Cloning and expression of S-Adenosyl Methionine Synthetase gene in recombinant *E. coli* strain for large scale production of SAMe

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Abbreviations: ATP: Adenosine Tri Phosphate
CNS: Central Nervous System
DNA: Deoxyribo Nucleic Acid
HIAA: Health Insurance Association of America
IPTG: Iso Propyl β-D-1-Thio Galactopyranoside
MAT: *Methionine-adenosyl-transferase*
NBSC: New Brunswick Scientific Corporation
NSAID: Nonsteroidal Anti-Inflammatory Drugs
NTP: Nicotine Tri Phosphate
PCR: Polymerase Chain Reaction
RNA: Ribo Nucleic Acid
SAH: S-Adenosyl Homocysteine
SAMe: S-Adenosyl Methionine
SDS PAGE: Sodium Dodecyl Sulfate Poly Acrylamide Gel Electrophoresis
Taq: *Thermus aquaticus*
TE: Tris EDTA

S-Adenosyl Methionine (SAMe) Synthetase is an enzyme which catalyses the synthesis of S-Adenosyl **Methionine using methionine and ATP. It is also known as *AdoMet* which is well known methyl donor, which**

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modifies DNA, RNA, histones and other proteins, dictating replicational, transcriptional and translational fidelity, mismatch repair, chromatin modeling, epigenetic modifications and imprinting. The objective of the present work is to clone the *SAMe Synthetase* gene in recombinant *E. coli* strain in order to express, characterize and purify it for further synthesis of SAMe in a large scale fermentation. Expression was induced by 1 mM IPTG and expressed protein was characterized by SDS-PAGE. The recombinant *E. coli* cells were used for the production of SAMe through batch and fed batch fermentation operations. The produced SAMe was purified through paper chromatography in order to use it in our future studies.

S-Adenosyl Methionine (SAMe) Synthetase (or) *Methionine-adenosyl-transferases* are enzymes involved in transmethylation reactions. They can broadly be classified into 2 categories namely, liver specific and non liver specific Methionine-adenosyl-transferases. These are products of two genes, MAT1A and MAT2A respectively. Mature liver expresses MAT1A while MAT2A is expressed by extra hepatic tissues. *Methionine-adenosyl-transferase* plays a vital role in catalyzing the synthesis of S-Adenosyl Methionine (SAM) using methionine and ATP (Lu et al. 2001).

SAMe (known formally as S-adenosylmethionine) is not an herb or a hormone, it is a molecule that all living cells, including our own, produce constantly (Wang et al. 2002; Wein et al. 2002). To appreciate its importance, one needs to understand the process called methylation. It is a simple reaction in which one molecule donates four-atom appendage -a so-called methyl group- to a neighboring molecule. Both the donor and the recipient change shape in the process, and the transformations can have far-reaching effects. Methylation occurs a billion times in a second throughout the body, affecting everything from fetal development to brain function. It regulates the expression of genes. It preserves the fatty membranes that insulate our cells. And it helps regulate the action of various hormones and neurotransmitters, including serotonin, melatonin, dopamine and adrenaline.

"Without methylation there could be no life as we know it and without SAMe, there could be no methylation as we know it". Though various molecules can pass methyl groups to their neighbors, SAMe is the most active of all methyl donors. Our bodies make SAMe from methionine, an amino acid found in protein-rich foods, then continually recycle it. Once a SAMe molecule loses its methyl group, it breaks down to form homocysteine (Ying et al. 1999; Ye et al. 2001).

Homocysteine is extremely toxic if it builds up within cells. But with the help of several B vitamins (B6, B12 and folic acid), our bodies convert homocysteine into glutathione, a valuable antioxidant, or "remethylate" it back into methionine. SAMe and homocysteine are essentially two versions of the same molecule-one benign and one dangerous. When our cells are well stocked with B vitamins, the brisk pace of methylation keeps homocysteine levels low. But when we're low on those vitamins, homocysteine can build up quickly, stalling the production of SAMe and causing countless health problems (Zubieta et al. 2001; Zubieta et al. 2003).

SAMe is also involved in over 40 transmethylation reactions, where it contributes in an enzyme-catalyzed reaction, a single carbon unit as a methyl group to another molecule. A typical adult can produce 6 to 8 grams of SAMe daily, with most of it being made in the liver. Since significant amounts of SAMe are not readily available through the diet, the body is responsible for making its own SAMe. For many reasons, it is important to maintain proper levels of SAM-e in the body. Levels of SAMe tend to decline with age, in humans (Baldessarini, 1987; Yoon et al. 2000; Zhang et al. 2004).

On considering various benefits and applications of Methionine-adenosyl-transferases,which play a vital role in the synthesis of SAMe, the present study is focused on cloning the gene of Methionine-adenosyl-transferases in *E. coli* strain in order use it for the further expression and characterization studies in the future.

Background

In 1951, an Italian scientist first brought SAMe to attention. It was an unstable molecule and could not be studied in clinical trials until it became commercially available in 1974. An Italian group devised a way of stabilizing S-adenosylmethionine by producing a derivative stabilized with p-toluene sulfate. Since then numerous studies have been carried out showing that SAMe participates in a variety of biological reactions. SAMe has been used clinically in Europe since the 1970's and is today the most prescribed natural supplement for stress, nervous tension, anxiety and low mood in Europe (Cantoni, 1952).

In 1989, it was reported that, the liver is actively involved in the metabolism of the sulphur-containing essential amino acid, methionine. Methionine is transformed into S-adenosyl-L-methionine (SAMe) and then into sulphur-containing metabolites (cysteine, taurine and glutathione) via the trans-sulphuration pathway (Vendemiale et al. 1989).

SAMe is a ubiquitous metabolite present in all cells and biological fluids, and serves as a methyl donor in a multitude of different methylation reactions involving proteins, phospholipids, catecholamines and DNA. Pharmaceutical preparations of some stable salts of SAMe are available for parenteral and oral use in humans, and have been shown to increase plasma and cerebrospinal fluid SAMe concentrations. In experimental studies administration of SAMe is associated with increases in brain monoamine neurotransmitters and b-adrenergic and
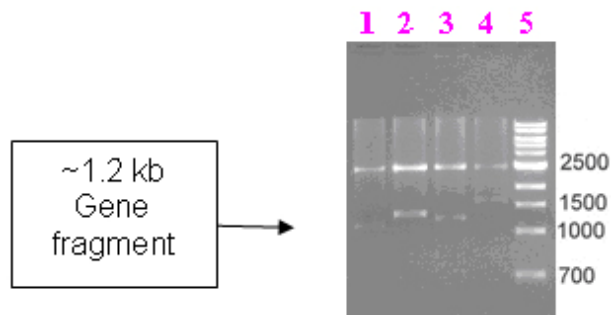
Figure 1. Double restriction digestion of recombinant plasmids.
1) Lane containing the plasmid DNA isolated from colony 1 which was subjected to double restriction digestion with Hind III and Nde I.
2) Lane containing the plasmid DNA isolated from colony 2 which was subjected to double restriction digestion Hind III and Nde I.
3) Lane containing the plasmid DNA isolated from colony 3 which was subjected to double restriction digestion Hind III and Nde I.
4) Lane containing the plasmid DNA isolated from colony 4 which was subjected to double restriction digestion Hind III and Nde I.
5) 1 kb ladder.

muscarinic receptor functions. These neuropharmacological effects are postulated to be involved in the antidepressant activity of SAMe which has been confirmed in numerous controlled studies (Bottiglieri et al. 1990). Preliminary studies indicate that SAMe has therapeutic potential in the treatment of other CNS disorders including dementia, acquired immune deficiency syndrome (AIDS)-associated myelopathy, and brain ischemia. This review will focus on recent experimental and clinical aspects of SAMe in the central nervous system, and the therapeutic use in psychiatric and neurological disorders.

A decrease in the S-adenosyl methionine/S-adenosylhomocysteine (SAMe/SAH) ratio occurs in the liver of rats fed a methyl deficient diet, which is a carcinogenic treatment, and in preneoplastic liver tissue, developing in initiated/promoted rats fed an adequate diet. The role of low SAM/SAH ratio in carcinogenesis is substantiated by the tumor chemopreventive effect of lipotropic compounds. Treatment with exogenous SAM prevents the development of preneoplastic and neoplastic lesions in rat liver. This is associated with recovery of SAM/SAH ratio, DNA methylation and inhibition of growth-related gene expression. People suffering from cirrhosis, chronic liver disease, alcoholic liver damage, toxic chemical exposure, NSAID-liver damage, estrogen induced liver problems, bile disorders, and environmental chemical hypersensitivity may all benefit from SAMe, as well as possibly suffer from SAMe metabolism bottlenecks (Frezza et al. 1988).

Homocysteine does not occur in the diet but it is an essential intermediate in normal mammalian metabolism of

methionine. Each compound, methionine or homocysteine, is the precursor of the other. Similarly, the synthesis of one is the mechanism for the detoxification of the other. The ubiquitous methionine cycle is the metabolic basis for this relationship. In some tissues the transsulfuration pathway diverts homocysteine from the cycle and provides a means for the synthesis of cysteine and its derivatives. Methionine, (or homocysteine) metabolism is regulated by the disposition of homocysteine between these competing sequences. Both pathways require vitamin-derived cofactors, pyridoxine for transsulfuration and both folate and cobalamin in the methionine cycle. The clinical consequences of disruption of these pathways were first apparent in rare inborn errors of metabolism that cause homocystinuria, but recent studies focus on hyperhomocysteinemia - a lesser metabolic impairment that may result from genetic variations, acquired pathology, toxicity and nutritional inadequacy. Hyperhomocysteinemia is an independent risk factor for thrombovascular diseases however it is not clear whether the minimally increased concentration of the amino acid is the causative agent or merely a marker for the pathology. Until we resolve that question we cannot predict the potential efficacy of therapies based on folate administration with or without additional cobalamin and pyridoxine (Finkelstein and Martin, 2000).

In 2000, scientists came up with the reactions that produce, consume, and regenerate SAMe are called the SAMe cycle. In the first step of this cycle, the SAMe-dependent *methylases* that use SAMe as a substrate produce S-adenosyl homocysteine as a product. This is hydrolyzed to homocysteine and adenosine by S-adenosylhomocysteine hydrolase and the homocysteine recycled back to methionine through transfer of a methyl group from 5-methyltetrahydrofolate, by one of the two classes of methionine synthases. This methionine can then be converted back to SAMe, by completing the cycle (Födinger et al. 2000).

Depression has always been a great problem for people who cannot tolerate standard antidepressant drugs, or who have minimal or no response to them. SAMe has been shown to significantly increase cerebrospinal fluid levels of HVA and 5HIAA, the chief metabolites of dopamine and serotonin, two key biogenic amine antidepressant neurotransmitters. This is the evidence for SAMe's enhancing brain biogenic amine metabolism and activity. SAMe has also shown considerable efficacy in treating depression secondary to chronic diseases such as arthritis, fibromyalgia, liver disease and alcoholism SAMe helps maintain youthful neuronal membrane ratios of phosphatidyl choline; cholesterol. This promotes more optimally fluid membranes, which in turn promotes optimal hormonal, neurotransmitter and electrical neuron signal reception and processing, there by protecting brain from ageing (Jamison, 2004).

It was observed that another major role of SAMe, which is in polyamine biosynthesis. Here, SAMe is decarboxylated by adenosylmethionine decarboxylase to form S-adenosyl-5'-3-methylpropylamine. This compound then donates its n-propylamine group in the biosynthesis of polyamines such as spermidine and spermine from putrescine (Roje, 2006).

It was reported that SAMe is required for cellular growth and repair. It is also involved in the biosynthesis of several hormones and neurotransmitters that affect mood, such as dopamine and serotonin. Methyltransferases are also responsible for the addition of methyl groups to the 2' hydroxyls of the first and second nucleotides next to the 5' cap in messenger RNA (Loenen, 2006).

MATERIALS AND METHODS

E. coli ESS and *E. coli* DH5α strains purchased from Medox Biotech, India, were used for genomic DNA isolation and for transformation.

Isolation of genomic DNA

Genomic DNA was isolated from *E. coli* ESS strain as per the standard protocol (Ausubel et al. 2002). 1 mL of overnight *E. coli* culture was centrifuged at max speed (13,000 rpm) for 2 min. 1 mL of 0.5 M NaCl was added to the pellet and again centrifuged for 2 min. This step was repeated again and 100 µL of TE buffer [Tris.Cl (10 mM), EDTA (1 mM), pH 8.0] and 100 µL of 2% SDS - (2 g/L)] were added to the pellet and vortexed for 2-5 min. 1 mL of proteinase K [(20 mg/mL), stored at 4ºC] was added to the mixture. The tube was kept at 65ºC for 30 min. 20 µL of NaCl and 0.6 mL of 95% ethanol were added to the mixture. The DNA was removed using micropipette and

Figure 2. 10% SDS PAGE results.
1) Lane containing the Molecular Weight Ladder.
2) Lane containing un-induced cells with pET vector.
3) Lane containing induced cells with IPTG containing pET vector.
4) Lane containing un-induced cells containing cloned DNA.
5) Lane containing induced cells with IPTG containing cloned DNA.

1mL of 70% ethanol was added in order to remove the salts. The tube was kept in ice for 15 min. The tube was spun down at 4ºC at neutral pH for 5 min and the pellet was allowed to dry. The pellet was resuspended in 0.5 mL of 10 mM Tris. 20 µL of *RNase* (Medox Biotech, India) was added and the tube was incubated at 37ºC for 2 hrs. The tube was centrifuged at maximum speed for 2 min and the supernatant was transferred to another fresh tube. 0.5 mL of phenol was added to the supernatant and centrifuged for 2 min. The upper layer of the mixture was taken without disturbing the lower layer and 0.5 mL of phenol-chloroform was added to it and again centrifuged for 2 min. The upper layer of the mixture was taken without disturbing the lower layer and 0.5 mL of chloroform was added to it and again centrifuged for 2 min. The upper layer of the mixture was taken without disturbing the lower layer and 20 µL of 5 M NaCl and 0.5 mL of 95% ethanol were added to the mixture. The tube was kept at room temperature for 30 min and centrifuged for 10 min. Supernatant was discarded and 1 mL of 70% ethanol was added and vortexed. The tube was centrifuged at 4ºC for 5 min. The supernatant was discarded and the tube was allowed to dry. The pellet was resuspended in 100 µL of TE. The ratio A_{260}/ A_{280} was examined and the purity of the sample was determined (Ausubel et al. 2002).

PCR technique

The gene of our interest, present in the isolated *E. coli* genomic DNA was amplified using the PCR. According to the N-terminal and C-terminal sequence of our gene of interest, forward primer (catatggcaaaacaccttttttacgtccg) and reverse primer (ccaacaccgagctggcctttgaacgc) were designed using the ProtParam^{TM} and Primer Blast^{TM} bioinformatics tools based on the already available sequence details of Methionine-adenosyl-transferase gene. Then a 10X PCR Amplification Buffer [KCl (500 mM), Tris.Cl (100 mM), Gelatin (1 mg/mL), pH 8.4, and Stored at -20ºC] was prepared. In combination with both the primers our gene of interest was amplified from the genomic DNA in 30 cycles of PCR using *Taq* DNA polymerase (Medox Biotech, India). The amplified fragments were analyzed on 1% agarose gel run along 1 kb ladder (Ausubel et al. 2002).

The following Primers were designed and used in our experiment.

Forward Primer (MD043)   :
"cat**atg**gcaaaacaccttttttacgtccg" (5' to 3')

Reverse Primer (MD044)   :
"ccaacaccgagctggcctt**tga**acgc" (5' to 3')

Purification of PCR product

Gen-elute^{TM} (Medox Biotech, India) mini preparation binding column (with a blue o-ring) was inserted into a provided collection tube. The purified PCR product was eluted and stored at -20ºC (Ausubel et al. 2002).
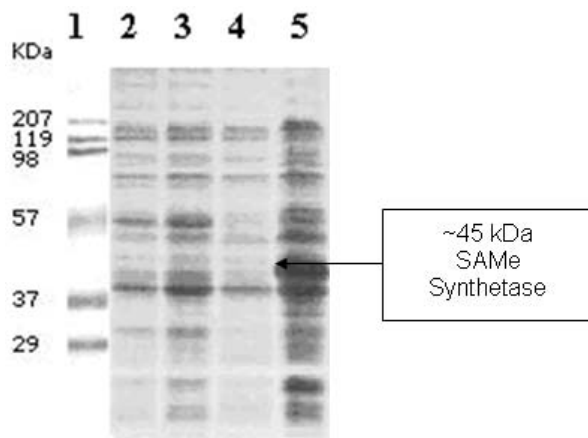
Colony PCR

In order to conduct a rapid detection of transformation success, colony PCR was performed. The plate, which was allowed for an overnight incubation, was taken out in the next day and blue and white (transformed) colonies were identified. Around 20 white colonies were numbered in the plate. From the numbered 20 colonies 5 colonies (say 1 to 5) were picked using different toothpicks and dissolved in 20 µL of sterile water in an eppendorf tube. Similarly remaining colonies were also picked and dissolved in different eppendorf tubes. All the tubes were incubated at 95ºC for 5 min and centrifuged for 1 min in order to remove the debris in the form of pellets. The DNA (in dissolved state) was in all the 4 tubes. PCR master mix [DNA 5.0 µL, 10X Buffer 5.0 µL, Forward Primer 1.0 µL, Reverse Primer 1.0 µL, dNTP (10 mM each) 1.0 µL, *Taq* DNA *Polymerases* 1.0 µL, H_2O 36.0 µL] was prepared using the 4 samples of DNA. 30 PCR cycles were carried out with the already. The fragments were checked on 1% agarose gel ran along 1 kb ladder. As per the standard protocols, competent cell preparation, transformation, plasmid DNA extraction, restriction and ligation reactions were carried out (Ausubel et al. 2002).

Production of SAMe from recombinant *E. coli* cells

The *E. coli* BL21 cells which carries the *SAMe Synthetase* genes were cultured in a medium consisting of 0.3% beef extract, 0.5% peptone, 0.15% L-methionine-S^{35}, and were supplemented with 1% glucose. Cultures were incubated at 37ºC for 72 hrs on a rotary shaker. Then the cells were harvested by centrifugation and washed twice with cold distilled water. The S-adenosylmethionine was isolated; the procedure consists essentially of extraction of the cells with perchloric acid, precipitation with Reinecke salt, and solubilization in methyl ethyl ketone, and removal of the precipitant from an acid solution with diethyl ether. This procedure was designed to recover S-adenosylmethionine; however, small amounts of methionine always appeared on the chromatograms. The volume of extraction solvents was adjusted to compensate for differences in cell yields obtained from the growth medium. Chromatograms were done on Whatman no. 1 filter sheets, and the papers were developed in n-butanol-acetic acid-water (60:15:25; v/v). Separated components were revealed by first surveying to observe ultraviolet light quenching by the adenine derivatives; duplicate sheets were then sprayed with ninhydrin reagents (Ausubel et al. 2002).

Extraction and analysis of SAMe

Extraction of SAMe was carried out briefly, cells growing in log-phase ($OD_{660} = 0.3$) were collected by centrifugation, washed, and then extracted with 1 ml of 10% perchloric acid for 1 hr at room temperature. The resultant supernatant was diluted with Milli Q-grade water, and the samples were filtered for capillary electrophoresis. Determination of SAMe was performed by capillary electrophoresis by using a Waters Capillary Ion Analyzer with an Accusep fused silica gel column (60-cm total length and 75 micrometer i.d.). The retention time of SAMe was 8.1. The SAMe was expressed as nmol per mg dry weight of cells.
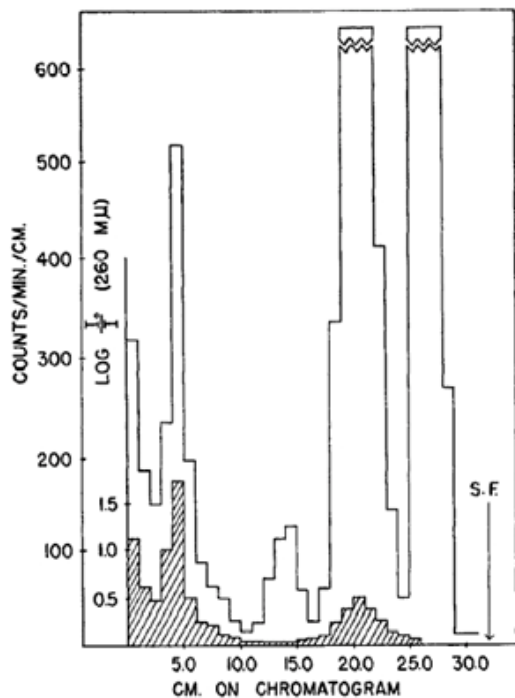
Paper chromatography

The extracted SAMe was purified by means of paper chromatography. It was found in preliminary experiments that, when L-methionine-S^{35} was added to the reaction mixture described for the preparation of SAMe, the intermediate was labeled with S^{35}. Thus, the task of separating SAMe from the residual methionine and other contaminants became greatly facilitated. SAMe-$S^{35,}$ was applied as a continuous band to one or more large sheets of Whatman no. 1 paper. The amount applied was not found to be critical; it was important, however, to keep the band narrow and of uniform width and this was accomplished by applying 0.005 cc. per each 0.5 cm. And repeating the application after the paper had dried. The large sheets were subjected to descending chromatography with 80% ethanol-5 percent glacial acetic acid. After development for 17 hrs, the paper sheet was allowed to dry in a current of air for 2 to 3 hrs. Next, a strip 20 mm wide was cut length wise from the paper sheet, lined off, and cut into consecutively numbered sections 1 cm long, and the radioactivity of each section was determined. After counting, each strip was placed in a test-tube and eluted with 3.0 ml of H_2O for 3 hrs at room temperature with occasional shaking.

Fed-batch fermentation

The following growth medium (per liter) was used for fermentation studies: $(NH_4)_2SO_4$ - 3 g; $K_2HPO_4 \cdot 3H_2O$ - 5 g; KH_2PO_4 - 10 g; $MnSO_4 \cdot 7H_2O$ - 0.1 g; $ZnSO_4 \cdot 7H_2O$ - 0.1 g; $MgCl_2$ - 0.2 g; $CaCl_2$ - 0.1 g; yeast extract - 3 g. Glucose was used as the sole carbon and energy source with an initial concentration of 10 g/L in the growth media. Six liters of growth medium were added to the 10-l fermentation vessel (NBSC BioFlo 415 Fermenter) and sterilized in situ at 121ºC for 30 min. The medium was cooled down and the initial pH value of the medium was adjusted to pH 5.0. All fermentations were performed at 37ºC. The pH was controlled at 5.0 via automated addition of ammonia. For fed-batch cultivation, the feeding glucose, with a concentration of 800 g/L, was sterilized separately at 121ºC for 15 min in 1 l bottles, and was fed into the fermenter with a peristaltic pump.

The process started with a short batch culture phase. Following the batch culture phase, a fed-batch culture phase with exponential feeding of medium was conducted to increase the concentration of recombinant *E. coli* cells. Then, L-methionine, a precursor of SAMe, was added into the fermentation broth with an amount of 1 g per 10 g dry weight of cells to improve the accumulation of SAMe in the cells. During transformation phase, the feeding of glucose was continued to provide necessary energy source and

Figure 3. Paper chromatography results for SAMe. Solvent was 80% Ethanol and 5% Acetic acid. The radioactivity (clear boxes) and ultraviolet absorption at 260 nm (shaded boxes) are indicated.

precursors for the synthesis of SAMe until the excessive glucose was detected in the fermentation broth.

RESULTS AND DISCUSSION

PCR

Thus the isolated genomic DNA was used for carrying out the PCR amplification reactions. The designed primers include the encoding sequence of *SAMe Synthetase.* With the designed primers and *Taq DNA Polymerases*, 30 cycles of amplification was carried out as per the protocol given earlier.

Cloning of gene of interest in recombinant strain *E. coli*

The double digested Plasmid DNA was run on 1% agarose gel with 1 kb ladder. Figure 1 shows the clear picture of the restricted gene of interest which can be eluted from the gel for storage and reuse for the future expression studies. Thus a compatible double digested plasmid DNA was prepared.

The plasmid DNA was used for cloning into *pET 24a (+)* expression vector which was already restricted by *Hind III* and *Nde I*. After transformation, the colony containing the recombinant plasmid was again inoculated which was later induced for the expression of *SAMe Synthetase* using IPTG. The expressed protein was analyzed using 10% SDS PAGE

(Figure 2), which confirmed that 45 kDa *SAMe synthetase* was expressed in the cell.

After confirming the presence of our gene of interest in *E. coli BL21*, it was used straight away for the production of SAMe through rotary shake flasks for 72 hrs and the cells were harvested in the late log phase and purified by capillary electrophoresis technique. The purified SAMe was used for further analysis with paper chromatography.

Typical results for such an experiment are shown in Figure 3. Three main radioactive peaks were observed. The radioactive component with the smallest RF was recognized as SAMe by examination of its activity as a methyl donor in the nicotinamide system. The fastest moving component was identical with methionine. The third component, with an RF of 0.55, has not been identified conclusively. It was thought to be a decomposition product of SAMe, but it is not a methyl donor. It can be seen from Figure 3 that there is satisfactory correlation between absorption in the ultraviolet and S^{35} activity in the SAMe peak.

Following the results of paper chromatography, the recombinant *E. coli* cells carrying the *SAMe synthetase gene* was used for fed batch fermentation studies for large scale production of SAMe.

CONCLUDING REMARKS

S-adenosylmethionine synthetase (SAMe synthetase) is an important enzyme for the synthesis of SAMe and lack of SAMe leads to various neurological diseases. Due to large requirement of SAMe for treatment, the Low cost fermentation process using novel strain is needed for development for SAMe based pharmaceuticals. The production of *SAMe synthetase* using biotechnological methods has been investigated in many cloning vectors such as *E. coli*, *Pichia pastoris* and *Kluyveromyces lactis, Catharanthus roseus*. In our present investigation, the Recombinant *E. coli* has been used for the cloning and expression of *SAMe synthetase* for the production of SAMe through fermentation method. The role of SAMe in human system and its various therapeutic benefits for human beings paved the way for undertaking this current work of Cloning and Expression of *S*-Adenosyl Methionine (SAMe) Synthetase gene in *E. coli* in order to produce SAMe in large scale through fed batch fermentation of engineered *E. coli BL21*. The results obtained from this work clearly suggested that the experiments and the protocols were followed correctly. This work will definitely help those researchers who are all working with SAMe world wide and also our future optimization and kinetic studies on SAMe.

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