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ORIGINAL PAPER

Production of L-tryptophan by enantioselective hydrolysis of D,L-tryptophanamide using a newly isolated bacterium

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Bacterial strain ZJB-09211 capable of amidase production has recently been isolated from soil samples. The strain is able to asymmetrically hydrolyze L-tryptophanamide from D,L-tryptophanamide to produce L-tryptophan in high yield and with excellent stereoselectivity (enantiomeric excess > 99.9 %, and enantiomeric ratio > 200). Strain ZJB-09211 has been identified as *Flavobacterium aquatile* based on the cell morphology analysis, physiological tests, and the 16S rDNA sequence analysis. Optimization of the fermentation medium led to an about six-fold increase in the amidase activity of strain ZJB-09211, which reached 501.5 U L^{-1}. Substrate specifity and stereoselectivity investigations revealed that amidase of *F. aquatile* possessed a broad substrate spectrum and high enantioselectivity.

Keywords: L-tryptophan, *Flavobacterium aquatile*, amidase, enantioselective biocatalysis

Introduction

L-Tryptophan (L-Trp) has a broad range of applications in the pharmaceutical and food industries, and as a precursor in the preparation of other chiral compounds (Azuma et al., 1993; Koçaba et al., 2006; Mateus et al., 2004). Many chemical and biological methods for L-Trp production have been developed. Current biological methods, which include fermentation and enzymatic synthesis, are less economically attractive than the chemical ones (Katsumata & Ikeda, 1993; Ikeda, 2006; Winnicka & Kańska, 2009; Eggers et al., 1988). The fermentation route suffers from low yields; whereas the enzymatic route requires a chiral substrate as the starting material and is hampered by the inhibitory activity of another substrate, indole. However, the chemical methods usually result in the formation of mixtures of L-Trp and D-tryptophan (D-Trp).

Therefore, improved biological methods for L-Trp production involving stereoselective enzymes are being sought for. Amidases possess excellent enantioselectivity and, therefore, have become promising tools for the synthesis of chiral carboxylic acids and their derivatives (Shaw et al., 2003; Martinkova & Mylerova, 2003; Wang et al., 2010). In particular, amino-acid amidases can steroselectively hydrolyze L- or D-amino-acid amide to the corresponding chiral amino acids and ammonia. Because racemic amino-acid amides can be easily synthesized from aldehydes, hydrogen cyanides, and ammonium (Komeda & Asano, 2008; Asano & Yamaguchi, 2005a, 2005b), amidase-catalyzed asymmetrical hydrolysis of amino-acid amides presents a promising method for the production of optically pure amino acids. Although various microbial amidases have been reported, their enzymatic characteristics vary remarkably (Wang et al., 2008). The amidase activity is also significantly affected by the culture conditions, which means that optimization of the culture medium is a crucial requirement (Açıkel et al., 2010).

Here, isolation of a microorganism that can produce L-Trp in high yields and with excellent enantioselectivity through asymmetric hydrolysis of the L-enantiomer present in racemic tryptophanamide

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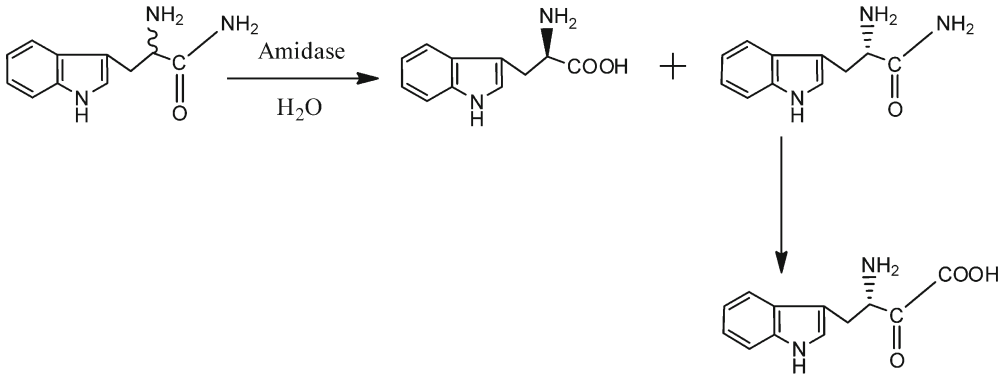

Fig. 1. Production of L-Trp by amidase-mediated stereospecific hydrolysis of D,L-tryptophanamide.

is reported (Fig. 1). Also, taxonomic study of the microorganism and the optimization of the culture medium for amidase production are presented. In addition, the substrate specificity of amidases was investigated.

Experimental

General

Sixty soil samples collected from different locations in China, were used for amide-degrading bacteria screening. D,L-tryptophanamides were purchased from HanHong Chemicals (China). L-Trp, D-Trp, and racemic tryptophan were from J&K Chemicals (China). Other chemicals were of analytical reagent grade and obtained from various commercial sources.

Media and culture conditions

Components of the enrichment medium were as follows: 1.0 g L^{-1} of K_2HPO_4, 1.0 g L^{-1} of KH_2PO_4, 0.2 g L^{-1} of $MgSO_4 \cdot 7H_2O$, 0.01 g L^{-1} of $FeSO_4 \cdot 7H_2O$, 1.0 g L^{-1} of NaCl, and 3.0 g L^{-1} of D,L-tryptophanamide. The enrichment agar medium contained 20 g L^{-1} of agar. pH was adjusted to 7.5 and the medium was then autoclaved at 121 °C for 20 min.

Components of the fermentation medium were as follows: 10.0 g L^{-1} of glucose, 2.0 g L^{-1} of pepton, 5.0 g L^{-1} of beef extract, 1.0 g L^{-1} of azepan-2-one (caprolactam), 1.0 g L^{-1} of K_2HPO_4, 1.0 g L^{-1} of KH_2PO_4, 0.2 g L^{-1} of $MgSO_4 \cdot 7H_2O$, 0.01 g L^{-1} of $FeSO_4 \cdot 7H_2O$, and 1.0 g L^{-1} of NaCl. pH of the fermentation medium was adjusted to 7.5 and the medium was then autoclaved as above.

Isolation of tryptophanamide-degrading bacteria

Microorganisms were isolated from soil samples by an enrichment method. Briefly, the soil samples were suspended in sterile water and then cultured on a rotary shaker at 30 °C, and 150 min^{-1} for three days. The resulting culture broth was transferred to a fresh enrichment medium with the inoculum size of 5 vol. %. After the enrichment procedure was performed three times, the resulting cultures were diluted, spread on the enrichment agar medium and incubated at 30 °C for three days.

Strains growing on the enrichment agar medium were three times purified by sub-culturing on the enrichment agar medium and then cultured aerobically in the fermentation medium at 30 °C for 48 h. The culture broth was centrifuged at 4 °C, 10000 min^{-1} for 10 min, and the pellets were washed with saline (0.85 mass % NaCl). The washed cells were used in the biotransformation.

Taxonomic characterization of strain ZJB-09211

Cell morphology of strain ZJB-09211 was investigated using a light microscope (Olympus CH20, Japan) and a transmission electron microscope (Philips-XL30, The Netherlands). Physiological characterization of the strain was carried out by indole production, starch hydrolysis, and nitrate reduction testing as well as by examining the carbon source utilization using a standardized micromethod (ATB Expression ID32 GN, bioMérieux, France).

16S rDNA sequence determination and phylogenetic analysis

Chromosomal DNA of a pure colony of stain ZJB-09211 was extracted according to the slightly modified procedure of Wilson et al. (1997). The PCR amplification of 16S rDNA was carried out with a TProfessional/Standard thermocycler (Biometra, Germany) using a universal primer set: p16S-8 (5'-AGAGTTTGATCCTGGCTCAG-3') and p16S-1510 (5'-AAGGAGGTGATCCAGCCGCA-3') (Shigematsu et al., 2003). The PCR products were cloned into the pMD-18T vector (Takara, Japan) using the T/A cloning procedure (Fournand et al., 1998; Jakoby & Fredericks, 1964). Related sequences were retrieved

from GenBank (National Center for Biotechnology Information, NCBI, USA) using the BLAST program, and were aligned with the CLUSTAL W algorithm, version 1.81 (Thompson et al., 1994). An unrooted phylogenetic tree of homologous 16S rDNA sequences was constructed by the neighbor-joining method using MegAlign (DNASTAR, Madison, USA).

Preparation of amidase-containing bacterial cells

Amidase-containing cells for biotransformation experiments were prepared by growing strain ZJB-09211 in 250-mL flasks containing 50 mL of the fermentation medium and 2 vol. % of inoculum. The flasks were incubated at 30 ℃ and 150 min^{-1} for 48 h. The resulting culture was centrifuged at 4 ℃ and 12000 min^{-1} for 15 min, and the cells were collected for further study.

Biotransformation

Hydrolysis of tryptophanamides to tryptophan was carried out in 50 mL Erlenmeyer flasks with a screw cap containing 10 mL of distilled water, 20 mM D,L-tryptophanamide and amidase-containing cells (prepared from a 30-mL culture as described above). The flasks were incubated at 150 min^{-1} and 30 ℃ for 10 min, and the reaction was terminated by adding 20 μL of 6 M HCl. The reaction mixture was centrifuged at 10000 min^{-1} and the tryptophan concentration in the supernatants was quantified by HPLC.

One unit of the enzyme activity was defined as the amount of cells required to form 1 μmol of L-Trp per minute under the experimental conditions.

Substrate specificity

Amidase activity of the strain towards various amides was examined in the biotransformation experiments in which the substrate concentration and the amount of wet cells were 20 mM and 0.5 g, respectively. The reactions were carried out as described above.

Optimization of fermentation medium

To select optimal composition of the fermentation medium for cell growth and amidase production, variations in the following elements were tested: carbon sources (glucose, lactose, sodium citrate, sorbitol, glycerin, starch, maltose, sucrose, mannitol, dextrin, and gelatin, all in 1.0 mass %, or 1.0 vol. % concentration), nitrogen sources (peptone, corn steep, beef extract, yeast extract, glutamate, NH_4Cl, $(NH_4)_2SO_4$, and NH_4NO_3, all in 0.7 mass %, or 0.7 vol. % concentration), amidase inducers (caprolactam, phenylacetonitrile, acetonitrile, butyronitrile, acetamide, and niacinamide, all in 1.0 g L^{-1} concentration) and ions (Mg^{2+}, Cu^{2+}, Co^{2+}, Mn^{2+}, Fe^{3+}, Ag^{+}, Ni^{2+}, Ca^{2+}, Ba^{2+}, Fe^{2+}, NO_3^-, Cl^-, SO_4^{2-}, HPO_4^{2-}, $H_2PO_4^-$, CO_3^{2-}, and SO_3^{2-}).

HPLC analysis

D,L-Tryptophanamide and tryptophan were identified and quantified by HPLC (U3000, Dionex, USA) using a C18 column (250 mm × 4.6 mm, Dionex) at 40 ℃. The column was eluted using the following solvents: acetonitrile/water (φ_r = 3 : 7) for 0–2 min; acetonitrile/10 mM KH_2PO_4 (φ_r = 1 : 4) for 2–5 min; and acetonitrile/water (φ_r = 3 : 7) for 5–12 min. The flow rate was 1.0 mL min^{-1}, and the detection wavelength was 278 nm. The retention times for L-Trp and D,L-tryptophanamide were 2.89 min and 5.42 min, respectively. Separation of L-Trp was carried out in a Chirobiotic column (250 mm × 4.6 mm, Astec, USA) at 30 ℃. The elution was performed with 80 vol. % acetonitrile containing 0.2 vol. % of acetic acid at the flow rate of 1.0 mL min^{-1}. The retention times for L-Trp and D-Trp were 9.28 min and 10.23 min, respectively.

Results and discussion

Screening of microorganisms

In recent years, the use of microbial amidases asymmetrically hydrolyzing racemic amides has become a promising method for the production of chiral carboxylic acids (Wang et al., 2010; Zheng et al., 2012; Yang et al., 2011). Amidase-producing microorganisms have usually been isolated by enrichment procedures using culture media containing a specific amide (target substrate or a structural analogue) as the sole source of nitrogen. In this study, a similar strategy was followed by subjecting 60 soil samples to enrichment cultivation in a medium containing D,L-tryptophanamide (3 g L^{-1}) as the sole nitrogen source. The initial screening resulted in the isolation of three amidase-producing microbial strains which were then tested in biotransformation experiments. In these experiments, the released L-Trp was monitored by HPLC analysis. Strain ZJB-09211 showed the highest activity in the biotransformation assays (81.45 U L^{-1}) and was selected for further investigation.

Identification of strain ZJB-09211

Colonies of the selected strain were primrose yellow, smooth, convex, translucent, and mucous on the enrichment medium agar. The cells were short, and rod-like, and their dimensions were 0.3–0.5 μm × 0.8–1.3 μm (Fig. 2). The microorganism was a Gram negative and non-sporulating bacterium able to produce indole, hydrolyze starch and reduce nitrate. Further physiological characterization of the strain was car-

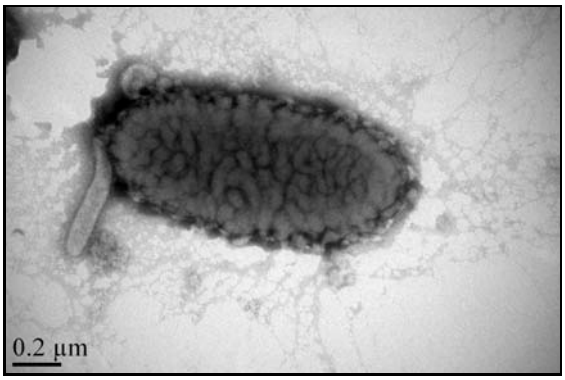

Fig. 2. Transmission electron micrograph of strain ZJB-09211 grown at 30 °C for 24 h.

ried out using the ATB Expression system (ID32 GN, bioMérieux, France); the results (Table 1) indicated that there was a 99.9 % probability that the microorganism belonged to the species *Flavobacterium aquatile*.

To confirm such an identification, a 16S rDNA fragment from the strain was amplified by PCR and its nucleotide sequence (1587 bp) was determined. Then, the 16S rDNA sequence obtained from ZJB-09211 was compared with similar sequences from other bacteria, and an unrooted phylogenetic tree was constructed by the neighbor-joining method (Fig. 3). This anal-

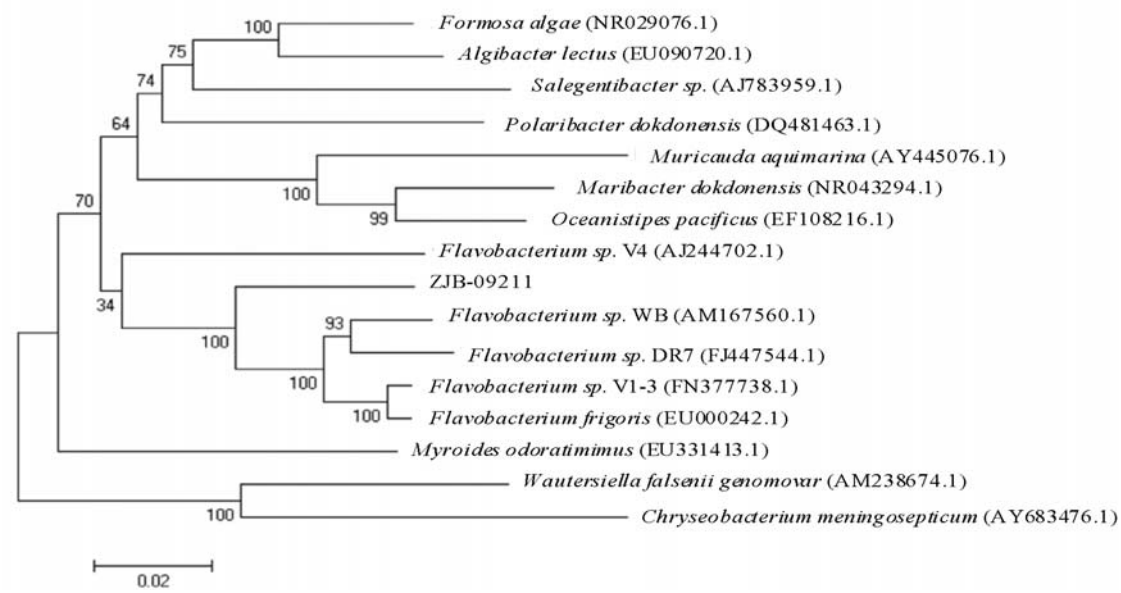

Fig. 3. Phylogenetic tree based on 16S rDNA sequences, constructed by the neighbor-joining method, showing the relationship between strain ZJB-09211 and representatives of some related taxa. Numbers in parentheses are accession numbers of published sequences.

Table 1. Characteristics of strain ZJB-09211

Characteristics	ZJB-09211	Characteristics	ZJB-09211
Gram-staining	−	D-Ribose	−
Oxidase	+	2-Ketogluconate	−
Citrate	−	Inositol	−
Itaconic acid	−	Glucogen	−
Histidine	+	L-Fucose	−
D-Maltose	+	Capric acid	+
D-Mannitol	−	Alanine	+
Arabinose	−	5-Ketogluconate	−
Sodium acetate	+	D-Glucose	+
3-Hydroxybutyric acid	−	Suberic acid	−
Propionic acid	+	Serine	−
D-Sorbitol	−	Sucrose	+
Salicin	+	L-Rhamnose	−
Lactic acid	+	Proline	+
Valeric acid	+	*N*-Acetylglucosamine	+
4-Hydroxybenzoic acid	−	Sodium malonate	+
3-Hydroxybenzoic acid	−	Nitrate reduction	−
D-Melibiose	+	Indole	−

+ – Positive, − – negative.

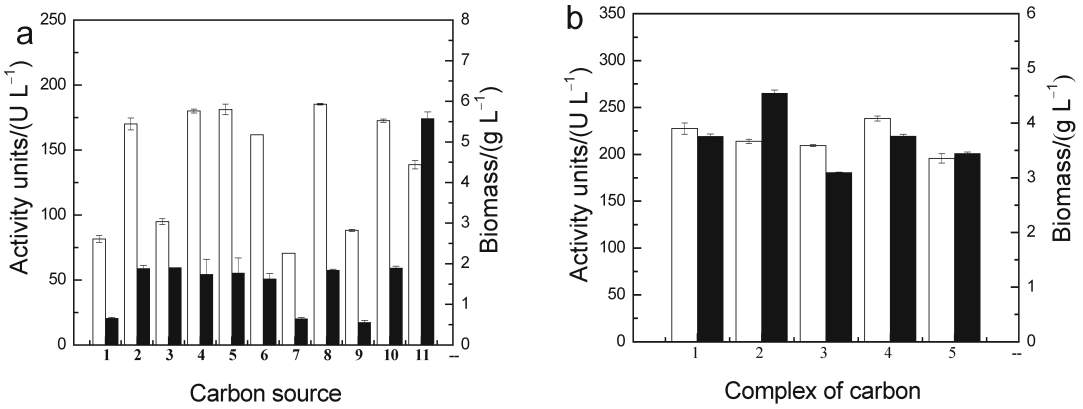

Fig. 4. Effects of different carbon sources (a) and combinations of two carbon sources (b) on amidase activity and biomass. Carbon sources (Fig. 5a); 1 – glucose, 2 – lactose, 3 – sodium citrate, 4 – sorbitol, 5 – glycerin, 6 – starch, 7 – maltose, 8 – sucrose, 9 – mannitol, 10 – dextrin, and 11 – gelatin. Combinations of two carbon sources (Fig. 5b); 1 – glycerin and gelatin, 2 – lactose and gelatin, 3 – starch and gelatin, 4 – sucrose and gelatin, and 5 – maltose and gelatin; □ – activity units, ■ – biomass.

ysis confirmed a strong relationship between strain ZJB-09211 and the members of the genus *Flavobacterium*. In particular, ZJB-09211 was closely clustered in the phylogenetic tree with *Flavobacterium* sp. WB (GenBank accession No. AM167560.1), and their 16S rDNA sequences were identical to 99 %. Results of the phylogenetic analysis were consistent with the physiological tests, the microorganism was therefore designated as *Flavobacterium aquatile* ZJB-09211. The bacterium was deposited in the China Center for Type Culture Collection (Wuhan, China) as strain CCTCC M 2011354.

Effect of different carbon sources on growth and amidase production of strain ZJB-09211

Carbon source has a crucial role in the determination of the growth and metabolic rates of microorganisms. Therefore, as a first step in the optimization of the fermentation medium, eleven carbon sources were tested for their effect on the growth and amidase activity of the ZJB-09211 strain (Fig. 4a). High enzyme activities were obtained with sucrose, sorbitol, or glycerin, whereas maximum cell growth was achieved with gelatin. The results suggest that a combination of different carbon sources leads to even higher yields of both cell growth and amidase production. Therefore, the effects of various combinations of gelatin with other carbon sources were investigated (Fig. 4b). The maximum enzyme activity (238.21 U L^{-1}) was achieved with sucrose and gelatin. To determine the optimum ratio of sucrose and gelatin for amidase production, the effects of different mole ratio of carbon atoms of the two components were tested. As shown in Fig. 5a, the highest amidase production (396.20 U L^{-1}) was obtained when the mole ratio of gelatin carbon atoms was 50 %. To determine the optimum concentration of the gelatin–sucrose combination for amidase production, different carbon concentrations

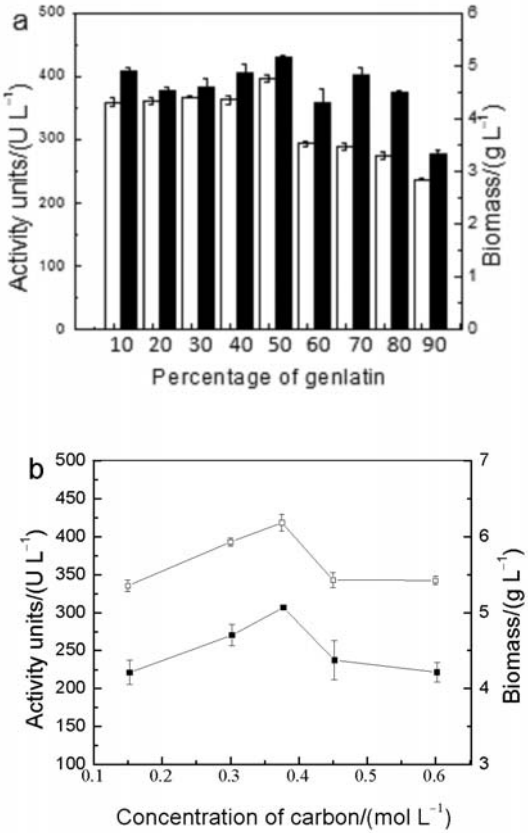

Fig. 5. Effect of different ratios of gelatin (a) and gelatin–sucrose carbon source concentrations (b) on amidase activity and biomass; □ – activity units, ■ – biomass.

(from 0.15 M to 0.60 M) were tested while keeping the 50 % mole ratio of gelatin carbon atoms. Maximum amidase production (418.66 U L^{-1}) was observed for carbon concentrations of 0.40 M (Fig. 5b), which corresponds to 5.48 g L^{-1} of sucrose and 6.25 g L^{-1} of gelatin. Therefore, these concentrations of sucrose and gelatin were used as carbon source in the following tests.

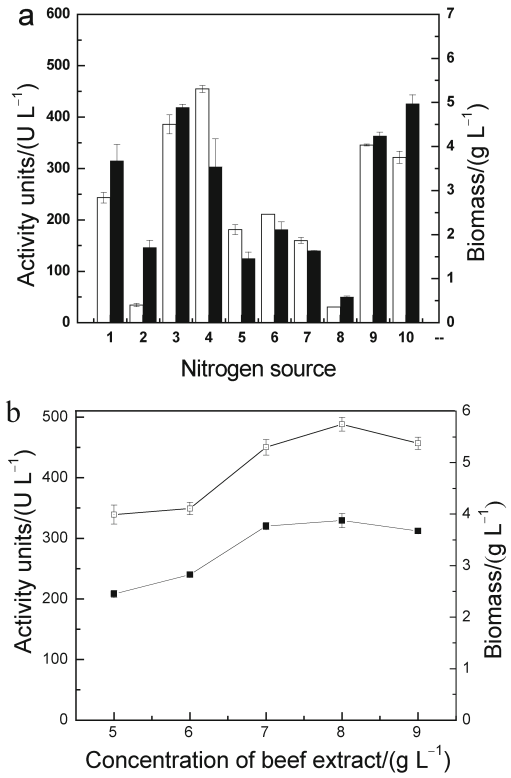

Fig. 6. Effects of different nitrogen sources (a) and beef extract concentrations (b) on amidase activity and biomass. Nitrogen source; 1 – peptone, 2 – corn steep, 3 – yeast extract, 4 – beef extract, 5 – NH_4Cl, 6 – $(NH_4)_2SO_4$, 7 – NH_4NO_3, 8 – glutamate, 9 – beef extract and peptone, and 10 – yeast extract and peptone; □ – activity units, ■ – biomass.

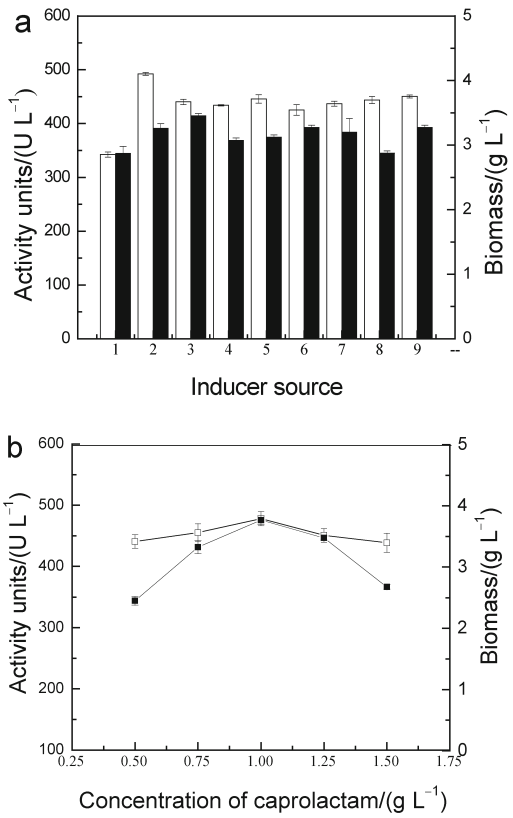

Fig. 7. Effect of different inducers (a) and caprolactam concentrations (b) on amidase activity and biomass. Inducer; 1 – control (no inducer), 2 – caprolactam, 3 – acrylamide, 4 – acetamide, 5 – benzamide, 6 – cyclopropane carboxamide, 7 – methionine amide, 8 – nicotinamide, and 9 – diamino-benzamide; □ – activity units, ■ – biomass.

Effect of different nitrogen sources on cell growth and amidase production

Because organic and inorganic nitrogen sources play an essential role in microbial production of enzymes, the effects of ten different nitrogen sources on the amidase activity were evaluated (Fig. 6a). The maximum enzyme activity (454.77 U L^{-1}) was obtained with beef extract, whereas both amidase activity and biomass were very low when corn steep or glutamate was used as the nitrogen source. In addition, the use of NH_4Cl, $(NH_4)_2SO_4$, or NH_4NO_3 as the nitrogen source led to low enzyme activity (below 210.98 U L^{-1}) and biomass (below 2.11 U L^{-1}), which indicates that *F. aquatile* ZJB-09211 cannot efficiently utilize inorganic nitrogen. The optimum concentration of beef extract for amidase production was found to be 8 U L^{-1}, which yielded the enzyme activity of 488.35 U L^{-1} (Fig. 6b). Therefore, 8 g L^{-1} of beef extract were used as the nitrogen source in the following experiments.

Effect of different inducers on cell growth and amidase production

It is known that some amides when added to culture media induce or enhance microbial amidase production. For this reason, the effects of eight different amides on the growth and amidase production of *F. aquatile* ZJB-09211 were examined. All the tested amides improved the enzyme production, and the maximum amidase activity was achieved with caprolactam (Fig. 7a). It was found that the optimum concentration of caprolactam for both cell growth and amidase production was 1.0 g L^{-1}, which resulted in the enzyme activity of 478.11 U L^{-1} (Fig. 7b). Therefore, 1.0 g L^{-1} of caprolactam was used as the inducer in the experiments.

Effect of various ions on cell growth and amidase production

Microorganisms require certain metal ions for cell growth and metabolite biosynthesis. It was found that the presence of Mg^{2+}, Cu^{2+}, Co^{2+}, Mn^{2+}, or Fe^{3+} leads to low amidase activity, whereas the presence of Ag^{+}, Ni^{2+}, Ca^{2+}, Ba^{2+}, and Fe^{2+} resulted in high enzyme activity (Table 2). The maximum enzyme activity was achieved with Ag^{+} (which was supplied in

Table 2. Effect of different metal ions on cell biomass and amidase activity

Metal ion	Biomass/(g L^{-1})	Activity units/(U L^{-1})	Relative activity/%
Blank	3.58	314.56	100.00
Mg^{2+}	4.00	228.32	72.58
Cu^{+}	3.45	249.01	79.16
Co^{2+}	3.43	95.63	30.40
Mn^{2+}	3.25	248.24	78.92
Fe^{2+}	3.48	382.26	121.52
Zn^{2+}	2.68	373.94	118.87
Ni^{2+}	3.23	464.85	147.78
Fe^{3+}	3.28	259.97	82.64
Ba^{2+}	3.20	401.71	127.70
Al^{3+}	2.88	361.47	114.91
Ag^{+}	3.28	478.17	152.01
Ca^{2+}	3.18	438.79	139.49

Table 3. Effect of different anions on cell biomass and amidase activity

Anionic	Biomass/(g L^{-1})	Activity units/(U L^{-1})	Relative activity/%
Blank	3.56	284.13	100.00
$AgNO_3$	3.27	468.23	164.80
$NaNO_3$	3.48	486.95	171.38
NaCl	3.18	298.10	104.92
Na_2SO_4	3.10	370.43	130.37
Na_2HPO_4	3.40	284.14	100.00
NaH_2PO_4	3.35	282.42	99.39
Na_2CO_3	3.20	328.82	115.73
Na_2SO_3	3.28	300.72	105.84

the form of $AgNO_3$). However, Ag^+ has not been previously described as an activator of amidase production in other microorganisms. Moreover, it inhibited cell growth in our experiments. Therefore, it is possible that the NO_3^- anions (and not the metal ion Ag^+) in $AgNO_3$ are promoting the amidase production. The effects of different anions (NO_3^-, Cl^-, SO_4^{2-}, HPO_4^{2-}, $H_2PO_4^-$, CO_3^{2-}, and SO_3^{2-}) on the enzyme activity were therefore examined and the results show that amidase production was significantly improved by $NaNO_3$ (Table 3). Optimum enzyme activity (501.50 U L^{-1}) was obtained with 20.0 mM $NaNO_3$ (Fig. 8), and this concentration was used in further experiments.

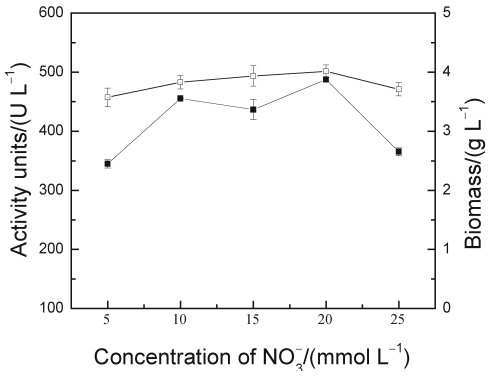

Fig. 8. Effect of different $NaNO_3$ concentrations on amidase production and biomass; □ – activity units, ■ – biomass.

Substrate specificity

Amidase produced by strain ZJB-09211 was found to be able to catalyze the hydrolysis of a variety of amide substrates (Table 4). High activity against aromatic, heterocyclic, and aliphatic amides was exhibited. For substrates in which the α-carbon is chiral, amidase displayed enantioselectivity. In addition, the amidase activity and enantioselectivity increased with the size of the substituent at the α-position, as indicated by the conversion rates and enantiomeric excess (e.e.) values obtained for 2,2-dimethylcyclopropane carboxamide (12.05 % conversion, e.e. = 60.01 %), methioninamide (28.02 % conversion, e.e. = 99.80%), and tryptophanamide (50 % conversion, e.e. > 99.90 %). The enantiomeric ratio (E), calculated according to the e.e. of the acidic fraction for tryptophanamide, was higher than 200 for tryptophanamide, methioninamide and mandelic amide, whereas it was only 4.3 for 2,2-dimethylcyclopropane carboxamide.

Conclusions

It has been shown here that *F. aquatile* ZJB-

Table 4. Substrate specificity of amidase produced by *F. aquatile* ZJB-09211

Substrates	Relative activity[a] /%	Enantioselective	e.e. value/%	*E*-value
Tryptophan amide	100.00	*S*	>99.9	>200
Butyl amide	15.28	–	–	–
Isobutyramide	261.17	–	–	–
Acetamide	17.40	–	–	–
Methionin amide	49.35	*S*	99.8	>200
Caprolactam	16.36	–	–	–
Levetiracetam	–	–	–	–
2,2-Dimethylcyclopro-panecarboxamide	12.11	*R*	60.01	4.334
2-Chloro-nicotinamide	–	–	–	–
Nicotinamide	–	–	–	–
Mandelic amide	298.25	*R*	>99.9	>200
Benzamide	277.66	–	–	–

a) Activity for tryptophanamide corresponding to 147.86 U g^{-1} was taken as 100 %.

09211 can selectively hydrolyze the L-enantiomer to produce optically active L-Trp in high yields when fed D,L-tryptophanamide. To the best of our knowledge, this is the first report on one strain of the genus *Flavobacterium* producing stereospecific amidase. The *F. aquatile* ZJB-09211 amidase exhibits strict *S*-enantioselectivity (e.e. >99.9 %, E >200) for tryptophan amides. Under optimized culture conditions, enzyme production reaches 501.5 U L^{-1}, an activity that is by about six times higher than that achieved before optimization. Our results indicate that this strain has great potential for L-Trp production in scientific and industrial applications.

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