and medical proteins due to its clear genetic background and sophisticated protein expression control tools. However, the protein expressed by *E. coli* is usually located intracellularly. Obtaining a desired product often requires a complicated purification process. The Sec and Tat are the typical protein secretion pathways in *E. coli*. These two pathways are dependent on typical signal peptide sequences at the N-terminus of the proteins [32]. However, no predictable signal peptide sequences were found in these *Lactobacillus* feruloyl esterases, indicating that they were transported by a novel protein secretion mechanism of *E. coli*. There were several researches concerning the atypical secretion of proteins. Novel secretory mechanisms have been excavated in *E. coli*, including Type III and Type VI secretion systems [33, 34]. The understanding of the atypical protein secretion pathway provides a new solution for the secretory expression of foreign proteins. Recombinant proteins had been successfully secreted by these pathways in *E. coli* [35]. Therefore, the secretion mechanism of feruloyl esterase can broaden the means of protein secretion in *E. coli*. The further work can be carried out by investigation of the structure or sequence basis of the feruloyl esterase for recognition, and exploration of the related protein required for the feruloyl esterase transportation in *E. coli*.

Conclusions

In summary, the ten *Lactobacillus* feruloyl esterases could be secreted into extracellular environment when expressed in *E. coli* BL21(DE3). However, they also showed difference in terms of secretion levels and extracellular accumulation. Recombinant *E. coli* strains expressing feruloyl esterase of *Lb. amylovorus*, *Lb. crispatus* and *Lb. helveticus* displayed high secretion level and stable extracellular activity. Furthermore, these recombinant strains could be used to release ferulic acid directly from agricultural waste. The maximal production was obtained by the *E. coli* expressing *Lb. amylovorus*

feruloyl esterase. These results provided a solid basis for the production of feruloyl esterase and ferulic acid.

Methods

Strains, plasmids, culture conditions and chemicals

The *Lactobacillus* strains of *Lb. acidophilus*, *Lb. amylovorus*, *Lb. crispatus*, *Lb. farciminis*, *Lb. fermentum*, *Lb. gasseri*, *Lb. helveticus*, *Lb. johnsonii*, and *Lb. reuteri* were anaerobically cultured at 37 °C in MRS (De Man, Rogosa and Sharpe) medium, which is composed (per liter) of typtone, 10 g; yeast extract, 5 g; glucose, 20 g; ammonium citrate, 0.58 g; $MnSO_4$, 0.25 g; $CH_3COONa{\cdot}3H_2O$, 3.12 g; Na_2HPO_4, 1.63 g; CH_3COOK, 2.25 g; beef extract, 10 g; Tween-80, 1 mL. The strain *E. coli* DH5α was used for recombinant plasmids construction. The strain *E. coli* BL21 (DE3) was used for heterologous feruloyl esterases expression. These two strains were aerobically cultivated in LB (Luria-Bertani) broth containing 10 g/L typtone, 5 g/L yeast extract, and 10 g/L NaCl. Plasmid pET-22b was used for the ligation of feruloyl esterase genes. When the pET-22b-derivative plasmids were transformed into *E. coli*, the LB medium supplemented with ampicillin at a final concentration of 100 μg/mL was used. To produce ferulic acid from the agricultural waste, the recombinant *E. coli* BL21 (DE3) strains were cultured in LB broth supplemented with 2 % (w/v) de-starched wheat bran.

Ferulic acid and ethyl ferulate were bought from Sigma Chemicals Industries., Ltd. (SanFrancisco, USA). Para-nitrophenyl ferulate (ρNPF) was procured from Shandong Chambroad Holding Co., Ltd. (Shandong, China). Bacterial genomic DNA extraction kit was purchased from Tiangen biotech Co., Ltd. (Beijing, China), and gel extraction kit, plasmid extraction kit, and cycle pure kit were purchased from Omega Bio-tek (Atlanta, USA). These kits were used by following the manufacture's recommended protocols. Phanta max super-fidelity DNA polymerase and Exnase®II were purchased from Vazyme Biotech Co., Ltd. (Nanjing, China). Restriction enzymes were purchased from TaKaRa Biotech Co., Ltd. (Tokyo, Japan). The wheat bran was procured from a local mill of Jinan city (Shandong, China). All other regents were bought from Solarbio Science & Technology Co., Ltd. (Beijing, China).

Cloning and expression of *Lactobacillus* feruloyl esterases

The *Lactobacillus* strains were cultivated in MRS medium at 37 °C for 12 h. The cells were collected by centrifugation at 6000×g for 5 min and washed twice by sterile water. Then the genomic DNAs of these *Lactobacillus* strains were extracted by using bacterial genomic extraction kit as described above. The amount and quality of the obtained DNAs was determined with a microspectrophotometer (Eppendorf, Hamburg, Germany), and then stored at -20 °C until to use. Based on the related reports and the genome sequences deposited in NCBI database, the primer sets were designed for amplification of these *Lactobacillus* feruloyl esterase coding genes, respectively. As shown in Table S1, the nucleotides pairing with the feruloyl esterase gene sequence are in uppercase letter, and the nucleotides pairing with the pET-22b vector are in lowercase letters. The PCR amplification procedure contained an initial denaturation at

95 °C for 3 min, followed by 30 cycles each of denaturation at 95 °C for 30 s, annealing at 50 °C for 30 s, extension at 72 °C for 45 s, and then a final extension at 72 °C for 5 min. The obtained feruloyl esterase genes were extracted from the gel after electrophoresis. The pET-22b vector was digested by *Nde* I and *Xho* I, and then purified. To ligate the gene into pET-22b, these two fragments were mixed with the molar ratio of 2:1, and the Exnase®II was used to activate the homologous recombination. After incubated at 37 °C for 30 min, the reaction mixture was transformed into the *E. coli* DH5α competent cells by heat shock method. The correct transformants were selected by colony PCR, and their plasmids were extracted and sequenced in Sangon Biotechnology Co. Ltd. (Shanghai, China). The putative signal peptides of these feruloyl esterases were predicted by using the signalP program (http://www.cbs.dtu.dk/services/SignalP/) and TatP program (http://www.cbs.dtu.dk/services/TatP/).

These generated pET-22b-derivative plasmids were further transformed into *E. coli* BL21 (DE3) cells. The transformants were picked up and inoculated into LB medium supplemented with 100 μg/mL ampicillin and cultivated at 37 °C in a shaker with 200 rpm. To express the feruloyl esterases, the inducer isopropyl-β-D-thiogalactopyranoside (IPTG) at a final concentration of 0.5 mM was added into the cultures when the growth of cells reached an OD_{600} of 0.5, and then the incubation was continued for an additional 72 h at 37 °C.

Feruloyl esterase activity determination

The LB plate-based assay was conducted to detect the feruloyl esterase activity of the recombinant strains and extracellularly secretory component. At the plate-pouring stage of LB medium, 100 μg/mL ampicillin, 0.5 mM IPTG, and 6.7 mM ethyl ferulate (dissolved in dimethylformamide) were added and fully mixed. The recombinant *E. coli* BL21 (DE3) strains were inoculated in the plates to detect the expression and activity of heterologous feruloyl esterases. In addition, a volume of 200 μL cell-free culture supernatant was added into the Oxford cup placed in the plate to preliminarily detect the extracellular feruloyl esterase activity. All these plates were incubated at 37 °C, and the formed transparent circle was observed and photographed.

The substrate ρNPF, which could be hydrolyzed by feruloyl esterase to produce the ρ-nitrophenol with a yellow color, was used to quantitatively determine the feruloyl esterase activity. The 1 mM substrate solution was prepared by adding 25 mM ρNPF (dissolved in dimethyl sulfoxide) into sodium phosphate buffer (100 mM, pH 7.0) which was supplemented with Tween-80 (1 %, v/v) previously. Then, 100 μL of sample was mixed with 900 μL substrate solution to initialize the reaction. After incubation at 37 °C for 10 min, 1 mL of acetic acid solution (50 %, v/v) was added into the mixture to terminate the reaction. Meanwhile, the control experiments were performed by using the inactivated sample. The released ρ-nitrophenol was determined at 410 nm using a spectrophotometer (Eppendorf, Hamburg, Germany). At the conditions described above, the required enzyme amount to produce 1 μmol ρ-nitrophenol in 1 min was calculated as one unit (U) of feruloyl esterase activity.

Protein analysis

The extracellular protein contents of *E. coli* cells cultured in LB medium were estimated by the Bradford protein assay in which the bovine serum albumin was used as standard [22]. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was used to detect the protein bands of the whole cell, cytoplasmic component, and extracellular component of *E. coli*. The gel was composed of a 5 % stacking gel and a 12 % separating gel. After electrophoresis, the Coomassie brilliant blue staining solution was applied to visualize the protein bands. The molecular mass was indicated by the standard protein molecular weight marker.

The whole cell, cytoplasmic component, and extracellular component of *E. coli* BL21 (DE3) were prepared as follows. The recombinant strain cultures after induction were centrifugated at 6,000×g, 4 °C for 5 min to separate the cells and supernatant. The supernatant was filtered through a 0.22-μm filter, and represented the extracellular component. The harvested cells were resuspended in an equal volume of sodium phosphate buffer (100 mM, pH 7.0). This represented the whole cell of recombinant *E. coli*. A ultrasonic breaker (Tenlin, Jiangsu, China) was used to break the cell suspension with the conditions of power 400 w, pulse 5 s, pause 5 s, cycle 49 at 4 °C. The cell-free extract was obtained by centrifugation at 17,400×g, 4 °C for 10 min and filtering through a 0.22-μm filter, representing the cytoplasmic component. For electrophoretic analysis, all the components were mixed with 5×SDS-PAGE loading buffer and then boiled for 10 min.

Release of ferulic acid from de-starched wheat bran

The ferulic acid releasing ability by these recombinant strains expressing *Lactobacillus* feruloyl esterases was investigated using de-starched wheat bran as substrate. A previously reported method was performed to prepare the de-starched wheat bran [23]. In brief, 100 g fresh wheat bran was treated with amylase (0.3 %, w/v) at 65 °C for 30 min, and then with papain (0.3 %, w/v) at 55 °C for 45 min. The reaction mixture was boiled for 20 min to inactivate these enzymes. After centrifugation, the wheat bran was collected and washed repeatedly using distilled water for removing starch completely. Subsequently, the de-starched wheat bran was dried to constant weight at 80 °C and milled to passing a 60-mesh sieve. For ferulic acid production, the medium was prepared by adding 0.1 g de-starched wheat bran into a tube containing 5 mL LB broth, and then treated by autoclave. The recombinant *E. coli* strains were inoculated in the medium and cultivated at 37 °C in a shaker of 200 rpm. The culture samples were taken out after 72 h induction by IPTG, and analyzed by high performance liquid chromatography (HPLC) as described below.

The HPLC (Shimadzu, Kyoto, Japan) was equipped with a CBM-20A communications bus module, a LC-20AT pump, a SIL-20A auto sampler, CTO-10A column oven, a reversed-phase WondaCract ODS-2 C18 cartridge, and a SPD-M10Avp photodiode array detector. This system was eluted by a mobile phase (methanol, water, and acetic acid as a ratio of 50:49.5:0.5) with a flow rate of 1 mL/min at 30 °C. Absorbance of the eluent was monitored at 320 nm. The cultures were boiled for 30 min and centrifuged at 10,000×g for 15 min. The supernatant was harvested and filtered through a 0.22-μm filter before HPLC analysis. The standard ferulic acid was used to qualitative and quantitative analysis of the samples.

Statistical analysis

Each experiment was done in triplicate. All statistical procedures were performed using the statistical packages for the social sciences (SPSS).

Abbreviations

IPTG: isopropyl-β-Dthiogalactopyranoside; SDS-PAGE: sodium dodecylsulphate polyacrylamide gel electrophoresis; HPLC: high performance liquid chromatography

Declarations

Authors' contributions

ZSX and JK conceived and designed the experiments, ZSX and SSZ carried out the experimental work. ZSX, JK, TW and XLL wrote and revised the manuscript. All authors read and approved the final manuscript.

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Not applicable.

Competing interests

The authors declare that they have no competing interests.

Availability of data and materials

All data generated or analysed during this study are included in this published article

Consent for publication

Not applicable.

Ethics approval and consent to participate

Not applicable.

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Tables

Table 1: Plasmids used in this study

Plasmids	Characteristics	References
pET22b-FaeLac	Amp^r, pET-22b vector ligated with feruloyl esterase gene of *Lb. acidophilus*	This study
pET22b-FaeLam	Amp^r, pET-22b vector ligated with feruloyl esterase gene of *Lb. amylovorus*	This study
pET22b-FaeLcr	Amp^r, pET-22b vector ligated with feruloyl esterase gene of *Lb. crispatus*	Our laboratory
pET22b-FaeLfa	Amp^r, pET-22b vector ligated with feruloyl esterase gene of *Lb. farciminis*	This study
pET22b-FaeLfe	Amp^r, pET-22b vector ligated with feruloyl esterase gene of *Lb. fermentum*	This study
pET22b-FaeLga	Amp^r, pET-22b vector ligated with feruloyl esterase gene of *Lb. gasseri*	This study
pET22b-FaeLhe	Amp^r, pET-22b vector ligated with feruloyl esterase gene of *Lb. helveticus*	This study
pET22b-FaeLjo1	Amp^r, pET-22b vector ligated with one feruloyl esterase gene of *Lb. johnsonii*	This study
pET22b-FaeLjo2	Amp^r, pET-22b vector ligated with the other feruloyl esterase gene of *Lb. johnsonii*	This study
pET22b-FaeLre	Amp^r, pET-22b vector ligated with feruloyl esterase gene of *Lb. reuteri*	This study

Figures

Figure 1

The transparent zones formed by the extracellular cell-free supernatant of the recombinant E. coli BL21(DE3) expressing different Lactobacillus feruloyl esterases. FaeLac was derived from Lb. acidophilus. FaeLam was derived from Lb. amylovorus. FaeLcr was derived from Lb. crispatus. FaeLfa was derived from Lb. farciminis. FaeLfe was derived from Lb. fermentum. FaeLga was derived from Lb. gasseri. FaeLhe was derived from Lb. helveticus. FaeLjo1 and FaeLjo2 were derived from Lb. johnsonii. FaeLre was derived from Lb. reuteri.

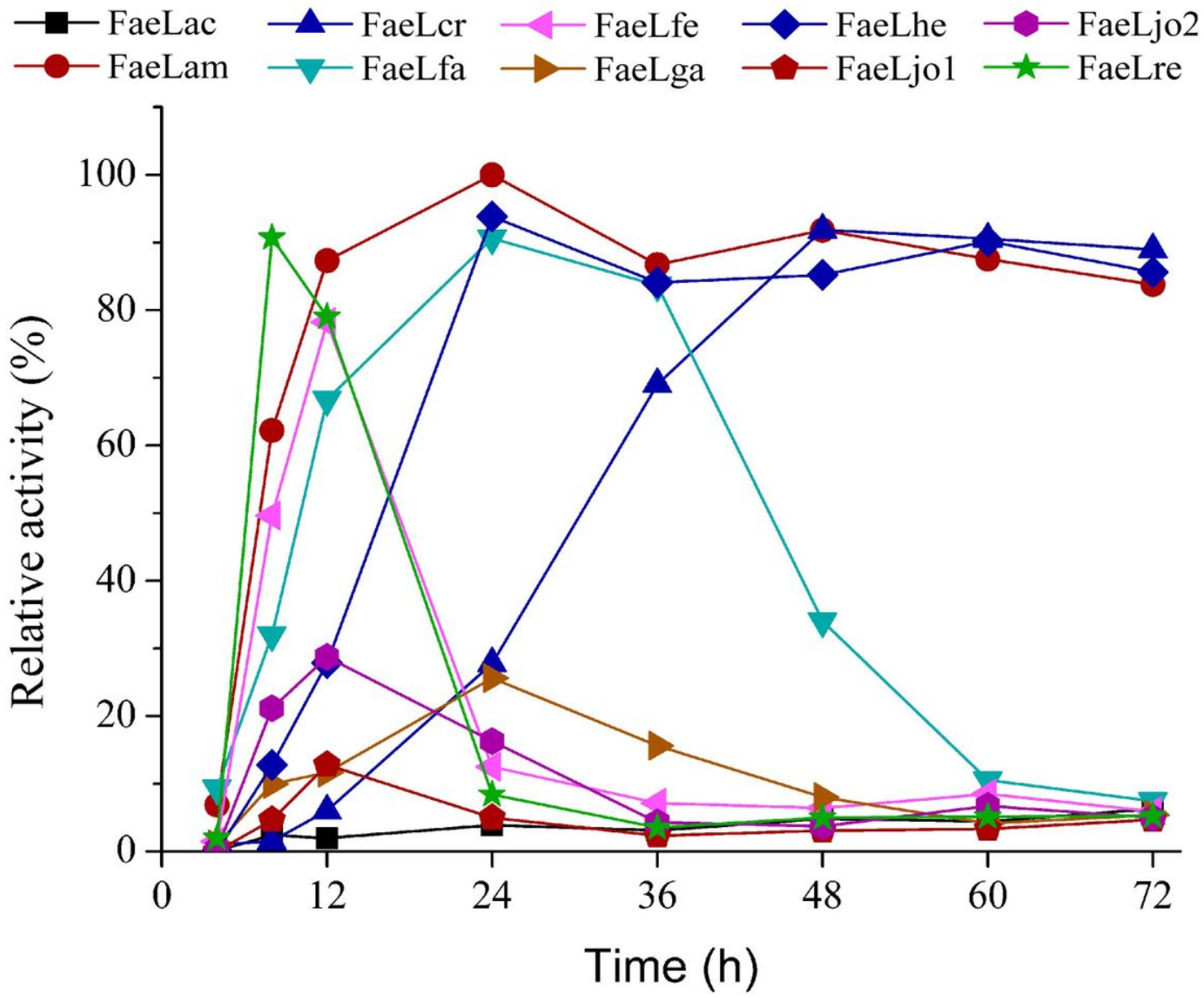

Figure 2

A time course study of extracellular feruloyl esterase activities of these recombinant E. coli BL21(DE3) strains. Activity was determined at 37 °C using ρNPF as substrate. Value are means, and the standard deviation not more than 3.45%. The detected maximal activities were defined as 100 %.

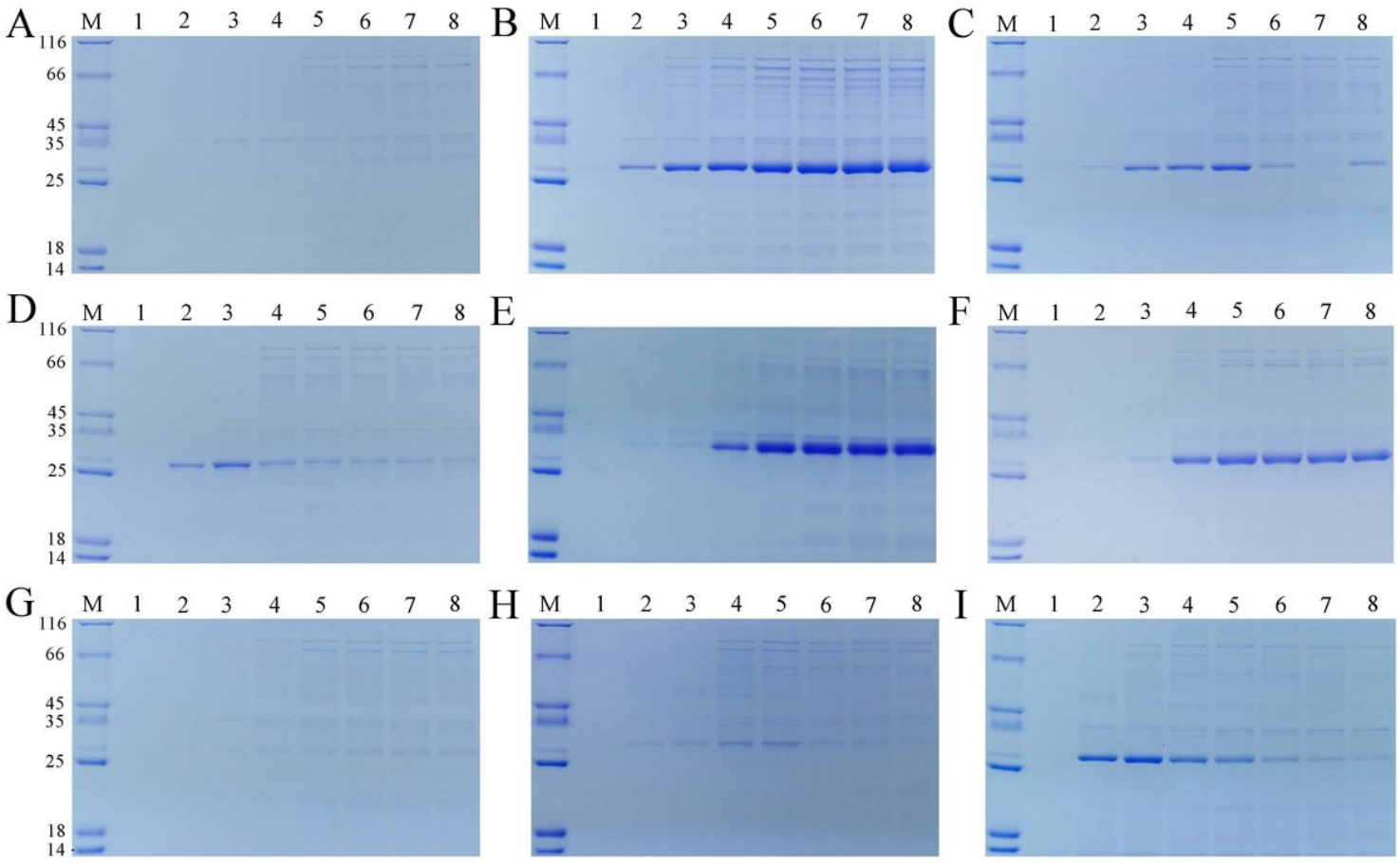

Figure 3

The extracellular protein profiles of the E. coli expressing feruloyl esterases derived from different Lactobacillus species, including Lb. acidophilus (A), Lb. amylovorus (B), Lb. farciminis (C), Lb. fermentum (D), Lb. gasseri (E), Lb. helveticus (F), Lb. johnsonii (G and H), and Lb. reuteri (I). Lane 1-8 represented the samples of 4, 8, 12, 24, 36, 48, 60, 72 h, respectively.

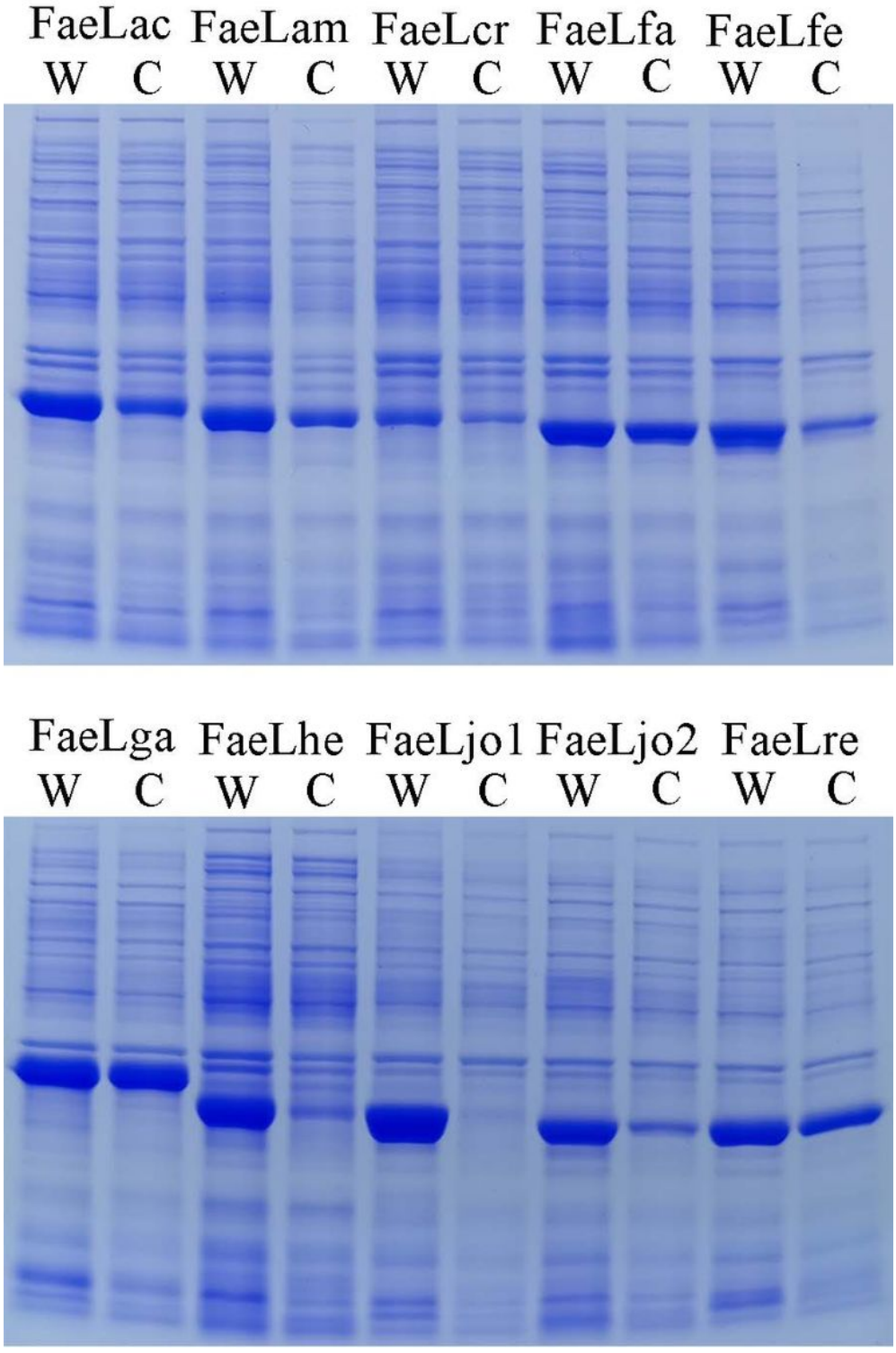

Figure 4

The whole cell (W) and cytoplasmic component (C) of recombinant E. coli expressing feruloyl esterases derived from different Lactobacillus species.

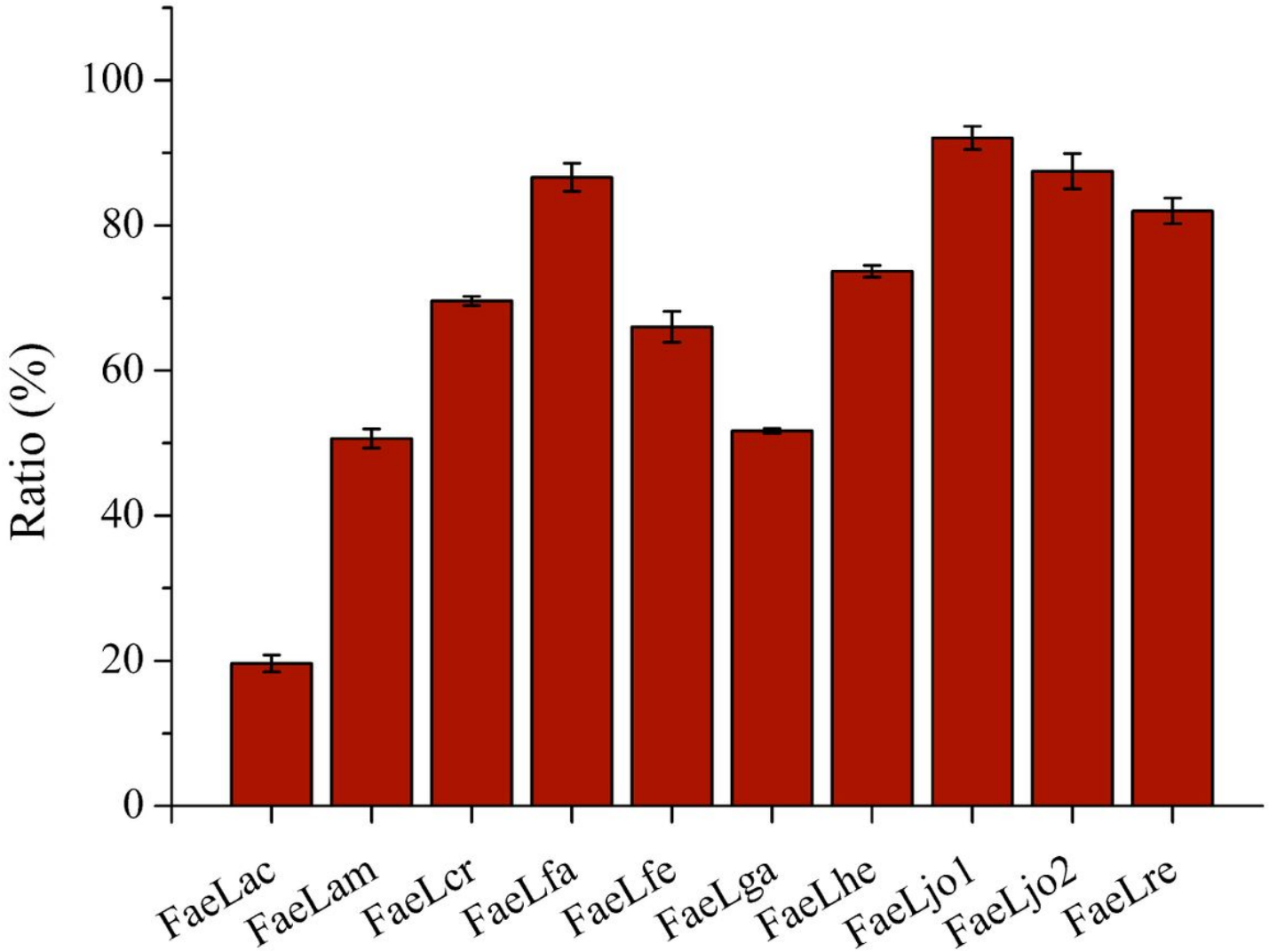

Figure 5

The ratio of extracellular feruloyl esterase activity to total (extracellular plus cytoplasmic) feruloyl esterase activity.

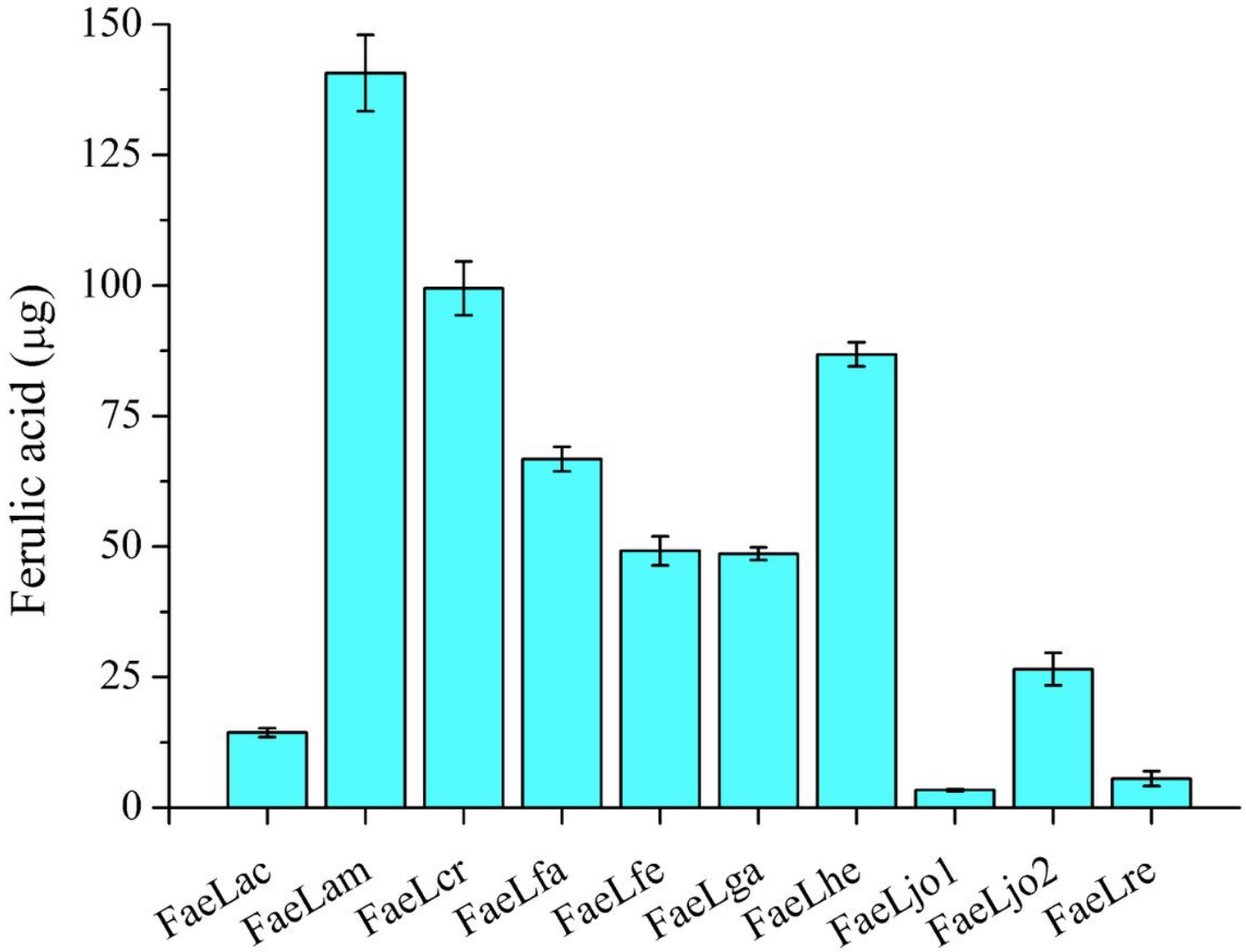

Figure 6

The releasing of ferulic acid from de-starched wheat bran by the recombinant E. coli strains expressing Lactobacillus feruloyl esterases.

Supplementary Files

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