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Original Research Article

Cloning and expression of Methionine-γ-lyase (MGL) of *Brevibacterium linens*

Kolasani Pavani[1]* and S. Vijaya Saradhi[2]

[1]Department of Biotechnology, Acharya Nagarjuna University, Nagarjuna Nagar, Guntur, Andhra Pradesh-522 510, India
[2]Department of Biotechnology, KL University, Vaddeswaram, Guntur, Andhra Pradesh-522 303, India
**Corresponding author*

K e y w o r d s

Brevi-bacterium linens;
L-Methionine γ-lyase;
16s rDNA.

A B S T R A C T

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 MGL gene was cloned into pRSET-A vector and expressed. The enzyme MGL was purified by precipitating with Ammonium sulphate, dialysis and anion exchange chromatography. 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 α-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.

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, clone MGL gene into pRSET-A vector, and assay the produced MGL enzyme from the recombinant clone.

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 B*revibacterium 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

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.

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* (Bio Leo Labs, Hyderabad) 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 (Bio Leo Labs, Hyderabad).

Sequencing and chimera checking

The eluted PCR product was directly sequenced using specific primers without GC-clamp (Bio Leo Labs, Hyderabad). 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).

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.

Isolation of Methionine γ-lyase gene of *Brevibacterium linens*

The DNA of *Brevibacterium linens* was prepared as per the protocol followed for isolation of DNA encoding 16S rRNA. The same DNA was used for amplification of Methionine γ-lyase gene. The following two upstream (5^{I}-GATCGTGGATCCTCGATCGGCACAG GCGATCGG -3^{I}) and downstream (5^{I}-GATCGTAAGCTTTCATGACGCCCGC AAGGTCGG-3^{I}) primers were used for amplification of the Methionine γ-lyase gene.

The PCR was performed by taking the different components in a 20µl reaction volume. Taqbuffer (5µl), TaqDNA polymerase(1µl), dNTP mixture(1µl), *MGL* forward primer(1.5µl), *MGL* reverse primer(1.5µl), templateDNA(2µl) and sterilized distilled water(8µl). After addition of all the components, the PCR tube was gently spun down in a centrifuge and placed in the thermocycler. PCR conditions were 94^{0}C for 2 min, and then 94°C for 1 min, 60°C for 1 min, 72°C for 3 min for a total of 30 cycles, with the extension at 72°C for 10 min (Volossiouk *et al.*, 1995). The PCR products were analyzed by agarose gel electrophoresis with 0.8 percentage agarose gel, visualized under UV light, photographed and documented with an Alpha Imager (Alpha Innotech, California,USA).

Sequencing of Methionine γ-lyase gene of *Brevibacterium linens*

The fragment of Methionine γ-lyase gene was excised with a clean razor blade. After removing the excess liquid, the agarose fragment containing Methionine γ-lyase gene was placed in the spin column. The tube was centrifuged at 5500rpm for 45 seconds. The fragment was eluted and checked with UV transilluminator for the presence of DNA and subjected for sequencing.

DNA manipulation

To insert an MGL gene into pRSET-A conveniently, multiple cloning sites were introduced into the *EcoRI* sites of the vector. This annealed product was cloned into pRSET-A by digesting the vector with *EcoRI*. The Promoter of pRSET-A vector is Lac Z. It was modified by inducing a strong promoter *gpd* so that the gene gets expressed faster. Then it was digested with S2 nuclease to remove the ATG translation start codon in the *gpd* promoter, and religated. Then the vector was subjected for the sequence analysis to

examine the orientation of the insert and to confirm that the ATG had been successfully removed. Then the pRSET-A was subjected for restriction digestion in the presence of restriction digestion mix by digesting the vector with *EcoRI*. The open reading frame (ORF) of the *Brevebacterium linens* gene coding for MGL product which was amplified by polymerase chain reaction was then inserted into pRSET-A and ligated in the presence of ligation mix.

After confirming that there was no mutant by sequence analysis, the open reading frame (ORF) of the *Brevebacterium linens* gene coding for MGL product was excised from pRSET-A with *EcoRI*.

Transformation

Transformation was performed for the introduction of our recombinant DNA into suitable host system i.e., *E. coli* DH5α by preparing competent cells. 10µl of DNA was added to 200µl of competent cells containing tube and gently swirled or tapped with finger for 10 seconds. The tube was kept on ice for 15 minutes. The tube was transferred to a rack placed in a preheated 42ºC water bath. The tube was stored for exactly 2 minutes. The tube should not be shaken. The tubes were rapidly transferred to an ice bath. The cells were allowed to chill for 10 minutes. 200µl of LB broth was added and the cells were incubated for 2 hours at 37ºc to allow the bacteria to recover and to express the antibiotic resistance. 100µl of fresh LB broth was added on top of transformed cells, mixed well and spread thoroughly using a spreader and the plates were incubated at 37ºC overnight. Control plates with competent cells that have not been transformed were also plated to rule out contamination of cells. The screening of the recombinants was done by blue-white screening.

Screening of the recombinant colonies

The competent cells were grown in the presence of X-gal. If the ligation was successful, the bacterial colony will be white; if not, the colony will be blue. This technique allows for the quick and easy detection of successful ligation, without the need to individually test each colony.

Gene Expression Studies

To analyze the production of MGL of *Brevebacterium linens*, the screened culture was transferred into LB broth. After 24 hours, 100 µg of protein from eluted fraction was taken and mixed with 10 µg of sample buffer in microfuge tube, boiled for 4 minutes and incubated at 40^0C for 30 minutes. The pellet was harvested by centrifugation and dissolved in 100 mM Tris-HCl buffer (pH 8.0). After dialysis against the same buffer, the crude extract was subjected to anion-exchange chromatography with a DEAE cellulose column (Bio Leo Labs, Hyderabad). 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.

Methionine γ-lyase 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 (Bio Leo Labs, Hyderabad). 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.

Methionine γ-lyase 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-methionine 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 *et al.*, 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, 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 MKP (pH 7.5), 0.02 mM PLP, 0.28 mM DTNB, and 0.1 to 40 mM Methionine. 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).

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).
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 the resulting PCR product was sequenced commercially. The amplified DNA was subjected to agarose gel electrophoresis (Figure 2).

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

GAGTTTGATCCTGGCTCGGACACATGCAAGTCGA
ACGCTGAAGCCAGAAGCTTGCTTCTGGTGGATGA
GTGGCGAACGGGTGAGTAACACGTGAGTAACCTG
CCCCTGATTTCGGGATAAGCCTGGGAAACCGGGT
CTAATACCGGATACGACCATCCCTCGCATGAGGG
TTGGTGGAAAGTTTTTCGATCGGGGATGGGCTCG
CGGCCTATCAGCTTGTTGGTGGGGTAATGGCCTA
CCAAGGCGACGACGGGTAGCCGGCCTGAGAGGGC
GACCGGCCACACTACTGAGACACGGCCCAGACTC
CTACGGGAGGCAGCAGTGGGGAATAAATGCACAA
TGGGGGAAACCCTGATGCAGCGACGCAGCGTGCG
GGATGACGGCCTTCGTGTAAACCGCTTTCAGCAG
GGAAGAAGCGTAAGTGACGGTACCTGCAGAAGAA
GTACGGCGGCTAACTACGTGCCAGCAGCCGCGGT
AATACGTAGGGTACGAGCGTTGTCTTCGGAATTA
TTGGGCGTAAAGAGCTCGTAGGTGGTTGGTCACG
TCTGCTCTGGAAACGCAACGCTTAACGTTGCGCG
TGCAGTGGGTACGGGCTGACTAGAGTGCAGTAGG
GGAGTCTGGAAGTCCTGGTGTAGCGGTGAAATGC
GCAGATATCAGGAGGAACACCGGTGGCGAAGGCG
GGATGGGCTGTAACTGACACTGAGGAGCGAAAGC
ATCTTTCCTCCACTAGGTCGG

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 phylogenetic analyses of these strains were confirmed using its 16S rDNA sequence.

Isolation of Methionine γ-lyase *gene* of *Brevibacterium linens*

Isolation and amplification of *Methionine γ-lyase* gene by Polymerase Chain Reaction (PCR) and sequencing was done as per the protocol. The amplified fragment of *Methionine γ-lyase* DNA when analyzed by agarose gel electrophoresis was found to be of good quality (Figure 4). The sample was eluted and sequenced. The sequence of the amplified product of *Methionine γ-lyase* gene was provided here under.

The sequence of Methionine γ-lyase *gene* of *Brevibacterium linens* is as follows.

GATCGTGGATCCTCGATCGGCACAGGCG
ATCGGATGCAAACGAGAAGGGTTGTGCT
CAAATCTGCGGCCGCGAGAACTCTGCTC
GGCGGCCTGGCTGGGTGCGCGACGTGGC
TGGATCGATCGGCACAGGCGATGCGATC
AATACGTGCGCGTCCTATCACAATCTCT
GAAGCGGGTTTCACACTGACTCACGAGG
ACATCTCGGCAGCTCGGCAGGATTCTTG
CGTGCTTGGCCAGAGTTCTTCGGTAGCG
CAAAGCTCTAGCGGAAAAGGCTGTGAGA
GGATTGCGCGCCAGAGCGGCTGGCGTGC
GAACGATTGTCGATGTGTCGACTTTCGA
TATCGGTCGCGACGTCAGTTTATTGGCC
GAGGTTTCGCGGGCTGCCGACGTTCATA
TCTGGCGGCGACCGGCTTGTGGTTCGAC
CCGCCACTTTCGATGCGATTGAGGTATG
TAGAGGAACTCACGCTAGTTCTTCCTGC
GGTGAGATTCAATATGGCATCGAAGTAC
ACCGGAATTAGGGCGGGCATTATCAAGG
TCGCGACCACAGGCAAGGCGACCCCCTT
TCAGGAGTTAGTGTTAAAGGCGGCCGCC
CGGGCCTCCTTGGCCACCGGTGTTCCGG
TAACCACTCACACGGCAGCAAGTCAGCG
CGATGGTGAGCGAGGCAGGCCGCCATTT

TTGAGTCCGAAGCTTGAGCCCTCACGGG
TTTGTATTGGTCACAGCGATGATACTGA
TGACTTGAGCTATCTCACCGCCCTGCTG
CGCGGATACCTCATCGGTCTAGACCACA
TCCCGCACAGTGCGATTGGTCTAGAAGA
TAATGCGAGTGCATCACCGCTCCTGGGC
ATCCGTTCGTGGCAAACACGGGCTCTCT
TGATCAAGGCGCTCATCGACCAAGGCTA
CATGAAACAAATCCTCGTTTCGAATGAC
TGGCTGTTCGGGTTTTCGAGCTATGTCA
CCAATATCATGGACGTGATGGATCGCGT
GAACCCCGACGGGATGGCCTTCATTCAC
TGAGATCGTAAGCTTTCATGACGCCCGC
AAGGTCGG

The amplified product of Methionine γ-lyase *gene* contained 1044 bases. The sequence on BLAST search revealed that it is having an open reading frame coding for the enzyme *Methionine γ-lyase* encoded by 1014 bases.

DNA manipulation

Restriction digestion of the vector was done by using a Restriction enzyme *EcoR1* in presence of *EcoR1* buffer and molecular grade distilled water by incubating at 72ºC temperature for 2 hours and thereby the multiple cloning site was excised. This excised vector pRSET-A was treated with the eluted amplified DNA. Thereby, ligation reaction was carried out in the presence of ligation buffer by incubating at 37ºC for 2 hours. The open reading frame (ORF) of the *Brevebacterium linens* gene coding for MGL product which was amplified by polymerase chain reaction was then inserted into pRSET-A upon ligation (Figure 5).

The presences of recombinant clones were screened by blue-white screening. Transformation was performed for the introduction of our recombinant DNA into suitable host system i.e., *E. coli* DH5α.

Screening of the recombinant colonies

The blue-white screen is a molecular technique that allows for the detection of successful ligations in vector-based gene cloning. DNA of interest was ligated into a vector. The vector was then transformed into competent cell. The competent cells were grown in the presence of X-gal. If the ligation was successful, the bacterial colony will be white. The plates were observed with white colonies which are recombinants (Figure 6).

Gene Expression Studies Analysis of MGL enzyme of *Brevebacterium linens* by SDS-PAGE

The enzyme MGL was precipitated by heating. This MGL enzyme fraction was checked by SDS-Polyacrylamide gel electrophoresis and the band corresponding to 24 KDa denotes that the eluent was MGL enzyme (Figure 7).

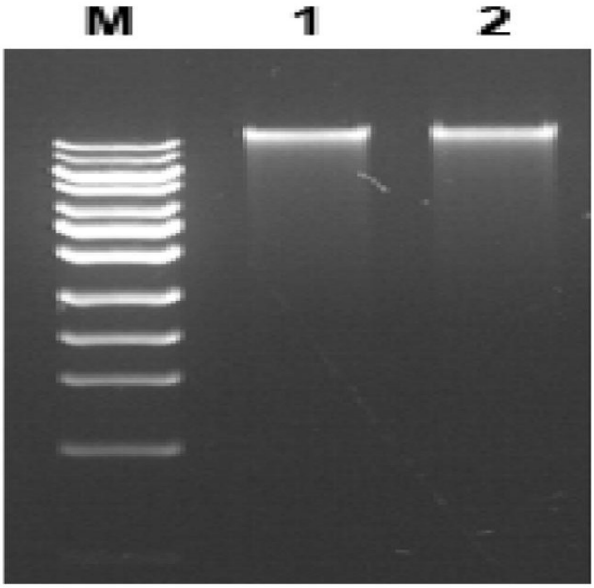
Enzyme analysis

The enzyme Methionine γ-lyase was precipitated by saturating with 35% and 70% Ammonium sulphate and the Methionine γ-lyase 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-

Table.1 Microscopic Examination and Biochemical Reaction of Bacterial Population

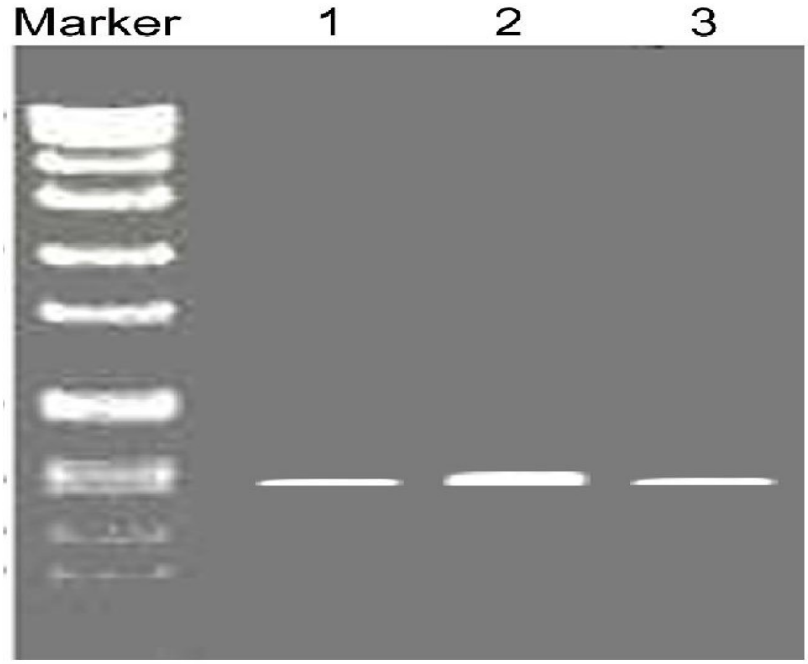
Microscopic Observations	
Simple staining	Rod Shaped
Gram staining	Gram positive bacilli
Motility Test	Motile
Biochemical Tests	
Indole production Test	Negative
Methyl Red test	Negative
Voges-Proskauer Test	Negative
Citrate utilization Test	Positive
Starch Hydrolysis Test	Negative
Gelatin Hydrolysis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

Figure.1 Agarose gel showing Whole Genomic DNA



Well M- Marker of 1kb.; Well 1 and 2 - *Brevibacterium linens* whole genomic DNA.

Figure.2 Agarose gel showing Amplified 16s rDNA



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

Figure.3 Phylogenetic tree of *Brevibacterium linens*

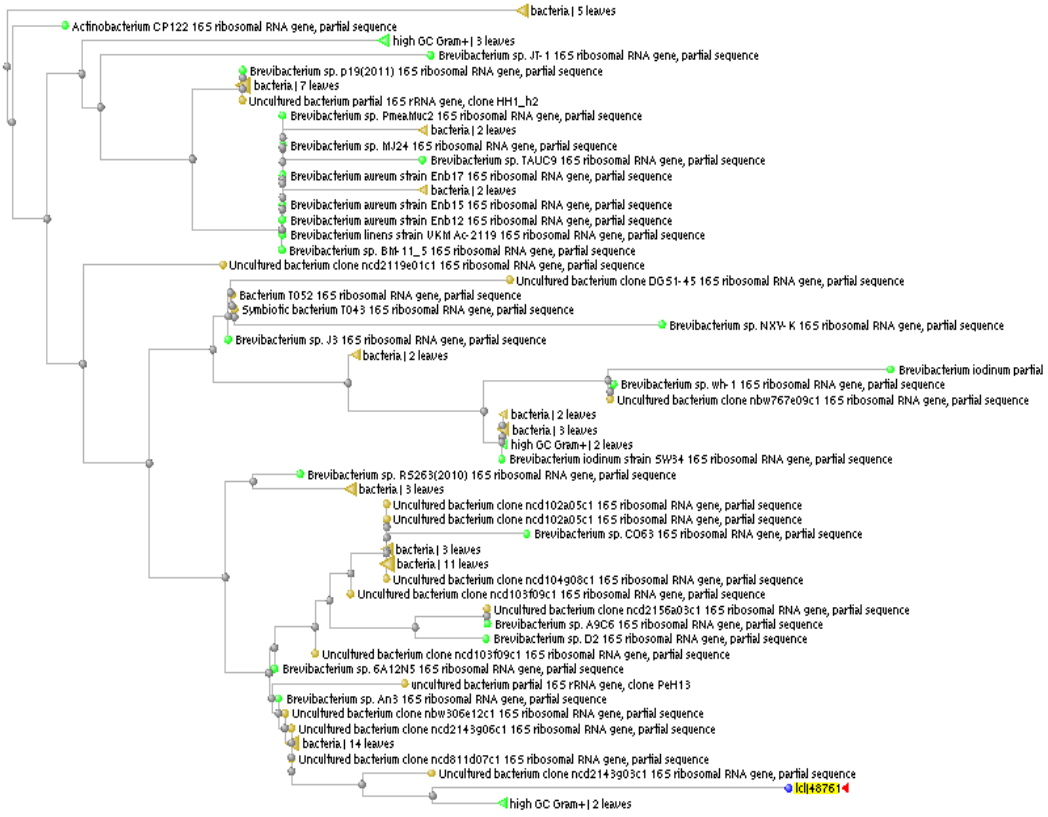


Figure.4 PCR amplification of Methionine γ-lyase *gene* of *Brevibacterium linens*

1-Amplified*Methionine γ-lyase* gene of*Brevibacterium linens*
Marker

Figure.5 pRSET A Vector cloned with *Brevebacterium linens* Gene coding for MGL enzyme

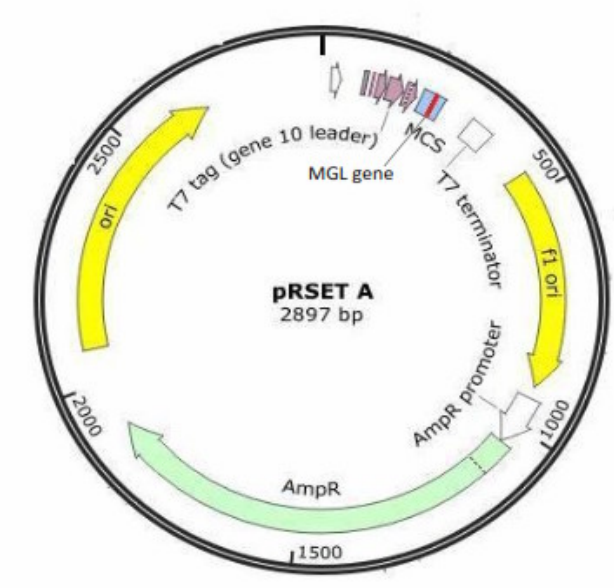


Figure.6 Plate showing white recombinant colonies

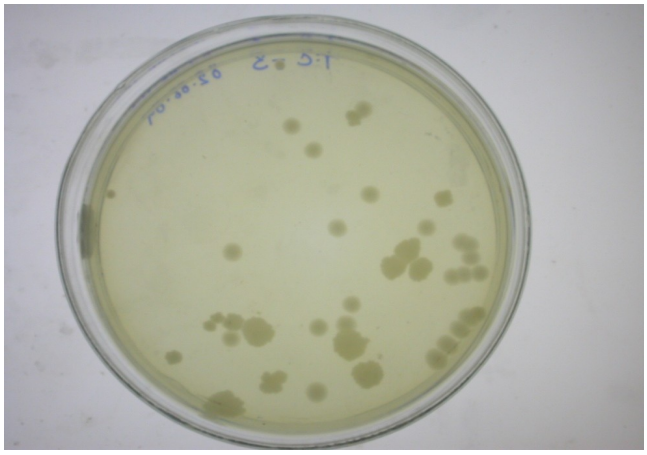
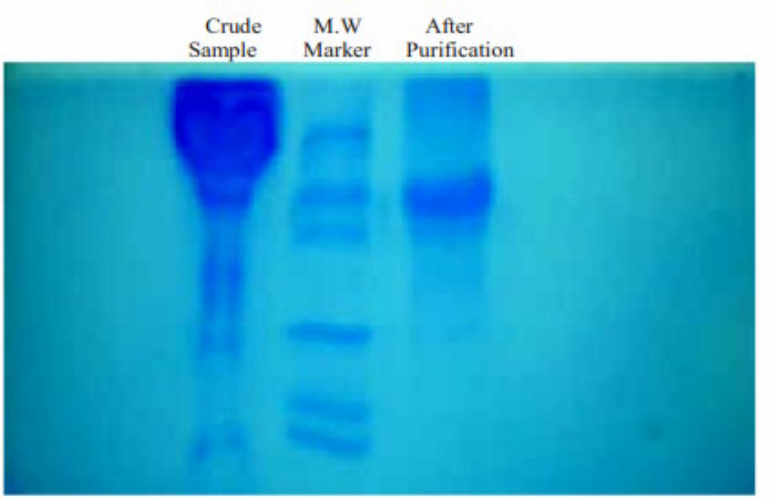


Figure.7 SDS-PAGE



Protein MW Marker: 14kDa, 20kDa, 25kDa, 35kDa, 45kDa, 66kDa

Figure.8 Infuence of pH on Methionine γ-lyase activity

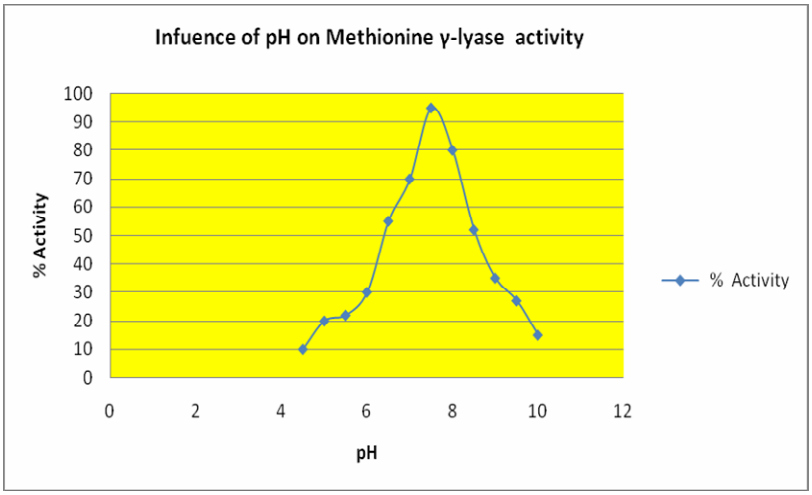
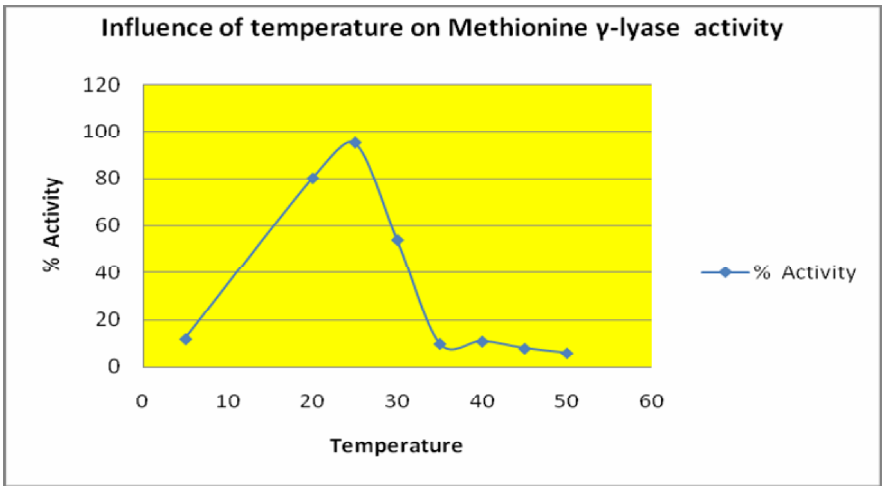


Figure.9 Influence of temperature on Methionine γ-lyase activity



PAGE, a single band with an approximate molecular mass of 24 kDa was observed (Figure 7). Analysis of the absorption spectrum of the purified enzyme demonstrated a peak at 420 nm in addition to a peak at 280 nm.

Substrate specificity

The activities of the purified enzyme on various substrates were determined by the production of α-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 8). 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 9). 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. Basing on the above studies, it can be concluded that the Methionine γ-lyase 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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