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ISOLATION, SCREENING AND ASSAYING OF METHIONINASE OF *BREVIBACTERIUM LINENS*

[1]Rajasekhar Pinnamaneni, [2]Sai Ramalinga Reddy Gangula, [1]Subramanyam Koona & [1]Ravindra Babu Potti

[1]Department of Biotechnology, Sreenidhi Institute of Science and Technology, Yamnampet, Ghatkesar, R.R.District, Andhra Pradesh, India-501 301

[2]Department of Microbiology, Sri Sai Baba National Degree and PG College, Anantapur Andhra Pradesh, India- 515 001

ABSTRACT

Brevibacterium linens is a normal flora present in the whey of curd, which is a rich source of L-Methionine γ-lyase (MGL). This enzyme can be utilized as a therapeutic agent in treating cancer. *Brevibacterium linens* is a gram positive bacterium and it was identified at the molecular level by ribotyping 16s rDNA along with the biochemical characterization, and finally concluded by BLAST analysis by constructing a phylogenetic tree. The enzyme methioninase was purified by precipitating with Ammonium sulphate, dialysis, Anion exchange chromatography and. The purity of methionase checked by SDS-Polyacrylamide gel electrophoresis and the band corresponding to the molecular mass of the native enzyme was estimated to be approximately 170 kDa. The activity of the crude enzyme was determined by the production of a-keto acids. The enzyme was stable at pH ranging from 6.0 to 8.0 for 24 h. Freezing and thawing the enzyme solution resulted in a loss of over 60% of the enzyme activity, and the enzyme was labile at temperatures greater than 30°C.

KEYWORDS: *Brevibacterium linens,* L-Methionine γ-lyase, 16s rDNA,

INTRODUCTION

Methanethiol is associated with desirable Cheddar-type sulfur notes in good quality Cheddar cheese (Aston and Dulley, 1995; Urbach, 1995). The mechanism for the production of methanethiol in cheese is linked to the catabolism of methionine (Alting *et al.,* 1995; Lindsay and Rippe, 1986). L-Methionine γ-lyase (EC 4.4.1.11; MGL), also known as methionase, L-methionine γ-demethiolase, and L-methionine methanethiollyase (deaminating), is a pyridoxal phosphate (PLP)-dependent enzyme that catalyzes the direct conversion of L-methionine to a ketobutyrate, methanethiol, and ammonia by an α,γ -elimination reaction (Tanaka *et al.,* 1985). It does not catalyze the conversion of D enantiomers (Tanaka *et al.,* 1985, 1983, 1976). L-Methionine γ-lyase is a multifunctional enzyme system since it catalyzes the α,γ - and α,β-elimination reactions of methionine and its derivatives (Tanaka *et al.*, 1983). In addition, the enzyme also catalyzes the β-replacement reactions of sulfur amino acids (Tanaka *et al.*, 1983). Many cancer cells have an absolute requirement for plasma methionine, whereas normal cells are relatively resistant to the restriction of exogenous methionine (Cellarier *et al.*, 2003). Methionine depletion has a broad spectrum of antitumor activities (Kokkinakis, 2006). Under methionine depletion, cancer cells were arrested in the late S-G2 phase due to the pleiotropic effects and underwent apoptosis. Thus, therapeutic exploitation of L-Methionine γ-lyase to deplete plasma methionine has been extensively investigated (Yoshioka *et al.,* 1998). Growth of various tumors such as Lewis lung carcinoma (Tan *et al.,* 1999), human colon cancer lines (Kokkinakis *et al.*, 2001), glioblastoma (Hu and Cheung, 2009), and neuro-blastoma (Miki *et al.*, 2001) was arrested by MGL. MGL in combination with anticancer drugs such as cisplatin, 5-fluorouracil, nitrosourea, and vincristine displayed synergistic antitumor effects on rodent and human tumors in mouse models (Spallholz *et al.*, 2004; Yang *et al.*, 2004; Sun *et al.,* 2003; Takakura *et al.*, 2006). Since its discovery in *Escherichia coli* and *Proteus vulgaris* (Onitake, 1983), this enzyme has been found in various bacteria and is regarded as a key enzyme in the bacterial metabolism of methionine. This enzyme has been partially purified and characterized from *Brevibacterium linens* (Collin and Law, 1989). *B. linens* is a nonmotile, non-spore-forming, non-acid-fast, gram-positive coryneform bacterium normally found on the surfaces of Limburger and other Trappist-type cheeses. This organism tolerates salt concentrations ranging between 8 and 20% and is capable of growing in a broad pH range from 5.5 to 9.5, with an optimum pH of 7.0 (Purko *et al.*, 1951). In Trappist-type cheeses, *Brevibacteria* depend on *Saccharomyces cerevisiae* to metabolize lactate, which increases the pH of the curd, as well as to produce growth factors that are important for their growth (Purko *et al.*, 1951). Interest in *B. linens* has focused around its ability to produce high levels of methanethiol (Boyaval and Desmazeaud, 1983; Ferchichi *et al.*, 1986; Hemme *et al.*, 1982; Sharpe *et al.,* 1977). *B. linens* produce various sulfur compounds, including methanethiol, that are thought to be important in Cheddar-like flavor and aroma (Boyaval and Desmazeaud, 1983; Ferchichi *et al.*, 1986; Hemme *et al.*, 1982; Sharpe *et al.,* 1977). In light of the importance of MGL and its production by *Brevibacterium linens*, which is a normal flora present in the whey of curd, hence an attempt was made to isolate *Brevibacterium linens,* screen the organism and assay the produced MGL enzyme.

MATERIALS AND METHODS
Collection of curd sample
Different curd samples were collected and the watery whey was taken from the curd samples and was subjected for serial dilution.

Isolation and screening of *Brevibacterium linens* for production of MGL enzyme
TGY media containing Tryptone 5g; Glucose 1g; Yeast extract 5g; K_2HPO_4 1g; Agar 1.5% per litre was prepared and sterilized. The serially diluted curd samples were inoculated on the solidified plates and kept for incubation of 48 hours.

Microbial and Biochemical characterization of the isolate
The isolated bacteria was analyzed using different staining techniques such as Simple staining, Gram staining, Motility Test and different biochemical techniques such as Indole production Test, Methyl Red test, Voges-Proskauer Test, Citrate utilization Test, Starch Hydrolysis Test, Gelatin Hydrolysis Test, Catalase Test, Nitrate reduction test, Caesin Hydrolysis, Gelatin Test and Oxidase Test.

MOLECULAR IDENTIFICATION
Primer design
Brevibacterium genus-specific primers (forward: 5[1]TACCAAACTGTTGCCTCGGCGG3[1] and reverse: 5[1]GATGAAGAAGGCAGCGAAATGCGATA3[1]) were designed based on the homologous regions specific to *Brevibacterium* genus.

DNA isolation and amplification of 16s RNA gene of *Brevibacterium linens* by Polymerase Chain Reaction (PCR)
The template genomic DNA from *Brevibacterium linens* was isolated following the protocol (Tsai and Olson, 1992). In Polymerase Chain Reaction, the specific primers *Forward* and *Reverse* (IBS, Vijayawada) were used to amplify the genomic sequence of the open reading frame (ORF) of the gene. PCR conditions were 94^0C for 2 min, and then 94^0C for 1 min, 60^0C for 1 min, 72^0C for 3 min for a total of 30 cycles, with the extension at 72^0C for 10 min (Volossiouk *et al.,* 1995).

Agarose gel electrophoresis
Required amount of agarose (w/v) was weighed and melted in 1X TBE buffer (0.9M Tris-borate, 0.002 M EDTA, pH 8.2). Then, 1-2 µl ethidium bromide was added from the stock (10 mg/ml). After cooling, the mixture was poured into a casting tray with an appropriate comb. The comb was removed after solidification and the gel was placed in an electrophoresis chamber containing 1X TBE buffer. The products were mixed with 6X loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol FF, 30% glycerol in water) at 5:1 ratios and loaded into the well. Electrophoresis was carried out at 60V (Sambrook *et al.,* 1989).

Eluting DNA from agarose gel fragments
Ethidium bromide stained agarose gel was visualized under a transilluminator. The fragment of interest was excised with a clean razor blade. After removing the excess liquid, the agarose fragment was placed in the spin column. The tube was centrifuged at 5500 rpm for not more than 45 seconds for the elution of DNA. The eluent was checked by running on an agarose gel and observed on a transilluminator for the presence of ethidium bromide stained DNA. The eluted DNA was used directly in manipulation reactions. This DNA fraction was subjected for sequencing (IBS, Vijayawada).

Sequencing and chimera checking
The eluted PCR product was directly sequenced using *Brevibacterium* genus-specific primers without GC-clamp at Ohmlina Centre for Molecular Research, Chennai. Sequencing reactions were carried out with ABI PRISM Dye Terminator Cycle Sequence Ready Reaction Kit (Applied Biosystems Inc., USA). All sequences exhibiting less than 95% sequence similarity to existing sequences in GenBank were checked using CHIMERA-CHECK program at the Ribosomal Database Project (RDP) using default settings (Cole *et al.,* 2003). All representative sequences corresponding to bands were *Brevibacterium* species.

Phylogenetic placement
The environmental sequences were compared to the sequences in GenBank using the BLAST algorithm (Altschul *et al.,* 1997) and RDP database (Cole *et al.,* 2003) to search for close evolutionary relatives.

GenBank accession numbers
The representative sequence of the soil *Brevibacterium* species was deposited in GenBank of National Centre for Biotechnology Information (NCBI). The GenBank accession number is JF747329.

METHIONINASE PURIFICATION
Filtration
Culture filtrate of culture grown for 48 hours was filtered through Whatman filter paper number 5 and the filtrate was subjected to precipitation with ammonium sulphate (75%, wt/vol). After dialysis against the same buffer, the crude extract was subjected to anion-exchange chromatography with a DEAE cellulose column (IBS, Vijayawada). After the column was washed with 3 column volumes of 20 mM Tris-HCl buffer (pH 7.8), it was eluted with a 100-ml linear gradient of NaCl (0 to 1 M) in the washing buffer at a flow rate of 1 ml/min. Then the samples containing equal amount of protein were loaded into the wells of 12% polyacrylamide gels. The medium ranged molecular weight marker mixed with the sample buffer was also loaded in one of the wells. Electrophoresis was carried out at constant voltage of 75 volts. The gels were stained with 0.2 percent coomassie brilliant blue solution overnight and then destained. Relative mobilities of each protein band were recorded.

Methioninase enzyme assay
Amount of free thiol groups were determined by the method of Laakso and Nurmikko, 1976. The assay mixture contained 50 mM potassium phosphate (KP; pH 7.2), 10 mM L-methionine, 0.02 mM PLP, 0.25 mM 5,59-dithio-bis-2-nitrobenzoic acid (DTNB), and the enzyme in cell extracts (CEs) or in pure form in a final volume of 1.0 ml. The reaction mixture was incubated quiescently at 25°C for 1 h and observed at 412 nm in a double-beam model UV2100U spectrophotometer (Shimadzu Scientific Instruments, Inc., Pleasanton,Calif.). The concentration of thiols produced was determined from a standard curve obtained with solutions of known concentrations of

ethanethiol. α-Ketobutyrate produced by the α,γ elimination of methionine was measured by derivatizing the reaction mix with 3-methyl-2-benzothiazolone hydrazone (Soda, 1967), The assay mixture contained 50 mM KP (pH 7.2), 10 mM L-methio-nine, 0.02 mM PLP, and .0.015 U of the enzyme in a final volume of 0.5 ml. The reaction mixture was incubated at 25°C for 1 h, and the reaction was stopped by the addition of trichloroacetic acid to a final concentration of 5%. After centrifugation at 16,000 3 *g* for 2 min, the α -ketobutyrate formed in the supernatant solution was determined with 3-methyl-2-benzothiazolone hydrazone (Soda, 1983).

Influence of temperature and pH
Optimum temperature for 1h assay was determined by assaying activity over temperatures ranging from 4 to 50°C in 0.05 M KP buffer (pH 7.5), with each buffer being made at each of the tested temperatures. Temperature stability of the enzyme was determined by incubating the enzyme in 0.05 M KP buffer (pH 7.5) for up to 1 h at temperatures ranging from 4 to 50°C. Aliquots were removed at various times, and residual activity was measured at 25°C in 0.05 M KP buffer (pH 7.5) by determining the amount of α -ketobutyrate produced with 10 mM Met as the substrate. The stability and optimum pH of the enzyme were determined at 25°C with 50 mM potassium citrate (pH 4.0 to 6.5), 50 mM KP (pH 6.5 to 8.0), and 50 mM potassium-glycine-NaOH (pH 8.0 to 10.0) buffers. The pH stability of the enzyme was determined by incubating the enzyme at each pH with 0.02 mM PLP for 24 h at 4°C. Residual activity was measured by incubating the enzyme in 0.05 M KP buffer (pH 7.5) at 25°C for 1 h and by determining the amount of α -ketobutyrate produced with 10 mM Met as the substrate.

Kinetic studies
Enzyme kinetics for the α, γ-elimination reaction was determined with methionine as the substrate and by measuring the amount of methanethiol produced with DTNB (Laakso and Nurmikko, 1976). The enzyme was incubated with 0.05 M KP (pH 7.5), 0.02 mM PLP, 0.28 mM DTNB, and 0.1 to 40 mM Met. The reaction was started by the addition of substrate, and product formation was monitored continuously at 412 nm with a model UV2100U double-beam spectrophotometer (Shimadzu Scientific Instruments, Inc.).

RESULTS AND DISCUSSION
Isolation and screening of *Brevibacterium linens* for production of MGL enzyme
The organism *Brevibacterium linens* species was isolated by plating the serially diluted samples on TGY media. The bacterial growth was observed in 10^{-6} dilution plate after incubation of 48hrs at RT which was reddish brown in colour.
Basing on the microbial and biochemical characterization of the test organism suspected as *Brevibacterium linens.* However, it cannot be concluded at this stand point itself and for the identification of the bacteria exactly upto the species level, it is evident to follow molecular based techniques and hence an attempt was carried out further for the characterization of the test bacteria based on the DNA coding for 16s rRNA sequences. All the biochemical tests were carried out based on Bergey's

Manual of Systematic Bacteriology (Krieg and Holt, 1984, Borjana *et al*, 2002., Holt *et al*., 1994) (Table 1).

TABLE 1: Microscopic Examination and Biochemical Reaction of Bacterial Population

Microscopic Observations	
Simple staining	Rod Shaped
Gram staining	Gram positive bacilli
Motility Test	Motile
Biochemical Tests	
Indole production Test	Negative
Methyl Red test	Negative
Voges-Proskauer Test	Negative
Citrate utilization Test	Positive
Starch Hydrolysis Test	Negative
Gelatin Hydrolysis Test	Positive
Catalase Test	Positive
Nitrate reduction test	Positive
Starch Hydrolysis	Negative
Caesin Hydrolysis	Positive
Gelatin Test	Positive
Oxidase Test	Positive

Molecular Characterization Based on 16s rDNA Sequence
The DNA isolated from bacteria suspected to be *Brevibacterium linens* when checked for purity exhibited an absorbance ratio of 1.9 (A_{260}/A_{280} ratio 1.8 to 2.0 to be pure), which can be concluded that the DNA isolated was pure and the same DNA samples, when run on an agarose gel also confirmed to be pure as the bands of DNA are single and distinct and no traces of contaminants were found when observed under the UV transilluminator (Figure 1).
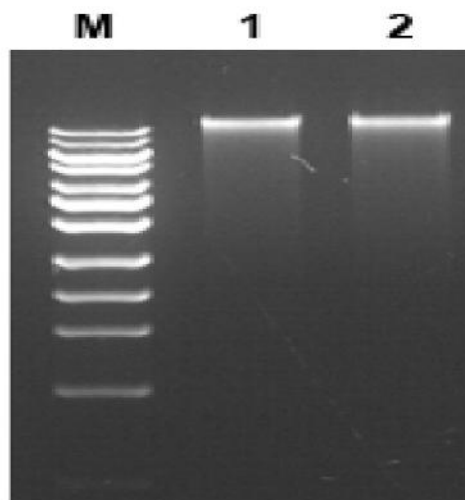

FIGURE 1. Agarose gel showing Whole Genomic DNA Well M- Marker of 1kb.
Well 1 and 2 - *Brevibacterium linens* whole genomic DNA.
The genomic DNA of the two organisms were isolated and gene (DNA) coding for the 16s rDNA was amplified by Polymerase chain reaction, yielded a DNA band of 735 bases for *Brevibacterium linens*.

Sequencing of the 16S rDNA gene of *Brevibacterium linens*

In order to characterize the strain, the nucleotide sequences of the 16S rDNA of the strain were determined. Phylogenetic tree was constructed by the neighbour-joining (N-J) method based on the 16S rDNA sequences. The 16S rDNA gene from the genomic DNA of the *Brevibacterium linens* (based on the Biochemical and staining properties) was enzymatically amplified by Taq DNA polymerase by using a universal eubacterial primer set, (Forward Primer) 5'-GAGTTTGATCCTGGCTCAG-3' (positions 9–27 [*Escherichia coli* 16S rDNA numbering]) and (Reverse primer) 5'-AGAAAGGAGGTGATCCAGCC-3' (positions 1542–1525 [*E. coli* 16S rDNA numbering]) were used. After purifying the PCR product with (Helini DNA purification kit) the resulting PCR product was sequenced commercially. The amplified DNA was subjected to agarose gel electrophoresis (Figure 2).
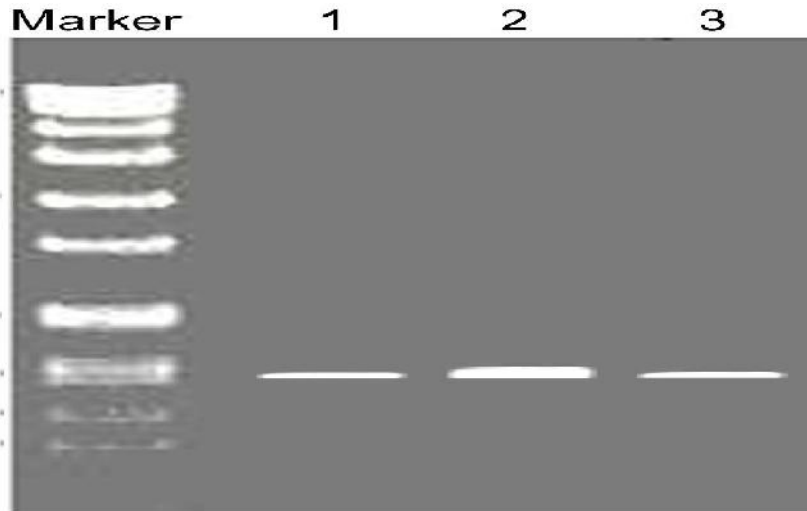

FIGURE 2. Agarose gel showing Amplified 16s rDNA

Well M- Marker of 1kb.
Well 1, 2 and 3   - *Brevibacterium linens* 16s rDNA

The sequence obtained was blasted in NCBI data base, and phylogenetic analysis of the *Brevibacterium linens* was carried out.
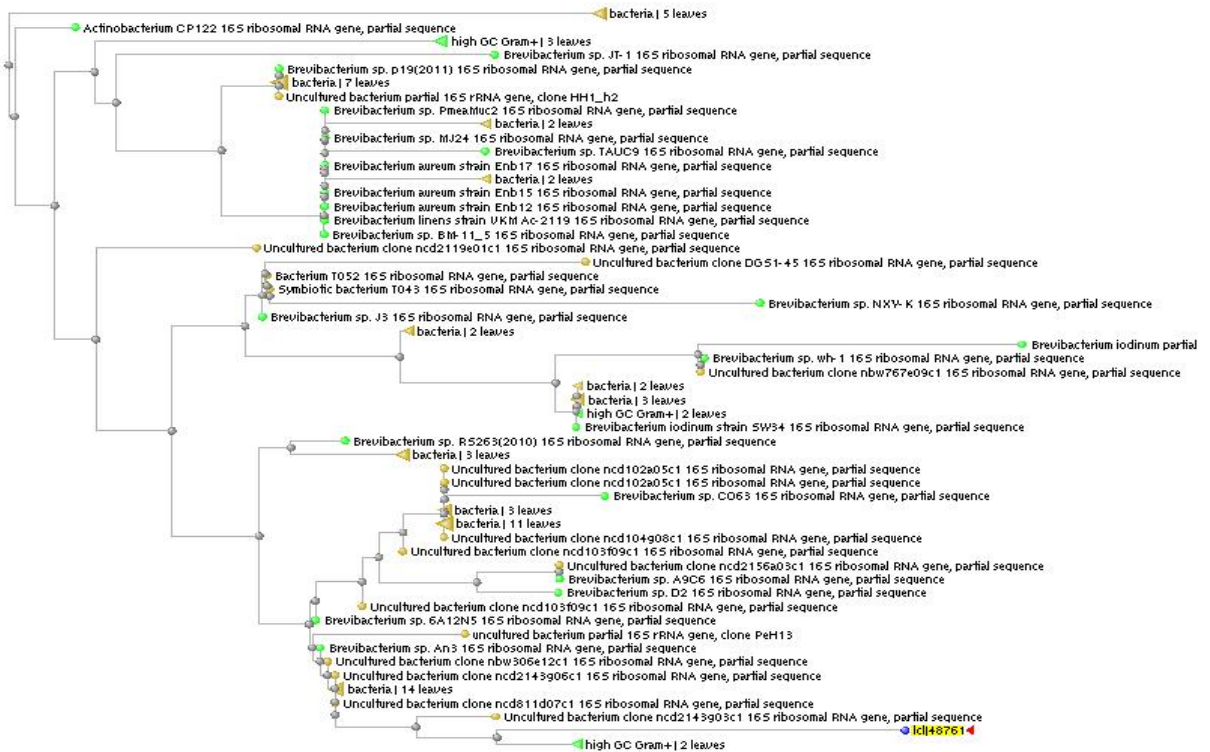

FIGURE 3. Phylogenetic tree of *Brevibacterium linens*
The phylogenetic analyses of these strains were confirmed using its 16S rDNA sequence.

GAGTTTGATCCTGGCTCGGACACATGCAAGTCGAACGCT
GAAGCCAGAAGCTTGCTTCTGGTGGATGAGTGGCGAACG
GGTGAGTAACACGTGAGTAACCTGCCCCTGATTTCGGGA
TAAGCCTGGGAAACCGGGTCTAATACCGGATACGACCAT
CCCTCGCATGAGGGTTGGTGGAAAGTTTTTCGATCGGGG
ATGGGCTCGCGGCCTATCAGCTTGTTGGTGGGGTAATGG
CCTACCAAGGCGACGACGGGTAGCCGGCCTGAGAGGGCG
ACCGGCCACACTACTGAGACACGGCCCAGACTCCTACGG
GAGGCAGCAGTGGGGAATAAATGCACAATGGGGGAAACC
CTGATGCAGCGACGCAGCGTGCGGGATGACGGCCTTCGT
GTAAACCGCTTTCAGCAGGGAAGAAGCGTAAGTGACGGT
ACCTGCAGAAGAAGTACGGCGGCTAACTACGTGCCAGCA
GCCGCGGTAATACGTAGGGTACGAGCGTTGTCTTCGGAA
TTATTGGGCGTAAAGAGCTCGTAGGTGGTTGGTCACGTC
TGCTCTGGAAACGCAACGCTTAACGTTGCGCGTGCAGTG
GGTACGGGCTGACTAGAGTGCAGTAGGGGAGTCTGGAAG
TCCTGGTGTAGCGGTGAAATGCGCAGATATCAGGAGGAA
CACCGGTGGCGAAGGCGGGATGGGCTGTAACTGACACTG
AGGAGCGAAAGCATCTTTCCTCCACTAGGTCGG

The above sequences were compared with the known sequences in the public databases in NCBI gave a BLAST results which are given in the form of a phylogenetic tree. Based on the 16s rDNA sequences, the above bacterium was confirmed as *Brevibacterium linens* (Figure 3). The

accession number of the sequence deposited at NCBI (National Centre for Biotechnology Information was JF747329

Enzyme analysis
The enzyme methioninase was precipitated by saturating with 35% and 70% Ammonium sulphate and the methioninase enzyme was obtained in 70% fraction. This enzyme fraction upon subjecting to dialysis, the pure fraction was obtained. This pure fraction was further purified by Anion exchange chromatography and the eluent was collected. This eluent was checked by SDS-Polyacrylamide gel electrophoresis and the band corresponding to the molecular mass of the native enzyme was estimated to be approximately 170 kDa and was determined during the final stage of purification with a Superose 12 gel filtration column. When the gel was electrophoresed under denaturing conditions by sodium dodecyl sulfate-PAGE, a single band with an approximate molecular mass of 43 kDa was observed (Figure 4). Analysis of the absorption spectrum of the purified enzyme demonstrated a peak at 420 nm in addition to a peak at 280 nm.
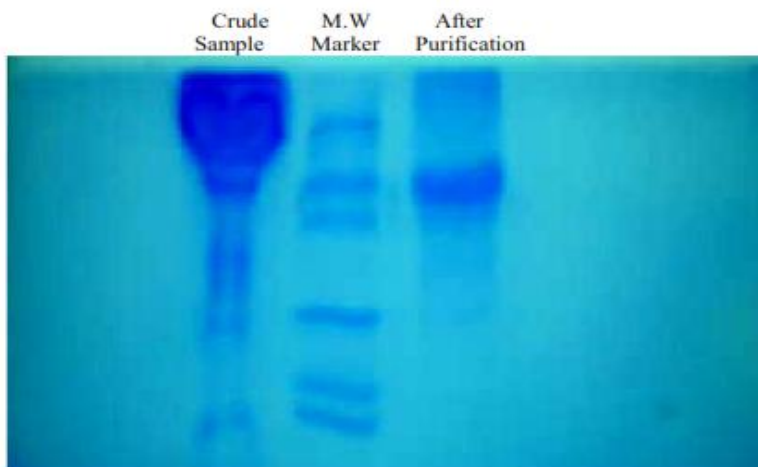

FIGURE 4. SDS-PAGE
Protein MW Marker: 14kDa, 20kDa, 25kDa, 35kDa, 45kDa, 66kDa

Substrate specificity
The activities of the purified enzyme on various substrates were determined by the production of a-keto acids. The production of thiols from KMTB was monitored. The purified enzyme was capable of catalyzing the a,g elimination of substrates L-Methionine and L-homocysteine.

Kinetic parameters
The *Km* for the catalysis of methionine as determined from the rates of methanethiol and α-ketobutyrate production was found to be 6.12 mM, and the maximum rate of metabolism as determined from Eadie-Hofstee plots was found to be 7.0 mmol /min/ mg.

Influence of temperature and pH
The pH optimum for the α,γ elimination of methionine was 7.5 to 8.0 (Figure 5). At pH 5.5 the enzyme retained

over 20% of its activity, at pH 4.5 its activity decreased to 10%, and at pH 4.0 it became inactivated. At pH 7.5 the enzyme had highest activity at 25°C (Figure 6). The enzyme was stable at pHs ranging from 6.0 to 8.0 for 24 h. Partially purified as well as pure enzyme could be stored on ice at 4°C in 0.05 M KP (pH 7.5) with 0.02 mM PLP without significant loss of activity for over 2 weeks. Freezing and thawing the enzyme solution resulted in a loss of over 60% of the enzyme activity, and the enzyme was labile at temperatures greater than 30°C. The denaturation reaction demonstrated first-order kinetics and had a standard free energy of activation of 186 kJ mol21.
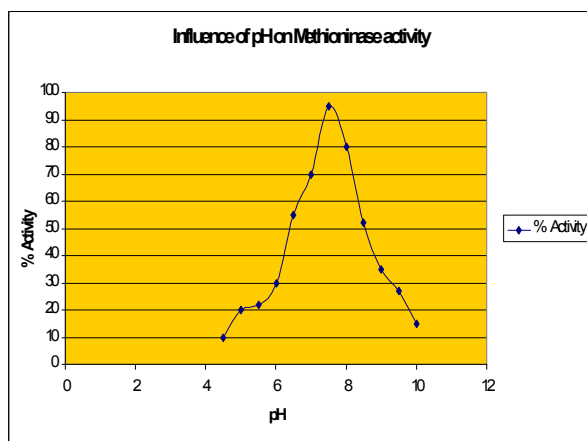
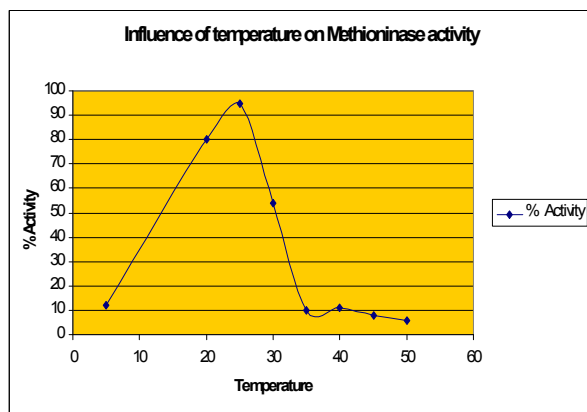**FIGURE 5.** Infuence of pH on methioninase activity

FIGURE 6. Influence of temperature on methioninase activity

CONCLUSION

Basing on the above studies, it can be concluded that the methioninase enzyme produced by *Brevibacterium linens* can be used for the treatment of cancer. MGL which is a rich source in *Brevibacterium linens* will provide a novel paradigm for cancer therapy. It can also be recommended to consume curd along with whey as it acts as a good substrate for the *Brevibacterium linens* and thereby the risk of being prone to cancer may be minimized.

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