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Full Length Research Paper

Purification and characterization of amidase from acrylamide-degrading bacterium *Burkholderia* sp. strain DR.Y27

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An amidase from a newly isolated acrylamide-degrading bacterium *Burkholderia* sp. strain DR.Y27 was purified to homogeneity by a combination of anion exchange and gel filtration chromatography. The purification strategy achieved 11.15 of purification fold and a yield of 1.55%. The purified amidase consisted of four identical subunits with a molecular weight 47 kDa and was active within the temperature range of 35 to 60°C, with optimum activity at 40°C and within the pH range of 7.5 to 8.0 with an optimum pH of 8.0. Aliphatic amides (acetamide and propionamide) were the best substrates for the amidase from *Rhodococcus rhodochrous* M8, whereas bulky aromatic amides were poor substrates of this enzyme. The amidase from *Burkholderia* sp. strain DR.Y27 was not sensitive to sulfhydryl agents such as β-mercaptoethanol and dithiothreitol (DTT).

Key words: Purification, characterization, amidase, *Burkholderia* sp.

INTRODUCTION

Acylamide amidohydrolase or amidase (EC 3.5.1.4) is an enzyme that catalyzes the hydrolysis of an amide to free carboxylic acids and free ammonium (Zabaznaya et al., 1998). This enzyme belongs to the family of hydrolases, which acts on carbon-nitrogen bonds other than peptide bonds. It is also involved in nitrogen metabolism in cells and widely distributed in nature. Amidase is often found in bacteria related to the group of Nocardia-like *Actinomycetes* (*Nocardia*, *Rhodococci* and *Arthrobacteria*). Previous studies have shown that this enzyme was purified from *Pseudomonas* sp. MC13434 (Komeda et al., 2004), *Rhodococcus* sp (Nawaz et al., 1994), and *Rhodococcus erythropolis* MP50 (Hirrlinger et al., 1996). This enzyme is of scientific interest in the bio-remediation field due to its ability to degrade acrylamide.Acrylamide is the building block used in the synthesis of polyacrylamide (PAM). It is known that commercial PAM preparations may be contaminated with its toxic monomer, acrylamide. Hence, regulations have been set on the amount of acrylamide that is present in PAM. These compounds are usually used as a sewage - flocculating agents (Myagchenkov and Poskurina, 2000), coagulants in the crude oil recovery process, and as adhesives (IPCS, 1991). Acrylamide is also known as a neurotoxicant, carcinogen and teratogen in animals (Prabu and Thatheyus, 2007), and now the presence of acrylamide in processed food products has become a very serious health issue (Singh et al., 2010; Shalini et al., 2010). High concentrations of acrylamide in the human body can also contribute to cancer risk (Tareke et al., 2002).

Under environmental conditions, PAM will degrade to acrylamide which has a half life ranging from weeks to months in aquatic conditions. However, acrylamide can also degrade faster with the help of microorganisms. Previous studies have shown that acrylamide-degrading bacteria isolated from the environment were confined to species within the genera *Pseudomonas*, *Bacillus*,

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Rhodococcus and *Enterobacter* (Shukor et al., 2009a, b; Buranasilp and Charoenpanich, 2011). These bacteria were shown to produce amidases which converted acrylamide to ammonia and acrylic acid (Shukor et al., 2009a, b; Buranasilp and Charoenpanich, 2011). In this study, we report the purification and characterization of a novel amidase from acrylamide-degrading bacterium *Burkholderia* sp. strain DR.Y27 that utilized acrylamide as its sole nitrogen source.

MATERIALS AND METHODS

All chemicals used were of analytical grade and they were purchased either from Sigma (USA), or Fermentas (USA), or Merck (Germany), or Agilent™ (USA).

Medium and culture condition

Burkholderia sp. strain DR.Y27 (DQ 851856), previously isolated from the grounds of a palm estate in Serdang, Selangor, was cultured at 30°C in mineral salt medium (MSM) containing (gL^{-1}): glucose, 10; NaH_2PO_4, 6.8; $MgSO_4.7H_2O$, 0.5; Yeast extract, 0.01 and 10 ml trace element, pH 7.5. The compositions of trace elements are (gL^{-1}): $ZnCl_2$, 0.03; $CoCl_3.6H_2O$, 0.003; $FeCl_2.6H_2O$, 0.002; $Cu(CH_3COO)_2.H_2O$, 0.01 and H_3BO_3, 0.05. The MSM were supplemented with 0.5 gL^{-1} acrylamide as nitrogen sources.

Purification of amidase

The purification procedure was carried out in 50 mM phosphate buffer, pH 7.5 at 4°C unless otherwise stated. Bacterial strain was grown in 8 L of MSM containing 0.5 gL^{-1} acrylamide. After 48 h incubation, the culture was centrifuged at 10,000x g for 10 min. Pellets were suspended in 50 mM phosphate buffer, pH 7.5 containing 10 mM PMSF. The cell suspension was sonicated using Branson Sonifier 450 for 1 min on an ice bath and followed by 4 min of cooling for a total sonication time of at least 20 cycles. The suspension was then ultracentrifuged for 60 min at 105,000x g.

The clear supernatant containing the crude extracts obtained after ultracentrifugation was directly applied onto different continuous ion exchange columns using DEAE cellulose (Pharmacia™; 1.6 × 10 cm) and Mono-Q 5/50 (Amersham-Bioscience; 0.5 × 5 cm). The Mono-Q fractions with amidase activity were then run through a Zorbax® Bioseries GF-250 gel filtration column connected to a HPLC system. The column was washed at the flow rate of 1 ml/min with 0.02 M sodium phosphate buffer (pH 7.5) containing 0.2 M NaCl. Amidase activity was assayed at room temperature by measuring the ammonia liberated from acrylamide degradation at 630 nm (APHA, 1998). Protein was assayed using the Coomassie dye-binding method (Bradford, 1976) using crystalline BSA as the standard. The mixture was incubated for 2 min before the absorbance readings were taken at 595 nm.

Determination of the optimum pH and temperature for amidase activity

An overlapping buffer system was used to study the pH profiles for the optimization of amidase activity. Two types of buffers were used in this system; phosphate buffer (pH 5.7, 6, 7, 7.5 and 8) and carbonate buffer (pH 8, 8.5, 9.5 and 10.5) at 0.5 mM. The optimum temperature for amidase activity was determined at temperatures from 20 to 70°C.

Determination of kinetic parameters and substrate specificity

The K_m values and substrate specificity were determined using acrylamide, acetamide, propionamide, 2-cloroacetamide, methacrylamide, urea and nicotinamide at concentrations of 0 to 100 mM.

Effect of sulfhydryl agents and metal ions on amidase activity

The amidase activity was measured under standard conditions after incubation at 30°C for 60 min with various sulfhydryl agents such as β-mercaptoethanol and dithiothreitol (DTT) at 0 to 10 mM and metal ions such as mercury, lead, silver, cadmium and copper at 1 mM.

Statistical analysis

The data obtained were analyzed statistically using one-way analysis of variance (ANOVA).

RESULTS

The acrylamide-degrading amidase was found in the soluble fraction of the crude cells extract of *Burkholderia* sp. strain DR.Y27. The elution profile of the DEAE cellulose Pharmacia™ column (1.6 × 10 cm), Mono-Q 5/50 Amersham-Bioscience column (0.5 × 5 cm) and Zorbax® Bioseries GF-250 gel filtration column are shown in Figures 1a, b and c, respectively. Elution by linear gradient produced a sharp peak with high amidase activity between 0.4 to 0.6 M NaCl for DEAE cellulose Pharmacia™ (Figure 1a) and 0.45 M NaCl for Mono-Q 5/50 Amersham-Bioscience column (Figure 1b). The Mono-Q fractions with amidase activity that were chromatographed through the Zorbax® Agilent Bioseries GF-250 gel filtration column produced a single peak with retention time of 8.5 min (Figure 1c). The amidase which was purified by 11.15 fold with a yield of 1.55% (Table 1) migrated as a single band in SDS-PAGE and has a molecular mass of 47 kDa (Figure 2). The molecular mass of the native protein estimated by using gel filtration on a Zorbax® GF-250 column was 186 kDa, indicating that the native enzyme is a homotetramer.

The optimal pH was determined by assaying hydrolytic activity in the pH range of 5.7 to 10.5. The amidase was active within the pH range of 7.0 to 8.5 with maximum activity at pH 8.0 (Figure 3). Beyond these pH limits, the enzyme activity was decreased. For example, at pH 5.7 and 9.5, the enzyme loses 39 and 33% of its activity respectively, and at pH 10.5, the loss in enzyme activity was at 70%. The optimum temperature for amidase activity was between 35 to 50°C with maximum activity at 40°C (Figure 4). The activity decreased sharply at higher temperatures.

The effects of substrate specificity, sulfhydryl agents and metal ions on amidase activity were also tested. Table 2 shows that the substrate preference of the enzyme for hydrolysis can be arranged in the following order; acetamide > 2-choroacetamide > urea >

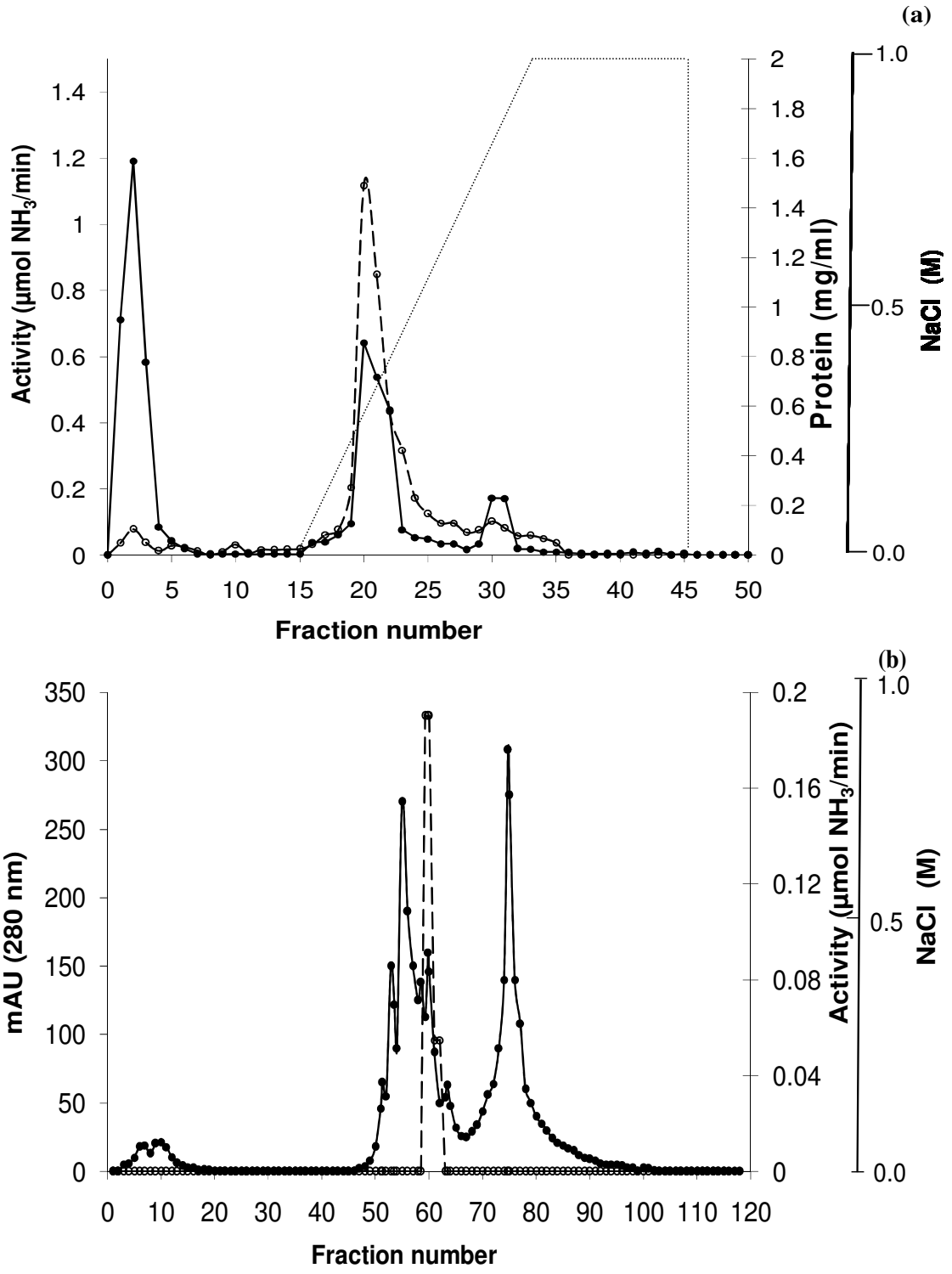

Figure 1. Amidase elution profile using (a) DEAE-cellulose anion exchanger column, (b) Mono-Q^{TM} strong-anion exchanger column and (c) Zorbax^{R} Bioseries GF-250 gel filtration column. -o-, Amidase activity; -•-, protein; ·····, gradient of 1M NaCl were measured during purification steps.

acrylamide > propionamide and no activity with methacrylamide and nicotinamide was detected. Sulfhydryl agents such as β-mercaptoethanol and DTT shows no increase in amidase activity (Figure 5), while all

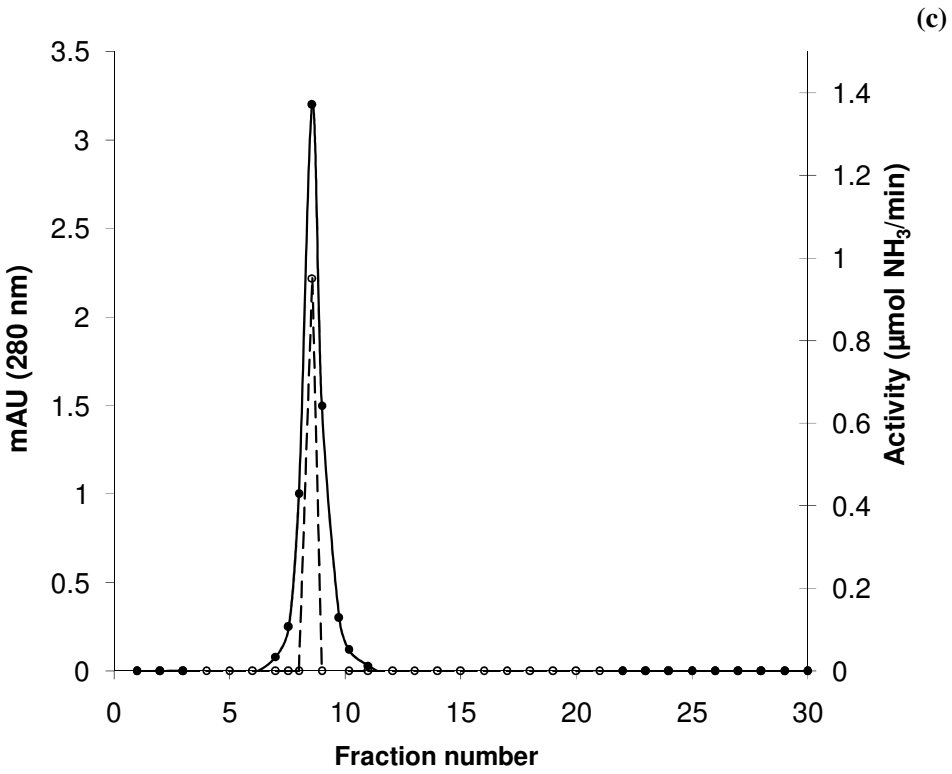

Figure 1. Contd.

Table 1. Purification scheme of amidase from *Burkholderia* sp. strain DR.Y27.

Parameter	Fraction			
	Crude	DEAE	MonoQ	GF
Total protein(mg/ml)	35.95	7.65	0.76	0.05
Activity(µmolNH_3/min/ml)	2.15	2.04	1.2	0.5
Total activity	32.24	18.38	2.40	0.50
Specific activity(µmol NH_3/min/mg)	0.90	2.40	3.16	10.00
Yield (%)	100	57.02	7.45	1.55
Fold	1.0	2.68	3.52	11.15

Crude = Crude extract; DEAE = DEAE-cellulose anion exchanger column; MonoQ = Mono-Q^{TM} - Mono-Quaternary ammonium (a trade name for a strong anion exchanger column); GF = Zorbax^{R} Bioseries GF-250 gel filtration column

the metal ions tested such as mercury, lead, silver, cadmium and copper showed significant inhibition of amidase activity (Table 3).

DISCUSSION

The present amidase is a homotetramer and has different properties compared to other microbial enzymes. This is in contrast to the amidase purified from *Pseudomonas* sp. MCI3434 (Komeda et al., 2004) and the thermostable amidase from *Klebsiella pneumoniae* NCTR1 (Nawaz et al., 1996) which are monomers with molecular weights of 36 and 62 kDa, respectively. The thermoactive amidase from *Pseudomonas thermophilia* (Egorova et al., 2004) and *Pseudomonas chlororaphis* B23 (Ciskanik et al., 1995) are dimers with molecular mass of 108, 105, 52 and 54 kDa, respectively.

The behavior of pH-dependence curve and the pH optimum were similar to amidases from *Sulfolobus tokodaii* strain 7 (Suzuki and Ohta, 2006) and *Rhodococcus rhodochrous* (Kotlova et al., 1999). The maximum temperature for amidase activity in *Burkholderia* sp. strain DR.Y27 was similar to the amidase from *Rhodococcus* sp. (Nawaz et al., 1994) where the maximum was 40°C, while the amidase from

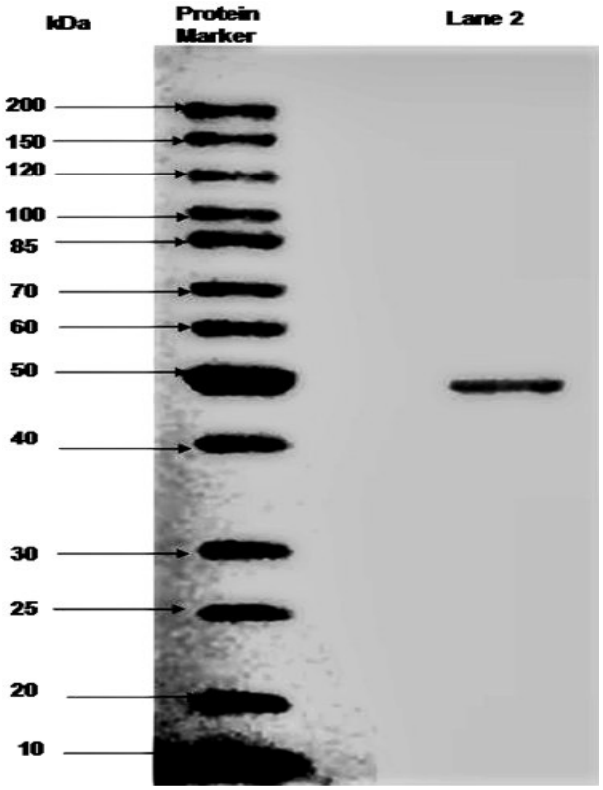

Figure 2. Reducing SDS-PAGE analysis of the purified amidase shown in Lane 2.

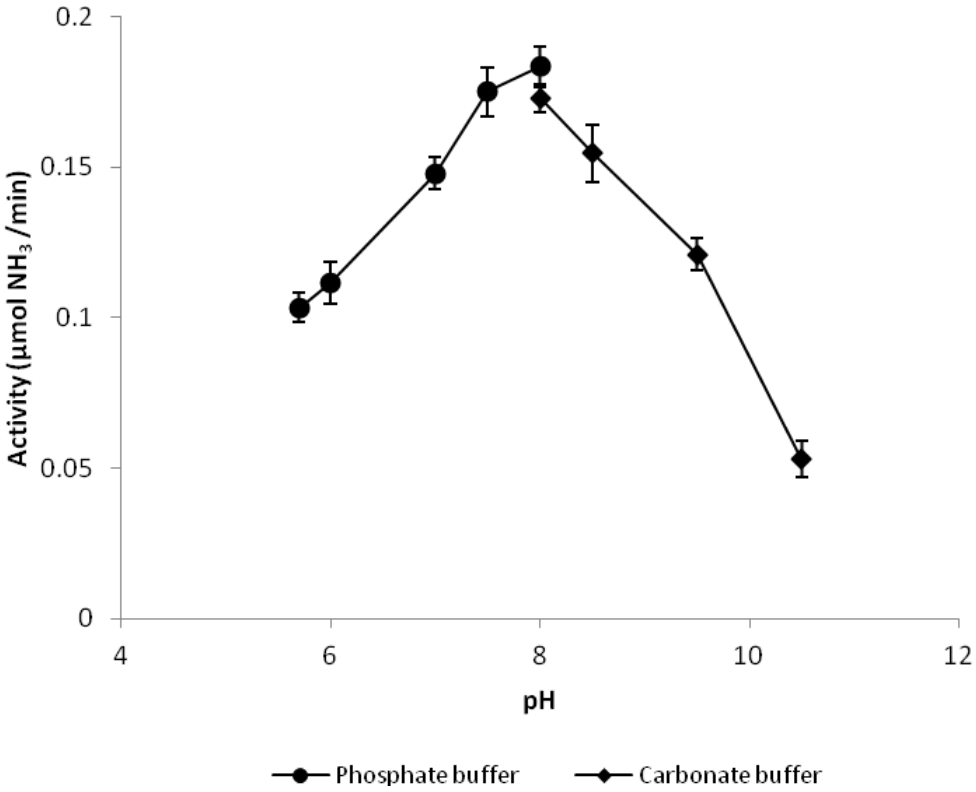

Figure 3. Effect of pH on the activity of purified amidase.

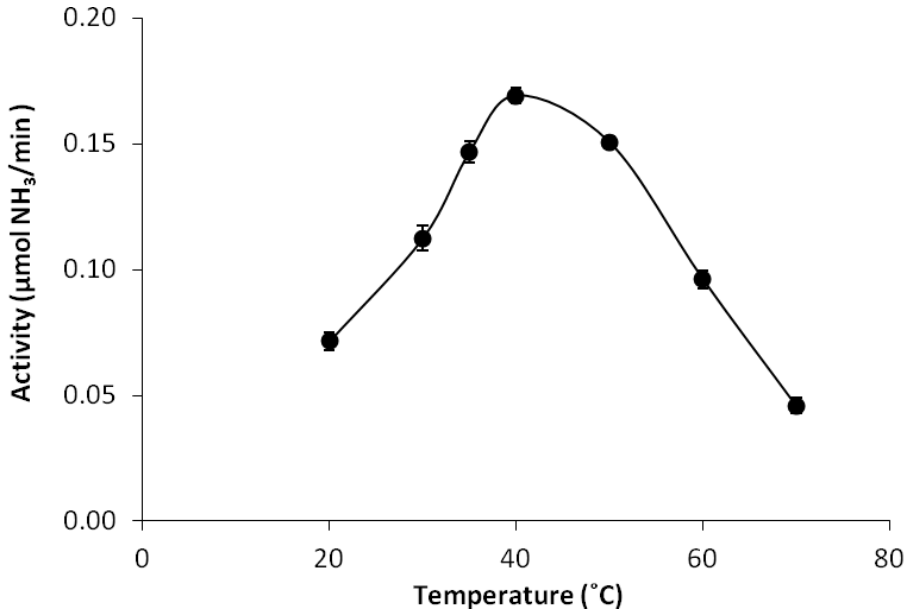

Figure 4. Effect of temperature on the activity of purified amidase.

Table 2. Substrate specificity of amidase from *Burkholderia* sp. strain DR.Y27.

Substrate	K_m (mM)	Chemical formula
Acrylamide	2.39 ± 1.84	CH_2=CH-$CONH_2$
Acetamide	0.27 ± 0.19	CH_3-$CONH_2$
2-Cloroacetamide	1.21 ± 0.13	Cl-CH2-CONH2
Propionamide	4.29 ± 0.87	CH_3-CH_2-$CONH_2$
Metachrylamide	0.00 ± 0.00	CH_2=CH(CH_3)-$CONH_2$
Nicotinamide	0.00 ± 0.00	$C_6H_6N_2O$
Urea	1.88 ± 0.28	NH_2-$CONH_2$

P. chlororaphis B23 was at 50°C (Ciskanik et al., 1995). In spite of the different subunit compositions and specificity, reported amidases were mostly active at relatively high temperatures of about 50°C. However, maximal activity at lower temperatures at 35°C has also been reported from *Comamonas acidovorans* KPO-2771-4 and *Blastobacter* sp. (Hayashi et al., 1997; Soong et al., 2000).

Kinetic studies using different substrates showed that the enzyme extracted from *Burkholderia* sp. strain DR.Y27 shows preference for aliphatic amides, especially those with short chain carbon atoms. The Michaelis constant values showed that amidase affinity was highest with the shortest aliphatic amide; acetamide (K_m = 0.27 ± 0.19) (Table 2). There was no activity with methacrylamide and nicotinamide. The carbon side chain and the cyclic form of methacrylamide and nicotinamide probably prevent substrate binding to the enzyme. Comparison of this present results with data from literature, places the amidase from *Burkholderia* sp. strain DR.Y27 in the group of short-chain aliphatic amidases.

The effects of sulfhydryl agents such as β-mercaptoethanol and DTT on amidase activity were tested to determine the role of sulfhydryl group in the catalytic activity. Figure 5 shows that no increase in amidase activity was detected at any concentrations of DTT and β-Mercaptoethanol tested, suggesting that the amidase from *Burkholderia* sp. strain DR.Y27 is possibly a non-sulfhydryl enzyme since it did not require DTT to restore the activity. In contrast, an increase in amidase activity in the presence of DTT was reported in *R. rhodochrous* M8 (Kotlova et al., 1999) and in the enantioselective amidase from *P. chlororaphis* B23 (Ciscanik et al., 1995). Furthermore, Skouloubris et al. (2001) reported that AmiE aliphatic amidase and AmiF formamidase from *Helicobacter pylori* produced in *Escherichia coli* required 10% sulfhydryl compounds as protective agents.

The effects of metal ions on amidase activity are shown in Table 3. Although all the metal ions tested showed significant inhibition of amidase activity, none of the metal ions caused more than 50% inhibition of the amidase in comparison to the control. Toxic metals such as mercury,

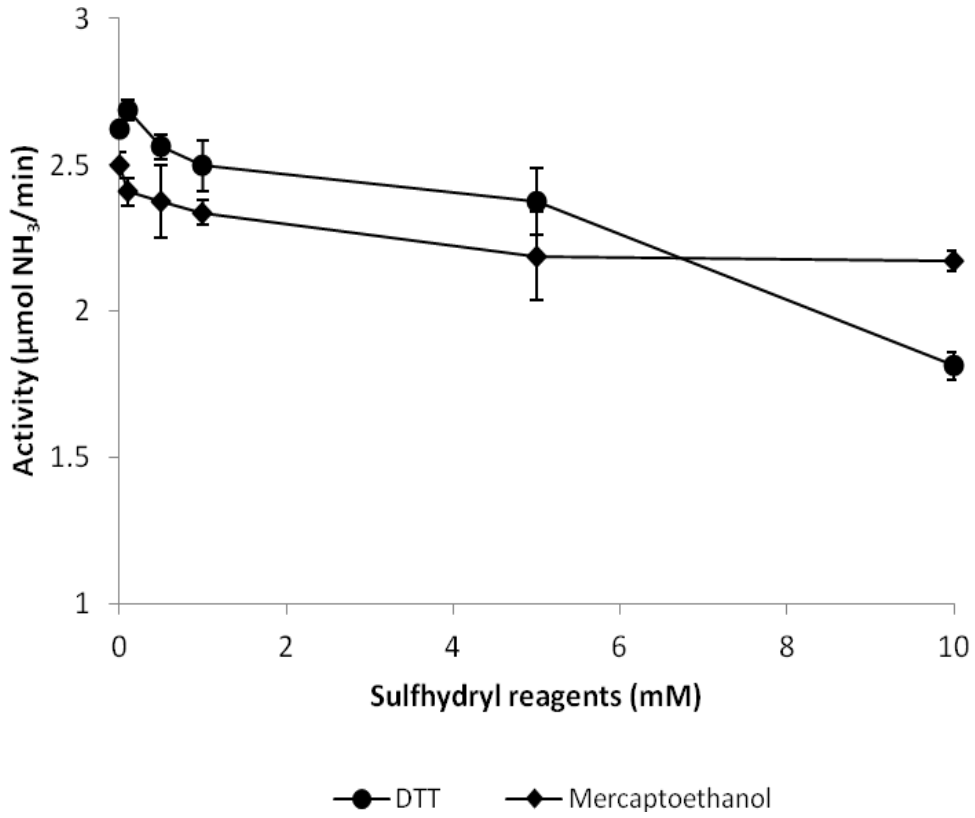

Figure 5. Effect of sulfhydryl reagents on amidase activity.

Table 3. Effects of metals ion on amidase activity.

Metal ion (1 mM)	Residual activity (%)
Control	100 ± 0.00[a]
$WO4^{2-}$	65.54 ± 1.66[b]
Li^{2+}	57.57 ± 2.36[c]
Fe^{2+}	61.26 ± 1.32[bc]
As^{4+}	68.10 ± 0.80[bc]
Ni^{2+}	69.69 ± 1.74[bc]
Se^{+2}	72.19 ± 1.50[bc]
Zn^{2+}	65.27 ± 1.17[bc]
Cs^{2+}	76.19 ± 3.00[b]
Cr^{2+}	70.84 ± 2.05[bc]
Al^{3+}	68.08 ± 1.47[bc]
Mn^{2+}	72.44 ± 1.29[bc]
Co^{2+}	77.85 ± 2.78[b]
Mg^{2+}	68.80 ± 5.39[bc]
Cu^{2+}	75.15 ± 6.46[bc]
Pb^{2+}	78.43 ± 2.36[b]
Cd^{2+}	66.19 ± 9.16[bc]
Ag^{2+}	70.08 ± 1.08[bc]
Hg^{2+}	71.02 ± 4.02[bc]

Residual activity (%) having different alphabets indicate significant differences ($P < 0.001$).

lead, silver, cadmium and copper usually bind to the sulfhydryl group of cysteine in the active sites of the enzyme leading to inactivation of the enzyme (Scopes, 1994). These results here suggest that cysteine is not present at the active site of the amidase from this strain. This is in contrast to earlier reports that showed that heavy metals inhibited amidase activity (Komeda et al., 2004; Nawaz et al., 1994). This further lends support to the earlier suggestion that the amidase extracted from the current strain is a non-sulfhydryl enzyme.

Conclusion

Burkholderia sp. strain Dr.Y27 was shown to produce an amidase which has different properties from the earlier reported enzymes from different microorganisms.

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