Discussion
The present study was undertaken to isolate and characterize acrylamide degrading microorganisms from soil. Acrylamide degradation is achieved biologically by the enzyme amidase produced by the bacteria. The culture conditions for the degradation of acrylamide were also optimized during the study.

Nitrogen Deficient Agar Medium (NDAM) was used for the culture of microorganisms. NDAM is a medium without any nitrogen source and acrylamide was the only source of nitrogen in the medium. The results of the present study revealed that the soil samples collected from the university garden in front of Zoology department and soil collected from outside the Kumarrappa handmade paper industry did not show any growth on NDAM plates. Soil collected from the Kumarappa handmade paper industry showed bacterial growth in NDAM. This may be because this industry uses lots of acrylamide for mucilage for making fine quality of handmade paper. Hence the presence of acrylamide is obvious in the soil and the acrylamide degrading bacteria easily grow in this soil and utilize the acrylamide as a nitrogen source for their survival.

The acrylamide degrading capacities of bacterial isolates were done by estimating the amidase activity in the culture medium. Amidase degrades acrylamide and releases ammonia and acrylic acid. The bacterial colonies were taken from various locations of NDAM plates. These NDAM plates showed evidence of production of amidase (0.4 units/ml). Some bacterial amidases that hydrolyzes acrylamide to acrylate have been purified and characterized.

The selected colonies of bacteria were streaked on NDAM plates to obtain pure culture. The morphological, cultural, biochemical and molecular characterization
of the isolate were performed. This revealed the bacteria isolated were Gram positive, rod shaped, spore forming and motile. Cultural morphology revealed smooth, round, whitish colonies on the agar plate. They hydrolyze acrylamide in the medium which is the sole source of nitrogen and release ammonia and acrylic acid.

Catalase test was performed to know the ability of the bacteria to detoxify the $H_2O_2$ which is produced as a result of aerobic respiration. The isolate was found to be positive for the catalase test and citrate utilization test. The isolate was found to be negative for starch hydrolysis, gelatin liquefaction, $H_2S$ production, oxidase activity and urea hydrolysis. The isolate was tested to be MR and VP negative. The isolate were also tested to determine indole production in tryptophan broth and was found to be negative. The isolate had the property to reduce nitrate into nitrite to determine indole production in tryptophan broth and was found to be negative. The isolate had the property to reduce nitrate into nitrite.

In addition, the isolate were also tested for their ability to use various carbohydrates as a sole source of carbon and for the presence of the gas production. In the present study, the isolate used glucose, sucrose, fructose, maltose, mannitol, xyloses, rhamnose, salicin and m-inositol as carbon source. The best carbon source for the growth of the isolate is glucose.

The genomic DNA was isolated and further subjected to PCR to amplify the 16S rRNA gene of bacterial genome. The PCR product was subjected to 1% electrophoresis and the amplified product was excised from agarose gel and purified. The purified fragment was subjected to sequencing. The nucleotide sequences obtained were subjected to BLAST at NCBI to look for homology of this nucleotide
sequences with the available nucleotide sequences of the database. The result of the BLAST analysis revealed that the isolated bacterium belongs to species *clausii* of genus *Bacillus*, family Bacillaceae order Bacillales, class Bacilli and the phylum Firmicutes. The phylogenetic tree was inferred by using the neighbour – joining method of BLAST at NCBI at a sequence difference of 0.75. The obtained isolate was deposited in the MTCC at IMTECH, Chandigarh with the strain identification 1779 (*Bacillus clausii*). The nucleotide sequence of bacteria showed similarity with bacteria *Bacillus clausii*, [DSM 8716(T)], *Bacillus pathogenesis* [PAT 05 (T)], *Bacillus lehensis* (AY 793550), *Bacillus oshimensis* [K11(T)], *S* (JSM 0810036).

The nutrients and growth conditions which support maximum growth of bacteria were determined. Reaction conditions were optimized with respect to substrate, pH, temperature, energy source and acrylamide concentration by growing the selected bacteria in the pre-optimized medium and the amidase activity was determined in changing conditions by changing one parameter at a time and keeping all the other parameter constant.

The effects of different pH range from 7.5 to 10.0 were also studied. The maximum growth of bacteria was obtained at pH 9.0. Miller and Gray (1982) reported that *Rhodococcus sp.* exhibit maximum enzyme activity at pH 7.0 whereas another *Rhodococcus* strain exhibit highest activity at pH 7.5 (Mayaux et al., 1991). Egorova et al., (2004) reported an optimum pH for the growth of *Psuedonocardia thermophilia* at pH 7.2. A slight departure from neutrality as the requirement of optimum growth was reported by Postec et al. (2005). They reported that the hyperthermophilic archaeon *Thermococcus hydrothermalis* requires pH 6.
The effects of different temperatures (20°C – 45°C) were studied for the optimal growth of bacteria. This revealed that maximum growth of bacteria was at 37°C. Miller and Gray (1982) reported that amidase of *Rhodococcus sp.* exhibited optimum activity at 25°C. Aromatic amidase from *Rhodococcus JJ* was active at 25°C (Kobayashi *et al.*, 1993). Most of the commonly studied acrylamide/aliphatic amide-degrading bacteria are mesophiles with optimum temperature for growth in the range of 25°C to 40°C. Ciskanik *et al.* (1995) reported an optimum temperature for the growth of *Psuedomonas aeruginosa* whereas Nawaz *et al.* (1994), Kotlova *et al.* (1999), and Wang and Lee (2001) reported 30°C as the optimum temperature for the growth of *Rhodococcus rhodochrous*.

Maintaining all other parameters studied constant, varying concentrations of acrylamide were studied for maximum growth. Maximum growth of these bacteria was observed at 1000 mg/l. A previous study reported that the *pseudomonas stutzeri* had an optimum growth at 440 mg/l (Wang and Lee, 2001).

The effect of different carbon sources on growth was also studied. The study indicates that the glucose is the best carbon source for growth of this bacterial growth. A previous study showed that *Klebsiella pneumoniae* NCTR1 (Ciskanik *et al.*, 1995; Nawaz *et al.*, 1996) demonstrated optimum growth at 1% glucose supplemented in the medium as a carbon source. Kotlova *et al.*, (1999) reported 2% glucose for an optimum growth. In contrast, Egorova *et al.*, (2004) used soluble starch as the carbon source of choice for the growth of *Psuednocardia thermophilia*. Maximum growth of these bacteria was shown in the presence of acrylamide compared to the other amides studied.
The nutrients and growth conditions which yielded maximum amidase enzyme production were determined. It was done in a sequential manner. This enzyme exhibited maximum activity within a range of pH 7.0 to 7.5 with optimum activity at pH 7.0. The increase or decrease in pH from 7.0 was found to decrease the enzyme activity probably because of alteration in the enzyme structure. Miller and Gray, (1982) reported that the *Rhodococcus sp.* exhibit maximum enzyme activity at pH 7.0 whereas Mayaux *et al.* (1991) reported that the *Rhodococcus* strain exhibit highest activity at pH 7.5. The maximum enzyme activity of aromatic amidases of *Rhodococcus rhodochorus* J1 is exhibited at pH 7.5 (Kobayashi *et al.*, 1993). Buffers are generally used to prevent a drastic change in reaction pH and to maintain the desired pH of the enzyme. The maximum activity of Enantioselective amidase of *Rhodococcus* strain is seen in 0.1 M Tris–HCL buffer at pH 7.5, containing 5Mm DTT (Mayaux *et al.*, 1991).

Temperature is a critical parameter which needs to be controlled for any biotransformation study. This usually varies from one organism to another. The optimum temperature is a crucial factor not only for the growth of cell, but also for the secretion of enzymes. The optimum temperature for the production of amidase was found to be 35°C. The optimum temperature of enzyme activity is dependent upon the activation energy at a given temperature and the rate of thermal denaturation of the enzyme. The activity of this enzyme was found to be reduced when the temperature was either increased or decreased below 35°C. The findings are attributed to be due to disturbances created in the enzyme structure when the temperature is increased. The majority of amidases are active at higher temperatures as compared to nitrile hydratases and nitrilases. Miller and Gray (1982) reported that
amidase of *Rhodococcus sp.* exhibited optimum activity at \(25^0\)C. Kobayashi *et al.* (1993) also reported that the aromatic amidase from *Rhodococcus J1* was active at \(25^0\)C.

Sonication time was also affected on amidase activity. Amidase exhibited maximum activity after 50 seconds of sonication. The effect of incubation time also has effects on the enzyme activity. This enzyme has maximum activity at 30 minutes of incubation. The changes in this time cause a decrease of enzyme activity. Different substrates effect on enzyme activity. Acrylamide showed maximum enzyme activity.

All the metal ions studied, were found to reduce the enzyme activity. These results were attributed to the fact that metal ions hindered the catalytic process either by preventing the exchange of functional groups between substrate and the enzyme or by blocking the enzyme activity by making complexes with essential functional groups. The minimum reduction in amidase activity was observed in the presence of \(\text{AgNO}_3\) where as maximum inhibition was observed in the presence of \(\text{MgCl}_2\cdot6\text{H}_2\text{O}\). Various studies were done to determine the effect of metal ions on amidase activity and they revealed that the inhibition pattern differed from organism to organism.

Amidase was purified from its native conditions by a two phase chromatography system using on ion exchange and gel filtration chromatography. The molecular weight of a purified amidase from *Bacillus clausii* 1779 was found to be 65 KDa in SDS PAGE. It is evident from literature that the molecular weight of amidase varies according to the organism and its nature of synthesis. The above variation in the protein size can be explained on the basis of its diversity or the
occurrence of various isoforms of the enzyme in different individuals. In addition to this, size is also dependent upon the location of the protein in the organism. Kotlova et al. (1998) purified amidase by precipitation of crude extract with isopropanol followed by anion exchange chromatography. It was also reported that amidase has 4 subunits with molecular weight 45KDa. A single band of amidase was also obtained when the crude extract was applied onto the anion-exchange column (Hirrlinger et al., 1996).

Thus the present investigation was an attempt to isolate and identify the bacteria which is able to metabolise the acrylamide. As a result of the study a bacterium was isolated and identified as *Bacillus clausii* 1779. Further, the enzyme amidase which helps in the degradation of acrylamide also isolated and characterised. The culture conditions for enzyme production were also optimized during the study.