ORIGINAL ARTICLE

Purification and characterisation of alliinase produced by Cupriavidus necator and its application for generation of cytotoxic agent: Allicin

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Abstract
Allicin, an extremely active constituent of freshly crushed garlic, is produced upon reaction of alliin with the enzyme alliinase (EC 4.4.1.4). A bacterium Cupriavidus necator with the ability of alliinase production was isolated from a soil sample and was identified by morphological, biochemical and 16S rRNA sequence. Alliinase production was optimised and it was further purified to apparent homogeneity with 103-fold purification and specific activity of 209 U/mg of protein by using DEAE Cellulose and Sephadex G-100 chromatography. The enzyme is a homodimer of molecular weight 110 kDa with two subunits of molecular weight 55 kDa each. The optimum activity of the purified enzyme was found at pH 7 and the optimum temperature was 35 °C. The enzyme exhibited maximum reaction rate (V_{max}) at 74.65 U/mg and Michaelis–Menten constant (K_{m}) was determined to be 0.83 mM when alliin was used as a substrate. The cytotoxic activity of in-situ generated allicin using purified alliinase and alliin was assessed on MIA PaCa-2 cell line using MTT assay and Acridine orange–ethidium bromide staining. This approach of in-situ allicin generation suggests a novel therapeutic strategy wherein alliin and alliinase work together synergistically to produce cytotoxic agent allicin.

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1. Introduction

Allicin (Diallyl thiosulphate) is the best-known active compound of freshly crushed garlic extract and is known to possess a wide range of pharmacological activities which include antimicrobial, anti-inflammatory, antithrombotic, antifungal, antiviral, antioxidant, anticancer, and antiatherosclerotic (Ilić et al., 2011; Oommen et al., 2004). This allyl-sulphur...
compound is synthesised as a result of C-S lyase activity of alliinase (alliin lyase; EC 4.4.1.14) on alliin (S-allyl-l-cysteine sulphonide), a naturally-occurring diastereomer molecule (Jones et al., 2004) (Fig. 1). Alliinase catalyses the release of volatile sulphur compounds which is responsible for the characteristic flavour and odour of Allium species.

Alliinase has been purified from garlic, onion, and other plants of the genus Allium (Schwimmer and Mazelis, 1963; Mazelis and Crews, 1968; Tobkin and Mazelis, 1979; Nock and Mazelis, 1987; Landshuter et al., 1994; Lohmuller et al., 1994; Rabinov et al., 1994; Manabe et al., 1998). The enzymatic production of allicin occurs in garlic, because of the injury of the plant tissue that enables interaction of the enzyme in vacuoles with alliin gathered in the cytosol (Yamazaki et al., 2002); hence allicin production has been discussed as a defense mechanism of the plant against microbial infection or insect attack (Slusarenko et al., 2008). Allicin generated from alliinase which was purified from garlic, has been studied for fungicidal activity against Magnaporthe grisea (Fry et al., 2005).

In the past, some microorganisms have also been studied for their alliinase producing ability, viz. Durbin and Uchytil (1971) reported the presence of enzyme in Penicillium corymbiferum and Yutani et al. (2011) reported the presence of an intracellular alliinase in Ensifer adhaerens; further alliinase generated from this enzyme exhibited potent fungicidal activity against Saccharomyces cerevisiae.

In the last few years, alliinase had found its application in agriculture, pharmaceutical and food industry. Alliinase has been immobilised on N-succinyl chitosan polymer (Zhou and Wang, 2009), calcium alginate beads (Anifantaki et al., 2010), Monoclonal antibody (Chhabria et al., 2015), porous silica beads can be further used for the development of a biosensor for monitoring cysteine sulphoxides which could prove valuable in several applications in the food industry.

Chemical instability and low miscibility of allicin in water obstruct its practical use; hence a binary system consisting of volatile sulphur compounds which is responsible for the characteristic flavour and odour of Allium species.

In the present study, we report for the first time, purification and characterisation of an intracellular alliinase enzyme from Cupriavidus necator. The isolated enzyme was further used for on-site generation of cytotoxic agent-allicin; which was assessed on pancreatic cancer (MIA PaCa-2) cell line using MTT assay and Acridine orange–ethidium bromide staining.

2. Materials and methods

2.1. Chemicals and reagents

Alliin & Allicin were purchased from LKT Laboratories, Inc. (St. Paul, MN). DEAE Cellulose, Sephadex G-100 and Pyridoxal 5’ Phosphate (PLP) were purchased from Sigma–Aldrich. Molecular weight markers for Sephadex G-100 chromatography were obtained from Bio-Rad laboratories, Inc., California, USA.

2.2. Isolation of alliinase-producing microorganism

Alliinase producing strain was identified by 16S rRNA sequence in addition to morphological and biochemical analysis, examined according to Bergey’s Manual of Systemic Bacteriology (Yabuuchi et al., 2005).

For 16S rRNA sequencing the DNA was extracted using the method described by Cheng and Jiang (2006). The 16S rRNA gene was amplified by polymerase chain reaction (PCR), using prokaryotic universal primers Bact 8F (Forward primer): 5’-AGATTGATCCCCGTGCAG-3’ and Bact 1391R (Reverse primer): 5’-GACGGGGCCGTGTCAG-3’. The PCR conditions were as follows: initial denaturation at 94 °C for 2 min, 30 cycles: 94 °C for 1 min, 55 °C for 1 min, 72 °C for 1 min and a final extension at 72 °C for 20 min. The amplified sequence was then sequenced using Applied Biosystems 3130 Genetic analyser. The acquired sequence was used for a gene homology search, with the 16S rRNA sequences accessible in the public databases from BLAST (http://www.ncbi.nlm.nih.gov/BLAST/), NCBI, Bethesda, MD, USA) (Altschul et al., 1997), and were identified to the generic level by the CLUSTAL-X Multiple Sequence Alignment Program (Strasburg, France), the 16S rRNA sequences of the isolated strain was aligned with sequences of related organisms obtained from Gen Bank (Thompson et al., 1997). A phylogenetic tree was constructed via the neighbour-joining method using the Tree View programme (Saitou and Nei, 1987).

2.4. Optimisation of growth conditions for alliinase production

Alliinase production by the isolate was optimised by varying glucose concentration (0.05–1%), alliin concentration...
(0.05–1%), temperature (25–55 °C), pH (3–11), incubation period (24–96 h) and incubation condition (shaking and static).

2.5. Purification of intracellular alliinase

2.5.1. Preparation of cell-free alliinase

Cells from 1000 ml of the medium were collected by centrifugation at 7000 g for 5 min. 0.2 g of harvested cells were suspended in 1 ml of lysis buffer and incubated at 4 °C for 10 min. The cell suspension was centrifuged at 12,000 g for 20 min at 4 °C and the supernatant obtained after centrifugation was used as a crude enzyme.

2.5.2. Ammonium sulphate precipitation

A typical batch was carried out using crude enzyme obtained from one litre of the medium. Solid ammonium sulphate was added to the supernatant at 4 °C, to reach a saturation of 40%. The mixture was kept overnight and then centrifuged at 10,000 g for 20 min at 4 °C. It was dialysed overnight with 3–4 changes of buffer and was further used for purification. Alliinase activity and protein content of the fraction were determined.

2.5.3. DEAE Cellulose chromatography

The 40% fraction obtained from ammonium sulphate precipitation was passed through DEAE Cellulose column (2 × 6 cm) eluted using a linear discontinuous gradient of 0.05–0.5 M KCl in 0.1 M phosphate buffer (pH 7). The active fractions were pooled and loaded onto the second column of DEAE Cellulose column (2 × 6 cm) and eluted using a linear continuous gradient of 0.1–0.3 M KCl in 0.1 M phosphate buffer, pH 7. The flow rate was maintained at 20 ml/h. Each fraction was analysed for protein content and enzyme activity.

2.5.4. Sephadex G-100 chromatography

The fractions exhibiting highest alliinase activity obtained from DEAE Cellulose continuous gradient column chromatography were pooled and loaded on Sephadex G-100 column (40.0 cm × 1.2 cm, bed volume 45 ml) equilibrated with 0.1 M phosphate buffer (pH 7). Flow rate maintained was 20 ml/h. Fractions of 2 ml were collected. The active fractions obtained were pooled and were used as the purified enzyme. The molecular weight markers used included Blue dextran (2000 kDa), Thyroglobulin (669 kDa), Ferritin (440 kDa), Alcohol dehydrogenase (150 kDa), Serum albumin (67 kDa) and Chymotrypsinogen (25 kDa).

2.6. SDS-PAGE analysis

Aliinase during the course of purification, was analysed using 10% SDS-PAGE. Subunits of alliinase were determined by electrophoresing it, in the presence and absence of β-ME (Gam and Latiff, 2005). Puregene broad range protein ladder was used as molecular weight standard. After separation, the proteins were detected using silver staining.

2.7. Effect of temperature and pH on alliinase activity and stability

The alliinase activity was determined at different temperatures (0–50 °C) and different pH (3–11) under standard assay conditions using alliin as a substrate to determine the optimum temperature and pH. The stability of alliinase enzyme was determined by pre-incubating the purified enzyme at different temperatures (0–50 °C) and pH (3–11) for 1 h and the alliinase activity was determined.

2.8. Effect of metal ions and chemicals on alliinase activity

The effect of different metals on alliinase activity was assessed by adding each metal ion to the reaction mixture and assayed for its activity. The final concentrations of the metal salts and Ethylenediaminetetraacetic acid (EDTA) in the reaction mixture were maintained at 1 mM, and Sodium dodecyl sulphate (SDS) was 0.1%. The metals used were chloride salts of Mn²⁺, Mg²⁺, Zn²⁺, Fe⁺⁺, Ca²⁺, Ag⁺⁺, Hg⁺⁺ and Na⁺ except for CaCl₂ (sulphate). The enzyme was incubated in the presence of various metal ions and chemicals, for 1 h and relative enzyme activity (%) was determined.

2.9. Determination of kinetic parameters

The Michaelis–Menten kinetic constants; the maximum reaction rate (Vmax) and Michaelis–Menten Constant (Km) of the purified alliinase were determined using the different concentrations of allin (0.5–5 mM). The kinetic parameters were determined using Lineweaver–Burk plot. Km and Vmax were calculated using Graph Pad PRISM software version 5.0.

2.10. Alliinase assay

Alliinase was synthesised intracellularly by the isolate, hence cells were lysed using lysis buffer (25 mM Tris, 0.15 M NaCl, 0.01 M Phosphate Buffered Saline, 0.1% Triton-X and 1 mM Phenyl methyl Sulphonyl Fluoride (PMSF) for 10 min at 4 °C and then after centrifugation the supernatant was assessed for alliinase activity. The standard alliinase assay mixture contained 40 mM allin, 20 mM PLP, 50 mM sodium phosphate (pH 7.0), and 100 μL enzyme in a total volume of 1.0 ml. Alliinase activity was determined at 515 nm in UV–Visible Perkin Elmer Lambda 25 spectrometer, by measuring the pyruvic acid produced in the reaction as described by Anthon and Barrett (2003). One unit of the enzyme activity was defined as the enzyme amount that catalysed the formation of 1 μmol of pyruvic acid per min.

2.11. Determination of allicin, ammonia, pyruvate and protein content

Ammonia and pyruvate are by-products of the reaction catalysed by alliinase on allin (Fig. 1), hence it was important to determine the concentration of allicin, ammonia and pyruvate generated, during the reaction. Alliinase (10 units) and 200 μM of allin were incubated in PBS in the presence of 20 mM PLP at 37 °C, which resulted in the generation of allicin, ammonia and pyruvate. Allicin, ammonia and pyruvate generated by 200 μM of allin were determined by adopting 2-nitro-5-thiobenzoate (Miron et al., 2002), Nessler’s reagent (Yuen and Pollard, 1954) and DNPH method (Anthon and Barrett, 2003) respectively. Protein concentration was determined according to the method of Lowry et al. (1951), using bovine serum albumin fraction V as a standard.
2.12. Procurement and maintenance of cell lines

Pancreatic carcinoma MIA PaCa-2 cell line was procured from National Centre for Cell Science (NCCS), Pune, India and Human dermal fibroblast HDF cell line was procured from V.G. Vaze College, Mumbai, India. Both the cell lines were cultured in high glucose Dulbecco’s Modified Eagle’s Medium (DMEM) (Sigma–Aldrich, St. Louis, MO, USA) with heat inactivated 10% foetal bovine serum (FBS) (Cell Clone, Genetix, India) and 1% antibiotic solution of penicillin–streptomycin (Sigma–Aldrich, St. Louis, MO, USA), and amphotericin (Himedia, Mumbai, India). Cells were maintained by passaging 1:3 at a regular interval of 48 h using 0.5% Trypsin-EDTA (Gibco, Paisley, UK) without phenol red. All reagents, media, buffers, chemicals and plastic-ware used were of cell culture grade.

2.13. Cytotoxic activity of in-situ generated allicin

Cytotoxicity of in-situ generated allicin was determined by using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay on pancreatic cancer (MIA PaCa-2) cell line and human dermal fibroblast (HDF) cell line. For each cell line, 1 × 10^5 cells were seeded in each well of a 96 well plate and incubated for 24 h. Growth medium was replaced with fresh medium containing 20 mM PLP. Alliinase (10 units) was added to the cells along with increasing concentrations of alliin (20–200 μM) for 24 h.

Cytotoxicity of reactants [alliin 200 μM, alliinase 10 U] and by-products of the reaction [ammonia (130 μM) and pyruvate (149 μM)] were also assessed on both the cell lines. After a 24 h incubation, 0.5 mg/ml of MTT (Himedia, Mumbai, India) was added to each well and incubated for 4 h. The purple MTT formazan precipitate was dissolved in 100 μl of DMSO. The absorbance was measured on a microplate reader (Bio-Rad, Hercules, CA, USA). Data were represented as mean ± S.D. of three replicates from three independent experiments. The values are represented as the percentage cell viability wherein viability of untreated cells was regarded as 100%. Data were analysed by a one-way ANOVA followed by Dunnett’s post hoc test.

2.14. Acridine orange–ethidium bromide staining

MIA PaCa-2 cells were cultured at a density of 1 × 10^6 cells per well in 6 well flat-bottomed plate and incubated for 24 h. Cells were then incubated with alliinase enzyme (10 U) in the presence of alliin (50, 100, and 200 μM) for 24 h in the presence of 20 mM PLP. Reactants [alliin (200 μM), alliinase (10 U)] and by-products of the reaction [ammonia (130 μM) and pyruvate (149 μM)] were also assessed after a 24 h incubation. Thereafter, cells were stained with 10 μl of Acridine orange–ethidium bromide mixture (10 μg/ml) (MP Biomedicals, USA) and cells were observed in an inverted fluorescent phase contrast microscope (Carl Zeiss, Jena, Germany).

2.15. Statistical analysis

All experiments were performed in triplicate and the results were represented with standard deviation calculated by Graph pad PRISM software version 5.

3. Results

3.1. Isolation & identification of alliinase-producing microorganism

Aliinase production was optimised by monitoring various nutritional and physiological parameters. Aliinase production increased with an increase in the glucose concentration up to 0.2% and then remained stagnant up to 0.4% whereas a sharp decline was observed at 0.5% and 1% (Fig. 2a). Increase in alliin concentration resulted in an increase in enzyme production up to 0.1% whereas higher concentrations of alliin did not enhance enzyme production by the organism (Fig. 2b).

Maximum alliinase production was observed at 37 °C (Fig. 2c) and at pH 7 (Fig. 2d). Organism when cultured under shaking condition increased the enzyme production by 300% as compared to when cultured under static condition (Fig. 2e).

Maximum enzyme production was observed at 48 h after its inoculation in the growth medium after which it started to decline (Fig. 2f).

3.2. Optimisation of growth conditions for alliinase production

The alliinase isolated from C. necator was purified using ammonium sulphate precipitation, DEAE Cellulose chromatography and Sephadex G-100 chromatography. The purification of alliinase enzyme, its SDS-PAGE analysis and its elution profile using chromatography techniques during the course of enzyme purification is summarised in Table 1, Figs. 3a and 4 respectively. The yield of alliinase enzyme was 1660 U per gram of the cell weight.

Aliinase in the presence of β-ME showed the presence of a single band at 55 kDa and in the absence of β-ME showed the presence of a single band at 110 kDa (Fig. 3b); indicating alliinase from C. necator is a homodimer consisting of two sub-units of 55 kDa each.

3.3. Purification of alliinase and molecular weight determination

The alliinase isolated from C. necator was purified using ammonium sulphate precipitation, DEAE Cellulose chromatography and Sephadex G-100 chromatography. The purification of alliinase enzyme, its SDS-PAGE analysis and its elution profile using chromatography techniques during the course of enzyme purification is summarised in Table 1, Figs. 3a and 4 respectively. The yield of alliinase enzyme was 1660 U per gram of the cell weight.

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3.4. Effect of temperature and pH on alliinase activity and stability

The enzyme was most active at 35 °C and it retained 80% of its activity at 30 °C and 40 °C (Fig. 5a). Aliinase exhibited maximum enzyme activity at pH 7 whereas more than 80% activity was retained at pH 6 and pH 8 (Fig. 5b). Optimum temperature and pH for enzyme activity was found to be 35 °C and 7 respectively. The alliinase enzyme from the isolate was stable at pH 7 and was more than 80% stable over a pH range of 6–8 (Fig. 5b). Aliinase was more than 80% stable at temperatures lower than 40 °C, whereas a further increase in
temperature resulted in a sharp decrease in the enzyme activity (Fig. 5a).

### 3.5. Effect of metal ions and chemicals on alliinase activity

The enzyme activity was enhanced in the presence of 1 mM of Mn$^{2+}$, Mg$^{2+}$, Zn$^{2+}$, Fe$^{2+}$ and Ca$^{2+}$. The highest activity was seen with Zn$^{2+}$ and Fe$^{2+}$. Cu$^{2+}$, Cs$^+$, Li$^+$, Ag$^{2+}$, SDS and Hg$^{2+}$ inhibited alliinase activity to 28%, 77%, 61%, 52%, 59%, and 27% respectively, whereas K$^+$, Na$^+$ and EDTA had no significant effect on alliinase activity (Fig. 6).

### 3.6. Determination of kinetic parameters

Michaelis–Menten kinetic parameters, $K_m$ and $V_{max}$ of the purified alliinase were calculated from Lineweaver–Burk double reciprocal plot (Fig. 7). The $K_m$ and $V_{max}$ values for the alliinase isolated from *C. necator* were found to be 74.65 U/protein mg and 0.83 mM respectively.

### 3.7. Ammonia, pyruvate and allicin determination

alliin (200 μM) on reaction with alliinase (10 U) resulted in the generation of 89.43 (±4.32) μM of allicin, 129.46 (±7.28) μM of ammonia and 148.45 (±8.98) μM of pyruvate.

### 3.8. Cytotoxicity of in-situ generated allicin

The cytotoxic effect of the in-situ generated allicin on MIA PaCa-2 and HDF cells was determined using MTT assay for 24 h. A concentration-dependent decrease in cell viability was observed for both the cell lines. IC$_{50}$ of alliin was found to be 74.054 (±4.545) μM for MIA PaCa-2 cells and 223.05 (±16.83) μM for HDF cell line. 140 μM of alliin in the presence of alliinase resulted in an 87.58% decrease in MIA PaCa-2 cell viability (Fig. 8) whereas the same concentration resulted in a 35.88% decrease in HDF cell viability.

Treatment of both the cell lines with reactants (alliin, alliinase) and by-products (ammonia and pyruvate) did not result
in any statistically significant difference in the cell viability when compared to untreated control.

3.9. Assessment of cytotoxicity by acridine orange–ethidium bromide staining

Acridine orange is a cell permeable dye and stains live cells green while ethidium bromide stains the cell that have lost their membrane integrity as red. As shown in Fig. 9f–h, cells treated with alliinase + 50, 100, and 200 μM alliin, exhibited orange–red colour signifying a loss of membrane integrity, cell shrinkage, nuclear fragmentation, membrane blebbing and condensed nuclei demonstrating cellular damage; whereas the untreated, alliin, alliinase, ammonia and pyruvate treated groups did not show any evident morphological changes (Fig. 9a–e).

4. Discussion

Recent years have seen significant progress in the study of the biological activity and application of sulphur containing compounds from garlic. Earlier, alliinases that have been purified to homogeneity and characterised include those from garlic cloves (Rabinkov et al., 1994), Onion bulbs (Schwimmer and Mazelis, 1963), leek (Lohmuller et al., 1994), Chinese chive (Manabe et al., 1998), and wild garlic/ramson (Landshuter et al., 1994). In this study, we have isolated alliinase producing bacterium from the soil, on the medium containing alliin as the sole nitrogen source. The alliinase producing organism was identified to be C. necator and soil is one of its natural habitats. C. necator is often used in bioremediation and is able to degrade a great number of chlorinated aromatic (Chloroaromatic) compounds and chemically related pollutants (Schenzle et al., 1999). This is the first report of the production of alliinase from C. necator. The synthesis of alliinase and its application for generation of allicin on demand, is a plant associated activity for defense purpose and the acquisition of this activity by a bacterium in the garlic grown soil is an important ecological development by plant-microorganism association and permits the organism to use alliin as a source of nitrogen.

Alliinase production from C. necator was lowest at 1% w/v glucose. A higher concentration of glucose inhibited alliinase production. Alliinase production from C. necator was highest during incubation under shaking condition which indicated that the organism requires aeration and it showed maximum production at 37 °C which was the average temperature of the area from where soil sample was collected. Alliinase isolated from C. necator is a homodimeric 110 kDa enzyme, consisting of two subunits of 55 kDa each, which is similar to the alliinase reported in bacterium E. adhaerens, which is a 96 kDa homodimeric enzyme consisting of two subunits of 48 kDa each (Yutani et al., 2011). Molecular weights of isolated alliinase vary depending on the source of the enzyme, ranging from 67 kDa in Chinese chive (Manabe et al., 1998) to 580 kDa in leek (Lohmuller et al., 1994). The number of enzyme subunits has been shown to differ depending on the source of the alliinase, ranging from 1 for Chinese chive (Manabe et al., 1998) to 12 for leek (Lohmuller et al., 1994). Musah et al. (2009) reported the presence of a heterotetrameric alliinase from Petiveria alliacea.

PLP is a cofactor essential for the C-S lyase activity of alliinase, so far reported (Lohmuller et al., 1994). Although each subunit of alliinase contains one tightly bound PLP, its exogenous addition enhances the enzyme activity as the purification proceeds (Schwimmer and Mazelis, 1963; Mazelis and Crews, 1968). Most metal ions had an effect on alliinase activity, amongst which, enzyme activity was enhanced by Mn²⁺, Mg²⁺, Zn²⁺, Fe²⁺ and Ca²⁺, whereas Cu²⁺, Cs⁺, Li⁺, Ag⁺, Hg²⁺ and SDS inhibited alliinase activity and K⁺, Na⁺ and EDTA had no significant effect. Stimulation of alliinase activity with Mg²⁺ and Mn²⁺ is in accordance with the findings by Kuettnert et al. (2002) with other Allium species. The increase in activity of alliinase due to Mn²⁺, Mg²⁺, Zn²⁺, Fe²⁺ and Ca²⁺ ions can be attributed to the vital role played by them in building active enzyme conformation. Inhibition of alliinase activity with Cu²⁺ has also been reported for alliinase from garlic (Kuettnert et al., 2002). In comparison to the results of Mazelis and Crews (1968), EDTA did not enhance enzyme activity of alliinase from C. necator, whereas EDTA appeared to enhance the activity of alliinase from garlic. The isolated alliinase preparation from C. necator may not have the presence of high metal ions, which is one of the factors responsible for EDTA activation of alliinase preparation of garlic.

The optimum reaction temperature and pH for alliinase from C. necator was found to be 35 °C and 7 respectively.
These conditions are similar to the study reported by others, wherein the optimum temperature of alliinase from garlic is 35 °C (Mazelis and Crews, 1968). On the other hand, it is higher than that established by Yutani et al. (2011) wherein the optimum reaction temperature for alliinase from E. adhaerens is 30 °C. A similar pattern was followed in pH, wherein

Figure 4 Elution profile of alliinase from C. necator. (a) Elution profile of alliinase in DEAE Cellulose discontinuous gradient chromatography. (b) Elution profile of alliinase in DEAE Cellulose continuous chromatography. (c) Elution profile of alliinase in Sephadex G-100 chromatography.

Figure 5 (a) Effects of temperature on alliinase activity and stability and (b) effect of pH on alliinase activity and stability. Relative activities were calculated by using the highest activity of free and immobilised alliinase as 100%, respectively.
alliinase from garlic was reported to be 6.5 (Mazelis and Crews, 1968) and alliinase from E. adhaerens was reported at pH 7 (Yutani et al., 2011). The alliinase enzymes from Allium sativum, Allium odorum and Allium chinense exhibited pH optima of pH 5.6–6.5 whereas alliinase from Allium tuberosum, Allium cepa, Allium porrum and Allium fistulosum exhibited pH optima of pH 8.0–8.5 (Manabe et al., 1998). The enzyme activity is stable over a narrow range of pH and temperature, a similar phenomenon is seen in most of the alliinases reported so far. Extreme temperature and pH might be having an effect on the structure of enzyme, rendering it inactive. This temperature and pH sensitivity of alliinase can be encountered during oral administration, by a method reported by Lianga et al. (2013) wherein an enteric coated double-layer tablet of alliin/alliinase was used, for optimum activity of alliinase on alliin under gastrointestinal condition.

The specific activity of alliinase isolated from C. necator was found to be 209 U/mg, which is the highest reported specific activity from microbial origin till date, whereas specific activity of alliinase reported from garlic varies from 218 to 660 U/mg depending on the source of garlic (Kuettner et al., 2002). Alliinase from C. necator appears to exhibit the highest affinity towards alliin as compared to alliinases reported from different sources. The $K_m$ of alliinase from C. necator was found to be 0.83 mM which is lower than the $K_m$ value of alliinase reported from garlic (1.1 mM) (Tobkin and Mazelis, 1979) and that from E. adhaerens (2.3 mM) (Yutani et al., 2011). A lower $K_m$ corresponds to the higher affinity of the enzyme towards the substrate. Purified alliinase from C. necator exhibited lowest $K_m$ reported so far, which indicates that the enzyme has the highest affinity towards alliin, hence it is an ideal candidate for immobilisation on a suitable matrix and can be exploited further for the generation of allicin.

MTT assay is a well-recognised in vitro technique, practiced to investigate cytotoxicity of compounds against cancer cell lines. It has been used as one of the conventional techniques for screening of compounds with potential cytotoxic properties.

**Figure 6** Alliinase was incubated with different metals and chemicals under standard assay conditions. The 100% enzyme activity was obtained in the absence of metal ions or chemicals.

**Figure 7** Lineweaver–Burk double reciprocal plot for kinetic analysis of alliinase extracted from C. necator using different concentrations of alliin.

**Figure 8** Cytotoxic effect of in-situ generated allicin on MIA PaCa-2 cells. Cells were treated with increasing concentrations of alliin in the presence of alliinase extracted from (C. necator) for 24 h. Data are represented as mean ± SD of three replicates from three independent experiments. The values are represented as the percentage cell viability where the viability of untreated cells was regarded as 100%. Data were analysed by a one-way ANOVA followed by Dunnett’s post hoc test. Asterisk above bars indicate statistically significant difference compared to untreated control (***$p < 0.001$, **$p < 0.01$, *$p < 0.05$).
In-situ generated allicin induced a concentration-dependent decrease in cellular viability in MIA PaCa-2 cells and IC$_{50}$ was determined to be 74.054 (±4.545) μM of alliin for MIA PaCa-2 cells and 223.05 (±16.83) μM of alliin for the HDF cell line which indicates that allicin is selectively more cytotoxic to MIA PaCa-2 cells as compared to HDF cells. Allicin-induced cytotoxicity was accompanied by morphological changes, including nuclear condensation, membrane blebbing and cell shrinkage which were confirmed using Acridine orange–ethidium bromide staining wherein a concentration dependent change in cell morphology was observed.

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