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Article

Heterologous Expression and Functional Analysis of *Exiguobacterium* Algin Lyase Gene by *Pichia Pastoris*

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Abstract: Algin is the most abundant substance in alga. Alginate lyase degrades algin and produces algin monosaccharides, disaccharides and oligosaccharides which widely used in bioenergy, food, medicine and other fields. In this study, one *Exiguobacterium* strain isolated from rotten kelp exhibited a robust ability to degraded the alga. The sequencing of this strain revealed the presence of three different types of algin alginate lyase. Nevertheless, the expression of three genes in the *Escherichia coli* revealed a lower alginate lyase activity compared to that of the original strain. After codon optimization, the gene with the highest activity of the three was successfully expressed in *Pichia Pastoris* to produce recombinant EbAlg664. The activity of the recombinant enzyme in 5L high-density fermentation reached 1306 U/mg protein, 3.9 times that of the original *Exiguobacterium* strain. The results of enzymatic analysis revealed that the optimal temperature and pH range of recombinant EbAlg664 were narrower compared to the original strain. Additionally, the presence of Cu^{2+} and Co^{2+} enhances the enzymatic activity, whereas Mg^{2+} and Fe^{3+} exhibit inhibitory effects on the recombinant alginate lyase. The study offers a theoretical and practical foundation for the industrial-scale production of engineered *Pichia Pastoris* with high alginate lyase activity.

Keywords: Alginate lyase; *Exiguobacterium*; heteroexpression; *Pichia Pastoris*; High-density fermentation

1. Introduction

Algin is a crucial component of the cell wall in algae [1]. Due to its excellent thickening, gelation, and biosecurity properties, algin has found extensive applications in the food, medicine, cosmetics, and other industries [2–4]. Algin primarily consists of β-D-mannuronic acid (M) and α-L-guluronic acid (G), which are isomers connected through β-1, 4-glucoside bonds. Depending on the monomer type, algin exists in three common forms: polyguluronic acid (poly G), polymannuronic acid (poly M), and a combination of both monomers alternately (poly MG) [5–7]. Through beta-elimination mechanism (β-eliminate), alginate lyase acts on the 1-4 glycosidic bond between monomers to degrade algin into various lengths of monosaccharide, disaccharide or oligosaccharide fragments [8]. Among these fragments, algin monosaccharides and disaccharides can be fermented to ethanol for use in bioenergy. Additionally, algin oligosaccharides produced by alginate lyase have been widely employed in medicine, food industry as well as agriculture due to their diverse biological activities such as immune regulation, anti-tumor effects, antioxidant properties, and anti-inflammatory capabilities that hold significant potential value within the medical field. Furthermore, in terms of food preservation, the antibacterial characteristics exhibited by aligin oligosacharides can effectively extend the shelf life of food products. Additionally, in agriculture, it also plays a role in promoting crop root growth [9–11].

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As a crucial enzyme for the production of algin oligosaccharides, alginate lyase features high reaction efficiency, mild reaction conditions and strong controllability [12]. It is convenient for the directional preparation of algin monosaccharides, disaccharides and oligosaccharides, and holds far-reaching application prospects. At present, more than 50 kinds of alginate lyase have been isolated from algae, marine mollusks and soil bacteria [13]. Most of the alginate lyase come from bacteria and fungi, especially from bacteria, such as *Pseudomonas*, *Microbulbifer*, *Flavobacterium* and so on [14–16]. In recent years, there have been many studies on cloning the genes of alginate lyase from different sources and heterologous expression in different host organisms. Masayuki et al. discovered a gene in the *Pseudomonas aeruginosa* genome that encodes a protein homologous to *Sphinsinomonas* alginate lyase A1-II (PA1167). Overexpression of this gene in *Escherichia coli* resulted in the degradation of sodium alginate and subsequent release of unsaturated sugars. [17]. Benwei hu et al. cloned and identified a novel dual-function enzyme FsAlgB from the deep-sea *Flammevirga* sp. The recombinant FsAlgB demonstrates both alginate lyase and endoenzyme characteristics, being capable of recognizing a tetrasaccharide as the smallest substrate and cleaving the glycosidic bond between related sites. [18]. Min Yang et al. cloned the gene *alym*, which encodes a new alginate lyase from *Microbulbifer* sp. Q7 and expressed it in *E. coli*. The recombinant AlyM demonstrates maximum activity at pH 7.0 and 55 ℃, and it shows a specific preference for PolyG [19].

In this study, a strain was isolated from rotten kelp and was found to possess strong alginate lyase activity. After identification, it was ascertained that the strain belongs to the *Exiguobacterium*, a deep-sea microbial species. *Exiguobacterium* is a genus of Gram-positive bacteria [20]. It originates from a wide array of sources and can be isolate in various environments such as soil, sediment, frozen soil, glacier, and others within seawater. *Exiguobacterium* can thrive in an environment ranging from -12 ℃ to 55 ℃ and demonstrates a strong tolerance to NaCl, with the maximum tolerance concentration reaching up to 15%. There exist numerous types of enzymes in *Exiguobacterium*, including amylase, cellulase, lipase, protease, pectinase, alginate lyase lyase, and so forth. However, there are relatively few studies on the alginate lyase of *Exiguobacterium* [21–23].

By means of whole-genome sequencing, it was discovered that the Exiguobacterium strain encompasses three types of alginates lyase, namely Ebalg660, Ebalg664, and Ebalg665. Subsequently, three genes of alginate lyase were cloned and heterologously expressed in E. coli separately. Through codon optimization, the gene with the highest alginate lyase activity was heterogeneously expressed in Pichia pastoris, and a recombinant enzyme with high alginate lyase activity was successfully obtained. By conducting enzymatic properties analysis on the recombinant enzyme, the optimal reaction temperature and pH conditions were determined, and the impacts of several metal ions on its activity were clarified. These discoveries will provide references for the industrial production and application of alginate lyase.

2. Materials and Methods

2.1. Culture Source and Medium

Exiguobacterium HH1 strain, isolated from rotten kelp in Weihai City, Shandong province, was stored in 2216E medium (peptone 5 g/L, yeast extract 1 g/L, iron phosphate 0.01 g/L, sodium chloride 35 g/L) [24]. *E. coli*, preserved in LB medium (yeast extract 5 g/L, tryptone 10 g/L, sodium chloride 10 g/L). Pichia X33 (Invitrogen), stored in YPD medium (yeast extract 10 g/L, peptone 20 g/L, glucose 20 g/L).

2.2. Culture and Optimization of Exiguobacterium

2216E and LB medium with different NaCl concentrations were used to culture *Exiguobacterium* strain. Firstly, the *Exiguobacterium* strain were inoculated into 2216E liquid medium for activation. When OD600 was adjusted to 1.0, the same volume of bacterial solution was transferred to 2216E and LB liquid medium with different NaCl concentrations (0.5-2%) for shake culture at 28 ℃ and 180 rpm. The OD600 value was monitored regularly, the growth curve was drawn, and the optimal culture conditions were determined.

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2.3. Cloning of Alginate Lyase Gene

The genomic DNA was extracted from *Exiguobacterium* using a Bacterial Gen DNA kit. Based on the data of genome sequencing of *Exiguobacterium*, specific primers were designed and synthesized (Table 1). These genes were amplified by polymerase chain reaction (PCR) from the genomic DNA. The PCR conditions were as follows: a hot start at 94℃ for 5 min, 35 repeated cycles of 94℃ for 30 sec, 62℃ for 30 sec, and 72℃ for 2-3 min, followed by one cycle of 72℃ for 10 min. The PCR products were purified from agarose gels. The purified DNA fragments were ligated to pEASY-Blunt (Transgen Biotech. Co., China) and the plasmids were transformed into *E. coli* DH5α cells. The resulting recombinant plasmids were isolated from a positive clone and sequenced.

Table 1. Sequences of the primers used in this study.

Primer Name	Primer sequences (5′→3′)
Ebalg660 F	GGATCCATGAAACGAATCTTACTCGTCCTCG
Ebalg660 R	CTCGAGTTAGATGGGCACACTGATCTGTCT
Ebalg664 F	GGATCCATGAAACCTTTGTATACACGATTATCG
Ebalg664 R	CTCGAGTCATTTTGGTACGTTGATCGTATTT
Ebalg665 F	GGATCCATGAGGGAACGAATGGAACATT
Ebalg665 R	CTCGAGTCAATATGGAATTCGGATGACG
664(*Pichia*) F	GAATTCATGAAACCTTTGTATACACGATTATCC
664(*Pichia*) R	CTCGACTCATTTTGGTACGTTGATCGTATTG

Restriction sites are underlined; F denotes forward primers; R denotes reverse primers;.

2.4. Expression of Alginate Lyase Genes in E. coli

The genes were excised from the pEASY-Blunt recombinant plasmids using one pair of restriction enzymes, BamHI and XhoI, and ligated with the pET-30a (+) vector, which was digested with the same pair of restriction enzymes. The ligation of the DNA insert was conducted overnight at 16℃ using T4 DNA ligase. *E. coli* BL21(DE3) cells were transformed with the ligation mixture and plated on Luria–Bertani (LB) agar containing kanamycin (50 μg/mL) [25]. Positive colonies were screened by direct colony PCR using vector-specific primers (T7 promoter and T7 terminator primers). *E. coli* BL21(DE3) cells, transformed with the recombinant plasmid pET-30a, were grown in LB medium containing 50 μg/mL kanamycin on a rotary shaker at 200 rpm at 37 ° C. When the absorbance at 600 nm reached 0.6, 0.1mM IPTG was added to the culture medium, and the cultures were incubated further at 25℃ for 12 h [26].

2.5. Heterologous Expression of Alginate Lyase Genes in Pichia Pastoris

The *Ebalg664* gene was entrusted to Tsingke Biological Company for codon optimization, and the optimized gene was used as the template to redesign primers (Table 1) to amplify linear fragments with restriction enzymes of *EcoR* I and *Sal* I. The fragment of *Pichia Pastoris* expression plasmid pPICZaA, which had also been digested by *EcoR* I and *Sal* I, was connected to construct the vector pPicZaA-*Ebalg664*, which was transformed into *E. coli* DH5α for further amplification. Then, the plasmid was extracted, digested with *Sac* I enzyme, linearized, and transferred into X33 cells of *Pichia pastoris* (methods are described in the operation manual of Invitrogen Company). The positive clones were selected by YPD culture medium containing 100 μg/mL Zeocin antibiotic.

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2.6. Extraction of Alginate Lyase and Determination of Its Activity

The recombinant *Pichia Pichia* strains were added to YPD medium containing 0.5% methanol for high density fermentation in a 5L fermenter. The alginate lyase activity in the fermentation solution was measured every 24 hours.

Take 10 mL of the culture solution with OD600 1.0, centrifuge at 5,000 rpm for 5 minutes at 4°C, separate the supernatant from the cells, and add 3.0 mL of 0.05 mol/L Tris-HCl (pH 7.0) buffer, 30 μL of lysozyme, and 30 μL of PMSF to the cell pellet. Sonicate the cell pellet using a sonicator. Subsequently, the liquid underwent ultrafiltration with 10K and 100K membranes to obtain the crude enzyme solution. Additionally, another 10mL tube were filled with the reaction mixture (3.8 mL of Tris at 2.42 g/L, 11.7 g/L of sodium chloride, and 10 g/L of sodium alginate), and 200 μL of fermentation supernatant or cell lysate extract were added separately. The reaction was carried out at 40°C for 20 minutes, and immediately 40 μL of 10 mol/L NaOH was added to terminate the reaction. Distilled water was used as the control and each sample was measured three times. The absorbance at 235 nm was determined, and one enzyme activity unit was defined as the increase in absorbance by 0.1 units per minute.

The molecular mass of the alginate lyase was estimated by SDS-PAGE, using a vertical gel electrophoresis system. SDS-PAGE was performed according to the procedure described by Laemmli [27].

2.7. Effects of pH and Temperature on Alginate Lyase Activity

The alginate lyase activity was determined in different pH buffers (sodium acetate (pH 2.0-5.0), sodium phosphate (pH 5.0-8.0), and Tris -- HCl (pH 8.0-10.0)). The effect of temperature on alginate lyase activity was determined at different temperatures ranging from 20℃ to 70℃ [26].

The activity of the above treated samples was assayed by the standard method, as given above. The relative activity was expressed as the ratio of alginate lyase activity under a certain condition to its maximum activity.

2.8. Effects of Metal Ions on Alginate Lyase Activity

The effects of metal ions (K^+, Ca^{2+}, Mg^{2+}, Mn^{2+}, Zn^{2+}, and Cu^{2+}) on alginate lyase activity were evaluated using sodium phosphate buffer (pH 7.0). The enzyme was incubated for 30 min at 37℃ with 5 mM of metal ions prior to the addition of substrate. The activity without added metal ions was taken as 100% activity [26].

2.9. Statistical Analyses

All data from the three replicates were expressed as the mean ± SD and analyzed by the LSD-t test at $p < 0.05$ using SPSS software (version 26.0; IBM, NY, USA) to assess the statistical difference between mean values. Different letters are used for identification.

3. Results

3.1. Media for the Growth and Alginate Lyase Activity of Exiguobacterium

In the selection and cultivation of *Exiguobacterium*, the 2216E medium is typically employed. In this study, we chose different NaCl concentrations of the LB medium and analyzed the growth of *Exiguobacterium* as well as the activity of alginate lyase to determine the optimum conditions for their growth and enzyme production. The results showed that (Figure 1), *Exiguobacterium* has the maximum growth of 4.41×10^8 CFU/mL in the 2216E medium during the stable period. Meanwhile, the highest growth rates in the LB medium with 0.5%, 1%, and 2% NaCl concentrations are 11.2×10^8 CFU/mL, 10.1×10^8 CFU/mL, and 10.2×10^8 CFU/mL respectively. It can be inferred that despite the varying NaCl concentrations in the LB medium, the growth rate of the *Exiguobacterium* in the LB medium is over 50% higher than that of 2216E medium. Additionally, by determining the enzymatic activity of alginate lyase in the supernatant and sediment of the *Exiguobacterium* fermentation, it was

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found that although the cells were treated with lysozyme and sonication, the enzymatic activity in the supernatant was significantly higher than that in the sediment, indicating that *Exiguobacterium* releases its alginate lyase through extracellular secretion. Furthermore, the results of the enzymatic activity of alginate lyase in different culture media revealed that under the same temperature and pH conditions, the best enzymatic activity was determined in 1% NaCl LB culture medium, reaching 336 U/mg protein. This indicates that although the *Exiguobacterium* lives in the marine environment and can tolerate a NaCl concentration of more than 15% [22], the enzyme activity of alginate is highest in 1% NaCl environment. Regarding the cause of the difference, it remains to be further explored whether it is the amount of enzymes production or the activity of enzymes that has an influence by NaCl.

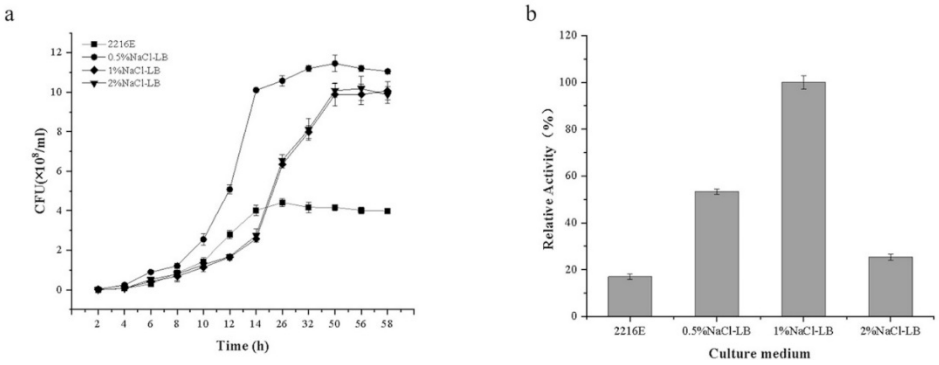

Figure 1. Growth of *Exiguobacterium* in different media and activity of alginate lyase. a: The growth of *Exiguobacterium*; b: Enzyme activity of *Exiguobacterium*.

3.2. Gene Sequencing and Protein Analysis of Exiguobacterium

The gene sequencing showed that there were three different types of algin lyase genes in *Exiguobacterium*, which we named as *Ebalg660*, *Ebalg664* and *Ebalg665* respectively. The total length of *Ebalg660*, *Ebalg664* and *Ebalg665* is 2085 bp, 2094 bp and 2082 bp respectively. The predicted molecular weight of three protein is about 75 KDa. The results of amino acid sequence alignment of the three algin lyase (Figure 2) show that the similarity between EbAlg660 and EbAlg 664 is 58%, between EbAlg 660 and EbAlg 665 is 36.9%, and between EbAlg 664 and EbAlg 665 is 48.6%. The above results indicate that, on the one hand, due to the existence of three different types of alginate lyase in *Exiguobacterium*, it has a very strong ability to hydrolyze algin; on the other hand, as to why the same *Exiguobacterium* strain contains three different types of alginate lyase, it is estimated that it is related to hydrolyzing different types of algin substrates, because algin usually exists in three different polymerization types: PolyG (Poly-α-L-guluronic acid), PolyM (Poly-β-D-mannuronic acid), and PolyMG (mannuronic acid and guluronic acid heteropolymer) [28,29]. These three alginate lyase may have distinct preferences in hydrolyzing the three diverse types of polymer substrates above.

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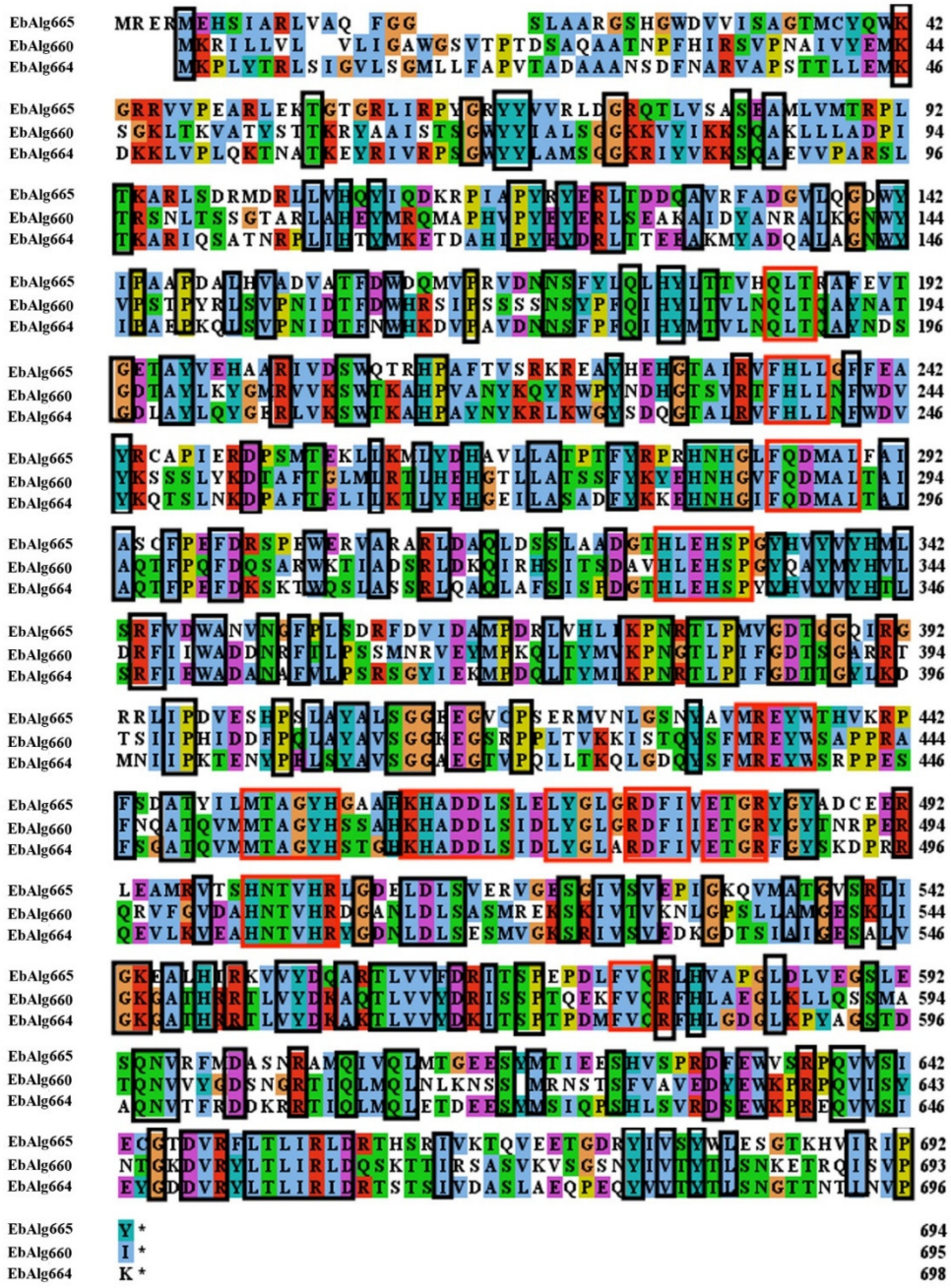

Figure 2. Amino acid sequence of EbAlg660, EbAlg664 and EbAlg665.

3.3. Expression of Alginate Lyase Gene in E. coli

In order to study the characteristics of three alginate lyase from *Exiguobacterium*, we cloned three genes, *Ebalg660, Ebalg664* and *Ebalg665*, respectively, which were inserted into expression vector pet30a and heterogeneously expressed in *E. coli* BL21. According to the results of sequencing the recombinant strains and the SDS-PAGE results of recombinant proteins (data not shown in this article), three different genes have been successfully transformed into *E. coli* BL21 and expressed. However, by measuring the alginate lyase activity of the recombinant protein (Figure 3), it was found that their activity was 76, 135 and 86U/mg protein, respectively, which were lower than that 336U/mg protein of the original *Exiguobacterium*. Regarding the reasons for this result, the first one is that the original *Exiguobacterium* might have interacted jointly with all three alginates enzymes, resulting in its higher activity compared to the recombinant *E. coli* strain with only one algin lyase. On the other

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hand, the recombinant strain has a higher enzyme yield; however, as it is an intracellular protein, it cannot be secreted outside the cell to exert its function. Even if we employ ultrasound and lysozyme to promote bacterial lysis, we cannot ensure that the enzyme activity reaches its maximum value. Another possibility is that the recombinant protein forms an inclusion body, which is disadvantageous for the binding of the enzyme and the substrate, thereby significantly affecting its enzyme activity.

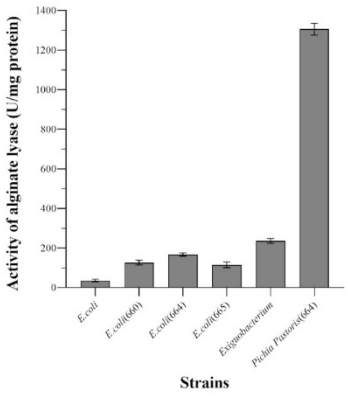

Figure 3. Alginate lyase activity of different strains. *E. coli* and *Exiguobacterium* are original untransformed strains; *E. coli* (660), *E. coli* (664), *E. coli* (665) and *Pichia Pastoris* (664) belong to the recombinant strains containing different algin lyases gene respectively.

3.4. Expression of Alginate Lyase Gene in Pichia Pastoris

In this study, *Ebalg664*, the gene with the highest alginate lyase activity in the heteroexpression of *E. coli*, was selected. Through codon optimization, the *Ebalg664* was inserted to the expression vector pPICZaA, and transformed to Pichia X33 for expression of recombinant alginate lyase. By culturing the recombinant *Pichia Pastoris* strain in a culture medium supplemented with methanol, the supernatant from 24h to 96h was collected for determined the alginate lyase activity. The supernatant of the original strain of *Exiguobacterium* was used as a control. The results showed that the activity of recombinant enzyme reached the highest value at 48 hours (Figure 4a) and the SDS-PAGE results indicated (Figure. 4b) that there were similar protein bands at the corresponding 75KDa position in both the fermentation supernatant and the purified protein, suggesting that the recombinant protein EbAlg664 had been successfully expressed. In recent years, the use of *Pichia pastoris* as a vector for expressing alginate lyase has been a research focus [30–32]. Yang et al. successfully expressed the cAlgM and its thermally mutated variant in *Pichia pastoris* [33]. Li et al. identified the alginate lyase gene *sagl* in *Flavobacterium sp*. H63 and optimized its codons for expression in *Pichia pastoris*, achieving a maximum yield of recombinant SAgl in the fermentation supernatant of 915.5U/mL [34]. Meanwhile, the recombinant enzyme activity in this research reached 1306U/mg protein, which was 3.9 times higher than that of the original *Exiguobacterium* strain (336U/mg protein). These results indicate that it is feasible to use the very mature transformation system of *Pichia pastoris* to heteroexpress the alginate lyase gene of *Exiguobacterium* and produce the recombinant alginate lyase.

a: Activity of alginate lyase of recombinant *Pichia pastoris* at different times; b: SDS-PAGE of *Pichia pastoris* fermentation, Lane M, low-molecular-weight standard; Lane 1, purified protein of recombinant Alg664; Lane 2, fermentation supernatant of recombinant *Pichia pastoris*; Lane 3, fermentation supernatant of original *Pichia pastoris*.

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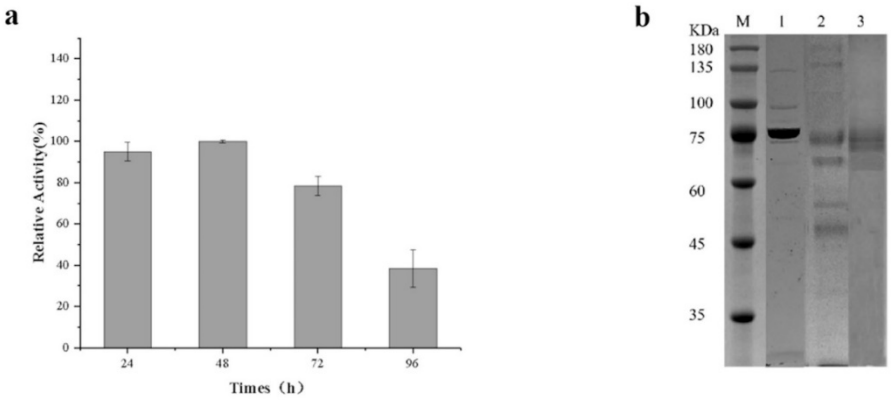

Figure 4. Enzyme activity at different times and SDS-PAGE of *Pichia pastoris* recombinant. a: Activity of alginate lyase of recombinant *Pichia pastoris* at different times; b: SDS-PAGE of *Pichia pastoris* fermentation, Lane M, low-molecular-weight standard; Lane 1, purified protein of recombinant Alg664; Lane 2, fermentation supernatant of recombinant *Pichia pastoris*; Lane 3, fermentation supernatant of original *Pichia pastoris*.

3.5. Enzymatic Properties of Recombinant Alginate Lyase

Given that the activity of *E. coli* recombinase is relatively low, this study focuses on the recombinase of *Pichia pastoris*, using the original *Exiguobacterium* strain as a control, to investigate the optimal pH, temperature, and the influences of different metal ions on their activity. It was found that the optimal temperature of alginate lyase for both the original *Exiguobacterium* and the recombinant *Pichia pastoris* Alg664 were 40℃ (Figure 5a,c), and the optimal pH were 6.0 (Figure 5b,d), which indicates that they were belong to acidic alginate lyase enzyme. However, the range of their optimal conditions varies slightly, for example, for original *Exiguobacterium* enzymes, the temperature range was 35℃-60℃, where 80% of the enzyme activity can be preserved, but for the recombinant enzyme, only the range of 38-45℃ achieves 80% of the highest enzyme activity. At pH 5.0-7.5, the enzyme activity of the original enzymes remains at 80%, but the recombinant enzyme only maintains 80% enzyme activity in the range of pH5.5-7.0, while enzyme activity drops to 60% at pH=5.0. The reason for the differences may be that the original strain contains three different alginate lyase, whose interactions result in a relatively wider range of adaptability.

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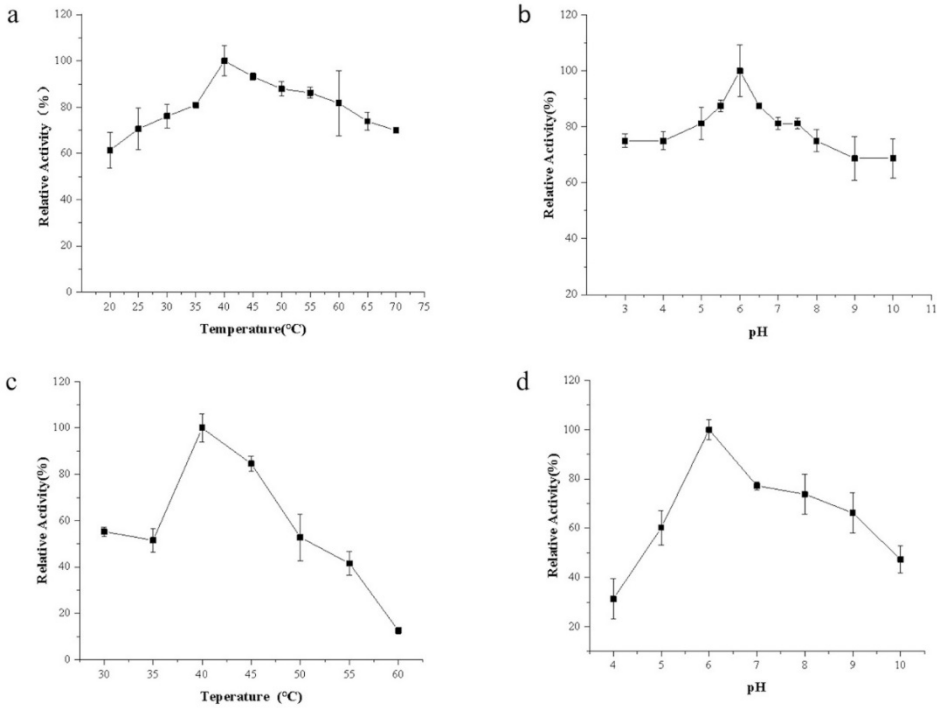

Figure 5. The optimum temperature and pH of original *Exiguobacterium* alginate lyase and recombinant *Pichia pastoris* EbAlg664. a, Optimum temperature of original *Exiguobacterium* alginate lyase; b, Optimum pH of original *Exiguobacterium* alginate lyase; c, Optimum temperature of recombinant *Pichia pastoris* EbAlg664; d, Optimum pH of recombinant *Pichia pastoris* EbAlg664.

The effects of various metal ions on the enzyme activity of recombinant EbAlg664 are shown in Figure 6. The figure shows that compared with the control without adding any ions, the enzyme activity of recombinant EbAlg was increased by 15.85% and 59.82% in 10 mM Cu^{2+} and Co^{2+} conditions, respectively. This suggests that Cu^{2+} and Co^{2+} at these concentrations have a significant promoting effect on the enzyme activity of EbAlg, with Co^{2+} having a more pronounced promoting effect. In contrast, the enzyme activity of EbAlg was not significantly influenced by 1 mM Cu^{2+} and Co^{2+}. Additionally, 1 mM and 10 mM of Mg^{2+} and Fe^{3+} exhibited obvious inhibitory effects on the recombinase，especially 10 mM of Mg^{2+} and Fe^{3+} could reduce the enzyme activity to 67.8% and 64.8%, respectively. Moreover,1 mM and 10 mM of EDTA had no significant influence on the enzyme activity. Furthermore, Mn^{2+}, Zn^{2+}, Ba^{2+}, Ca^{2+}, and Fe^{2+} showed inhibitory effects at a concentration of 1 mM, while the inhibitory effects at a 10 mM were weakened, but still lower than the control enzyme activity.

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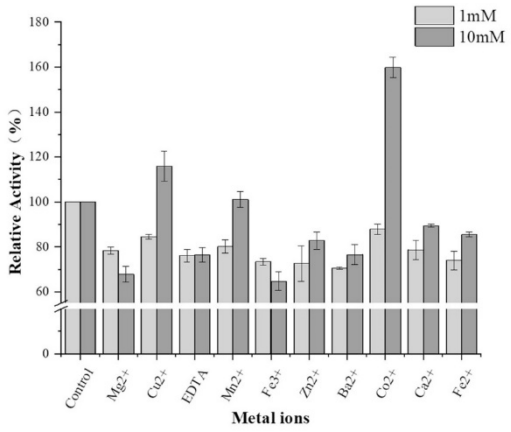

Figure 6. Effects of different ions on enzyme activity of recombinant EbAlg664 at different concentrations.

4. Conclusions

This study centered on a *Exiguobacterium* strain and revealed that it possesses three distinct types of alginates lyase genes, which are expressed through extracellular secretion for the degradation of alginate. The expression of these three genes in *E. coli* revealed that their enzyme activity was lower than that of the original strain. After optimizing the codon of the Ebalg664, which exhibits the highest enzyme activity among the three alginate lyase, in *Pichia pastoris*, a recombinant alginate lyase EbAlg664 was successfully expressed and produced. The activity of the recombinant enzyme reached 1306 U/mg protein, which is 3.9 times that of the original *Exiguobacterium* enzymes. The results of enzymatic analysis showed that the temperature and pH stability of recombinant EbAlg664 was lower than that of the original alginate lyases. Cu^{2+} and Co^{2+} can promote recombinase activity, while Mg^{2+} and Fe^{3+} can decrease the activity of recombinase. The successful expression of recombinant alginate lyase in *Pichia pastoris* offers the theoretical and practical foundation for the industrial production of this enzyme. In the future, we will attempt to further enhance the enzyme's activity and stability. For instance, we may modify key amino acid residues by site-specific mutation. Additionally, we can boost the yield of the enzyme protein by elevating the activity of the promoter and strengthening gene expression.

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Data Availability Statement: The original contributions presented in the study are included in the article, further inquiries can be directed to the corresponding author.

Conflicts of Interest: The authors declare no conflicts of interest.

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