

METHYL TRANSFERASE, A POLYKETIDE BIOSYNTHETIC ENZYME FROM *DRESCHLERA MONOCERAS*: PURIFICATION AND PROPERTIES

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ABSTRACT

Methyl transferase, a polyketide biosynthetic enzyme in monocerin biosynthesis was isolated and purified from *Dreschlera monoceras*. The enzyme was purified to near homogeneity with 11.1% recovery, using ammonium sulphate fractionation followed by ultra filtration, SP Sepharose chromatography and gel filtration chromatography. The molecular mass of the purified enzyme as determined by elution through Superdex TM gel filtration chromatography was found to ~ 165kDa. SDS-PAGE of the purified enzyme showed a single band at ~55kDa indicating that possibly enzyme could be a trimer of 3 subunits. The enzyme showed optimum pH at 7.5-7.7, whereas optimum assay temperature was 35-37°C. Enzyme was stable up to 45°C and above this temperature enzyme activity slowly declined and inactivated around 70°C. Apparent Km of the enzyme was found to be ~ 0.083mM.

Keywords : methyl transferase, monocerin biosynthesis, polyketides, *D. monoceras*.

INTRODUCTION

Polyketides are naturally occurring compounds, most often produced by micro-organisms such as fungi and the filamentous bacteria. These compounds are widely spread in nature and show a higher range of structural diversity.

In addition to their wide occurrence and structural diversity, polyketides display a very large range of biological activities both beneficial and harmful to man. These include antibiotics, anticancer agents, anti fungals, antiparasitics, immunosuppressive agents and cholesterol lowering agents (Simpson 1995).

The biosynthetic pathway of polyketides has been studied for many years, but recent advances at the genetic and biochemical level have revealed the pathway's unique potential for manipulation to give modified or totally novel compounds.

Despite their enormous structural diversity, all of the polyketides are related by their common biosynthetic origin. These metabolites produced via linear, highly functionalized 'polyketide' intermediates that are assembled from simple coenzyme A precursors by multifunctional enzyme complex called polyketide synthase (PKSs).

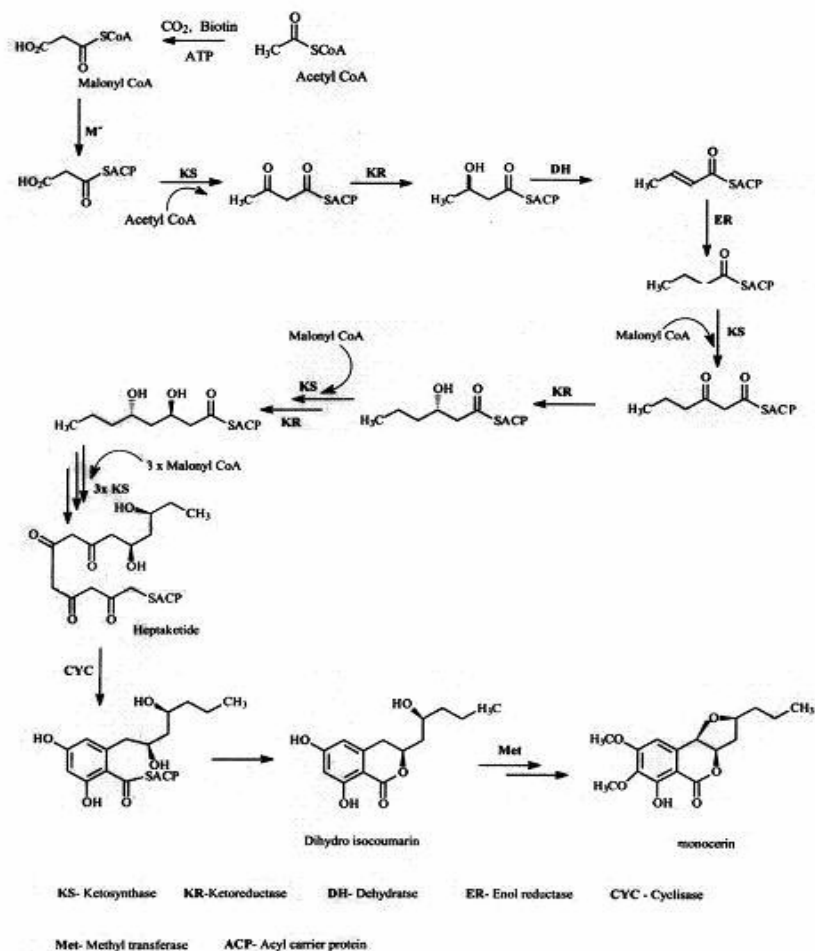
The exact nature of these PKS enzymes may vary according to their source; fungi, bacteria or higher plants. In fungi, all PKSs characterized to date, belong to Type I class and consists of single multi-domain protein encoded by single gene, but the isolation and characterization of fungal PKSs gene remains a significant challenge. There is much current interest in the engineering and heterologous expression of PKS genes from different sources to permit the rational production of novel structures. Monocerin PKS is likely to be of particular interest in this context as it produces an intermediate, with an initial high level of reductive modification and ending with a more classical β -polyketide moiety (Axford *et al.* 2004, Simpson 1995).

Monocerin is a polyketide fungal metabolite which exhibits antifungal, insecticidal and plant pathogenic properties. It has been isolated from several fungal species including *Dreschelera monoceras* (Aldridge & Turner 1970),

Dreschlera ravenelii (Scott *et al.* 1984), *Exserohilum turcum* (Robertson & Strobel 1982).

Studies on the biosynthesis of monocerin have established its heptaketide origin.

Dihydro isocoumarin has been found as the first PKS free intermediate which would be formed from the reduced heptaketide whose proposed assembly pathway is shown in scheme 1 (Dillon *et al.* 1992, Axford *et al.* 2004, Scott *et al.* 1984).



Scheme 1: Monocerin biosynthesis

Ketosynthase, ketoreductase, dehydrates, enol reductase and cyclisase are considered as domains of the Monocerin PKs. Methyl transferase is considered as the tailoring enzymes of the monocerin PKS (Staunton & Wisemann 2001).

Hence, Isolation and purification of these PKS protein will allow partial peptide sequencing and design of specific oligonucleotide probes for isolation of the monocerin PKS gene. Therefore, in our study we concentrated on methyl transferase which methylate dihydroisocoumarin to monocerin. This paper, describes the purification and properties of methyl transferase from *D. monoceras*.

MATERIALS AND METHODS

All media were autoclaved for 15 minutes at 120°C for 15 minutes at 15 p.s.i.

Monocerin (*Dreschlera monoceras*)

Dreschlera monoceras (ATCC 24641) culture was obtained from LGC Promochem, U.K.

Strain storage

Stock slopes were maintained on Potato dextrose agar (PDA) plates.

Culture conditions

Liquid cultures were grown under static conditions at 25°C for 14 days as described in Dillon *et al.* (Dillon *et al.* 1992).

A spore suspension was made from one PDA plate with sterilised water (8.00 ml) and filtered with glass wool. This was used to inoculate 4 Erlenmeyer flask each containing above liquid culture medium (100 ml).

Standard Enzyme assay

enzyme (100 µl, ~ 1.2 mg/ml)

2mM S-adenosyl methionine

1mM dihydro-isocoumarin incubates at 37°C for 30 min.

Detection of metabolites

After 30 min incubation period in each assay metabolites were extracted into ethyl acetate (300 µl x 3). Then all the ethyl acetate was evaporated, the

pellet was redissolved in 100 µl of HPLC grade ethyl acetate and analysed by HPLC under the following conditions.

<u>Time (min)</u>	<u>% acetonitrile in water</u>	<u>Flow rate (ml/min)</u>
0	5.00	1.00
5	5.00	1.00
35	100.00	1.00
40	100.00	1.00
45	5.00	0.8
50	5.00	0.8

C₁₈ reverse phase phenomenex column was used for HPLC analysis.

Kinetics of the enzyme activity with the culture growth

Cultures were grown in 500 ml flasks each contained 100 ml of culture media as described above. The mycelia were harvested, at 6, 7, 8, 9, 10, 12, 13 and 14 days of inoculation. Cell free extracts were prepared by grinding mycelia in a motor and pestle freezing with liquid nitrogen and assayed for the activity of methyl transferase as described above. Enzyme activity was quantified by the amount of monocerin produced in each day.

Purification

All the experiments were carried out at 0-4°C unless otherwise specified. Quantitative estimation of protein after each purification steps were carried out by using BSA protein assay kit supplied by Pierce Company, U.K.

Preparation of crude enzyme extract

Mycelia were harvested from well grown culture flasks after 9-10 days of inoculation, immersed in potassium phosphate (0.1 M, pH 7.66) buffer containing 1 mM EDTA, 5 mM mercapto-ethanol, Benzamidine (0.34 g/l) and 20% glycerol and then pulverised in a motor and pestle freezing with liquid nitrogen. Thus prepared crude extract was centrifuged at 18,000 rpm for 20 min at 2°C.

Fractional precipitation with streptomycene sulphate

Streptomycene sulphate (1% w/v) was added to the crude extract and stirred on ice for 30 min. The solution was centrifuged at 18000 rpm for 20 min at 2°C.

Fractionation with ammonium sulphate

Crude homogenate was fractionated with different saturations of ammonium sulphate and 60-90% ammonium sulphate cut which provides the highest enzyme activity was obtained and stored at -80°C until required for further analysis.

Ultrafiltration

Above ammonium sulphate cut was dissolved in phosphate buffer (0.1 M, pH7.66) containing 1 mM EDTA, 5 mM mercaptoethanol, Benzamidine (0.34g/l) and 20% glycerol and filtered with a millipore ultrafiltration membrane (Polyethersulphone, 10 kDa) using a stirred ultrafiltration cell (Amicon model 8050).

Dialysis

Above filtered solution was dialysed with 50mM potassium phosphate buffer (pH 7.66) containing 1mM EDTA, 5 mM mercaptoethanol, Benzamidine (0.34g/l) and 20% glycerol for 4 hours at 0°C. Two changes of buffer were given within 4 hour period.

Purification by FPLC

Chromatographic preparations were carried out under condition recommended by Pharmacia. All buffers were made with Milli Q quality water filtered with cellulose nitrate membrane filters (Whatman, 0.45 µm) and degassed under reduced pressure prior to use.

SP sepharose chromatography

Dialysed protein (3.7 mg/ml, 35.00 ml) was loaded on 26/10 Hiload SP sepharose column pre-equilibrated with Potassium phosphate (pH 7.66, 50 mM) containing 1 mM EDTA, 5 mM mercaptoethanol, Benzamidine (0.34 g/l) and 20% glycerol. Protein solutions were applied to the column via a 50 ml super loop followed by filtering through Whatman Puradisc filter devices (0.45 µm).

The proteins were eluted at a flow rate of 2 ml/min, with a linear gradient of 0-1M NaCl added to the Potassium phosphate (pH 7.66, 50 mM) buffer

containing 1 mM EDTA, 5 mM mercaptoethanol, Benzamidine (0.34 g/l) and 20% glycerol. Fractions (5.00 ml) were collected and assayed for methyl transferase activity. Blank experiments were also performed, assaying each enzyme fraction under same conditions but without substrates.

Superdex TM 200 gel filtration chromatography

Active fractions obtained from SP sepharose step were pooled and loaded (20 mg protein/ml, 2.0 ml) to Superdex TM 200 gel filtration column pre-equilibrated with Sodium phosphate (pH 7.66, 50 mM) containing 1 mM EDTA, 5 mM mercaptoethanol, Benzamidine (0.34 g/l), 20% glycerol and 0.15 M NaCl. The column was eluted at a flow rate of 0.35 ml/min. Fractions (1.00 ml) were collected and each fraction was assayed for methyl transferase as described above. Blank experiments were also performed, assaying each enzyme fraction under same condition but without the substrates.

Purity Assessment by SDS-PAGE gel electrophoresis (10%)

SDS-PAGE (10%) gel was run for the active fractions (fraction no 9 and 10) which obtained from above gel filtration step.

Sample preparation

TCA (72%, 10 μ l) was added to 100 μ l aliquots of each fraction to precipitate the protein. The pellet was dissolved in loading buffer (5 μ l) and tris base (3 μ l) and boiled for 15 min.

The slab gel was made of 4% stacking gel and 10% separating gel. Bio-rad Mini Gel III apparatus was used to run the gel. SDS high range Molecular Weight Marker (Sigma) was used as the gel marker.

Gel buffer; Tris-HCl (3 M, pH8.45) containing SDS (0.3% w/v)

Anode buffer; Tris-HCl (200 mM, pH8.9)

Cathode buffer Tris-HCl (100 mM, pH8.25) containing tricine (100 mM) and SDS (0.1%, w/v)

Samples was loaded to the gel and the gel was run at 35 V initially for 30 min and then at 80 V for another 2 hr. After running, the gel was stained with coomassie blue.

Calibration of Superdex™ 200 gel filtration column

The column was pre-equilibrated with sodium phosphate buffer (pH 7.66, 50 mM) containing 0.15 M NaCl, 1 mM EDTA, 5 mM mercaptoethanol, Benzamidine (0.34 g/l) and 20% glycerol. Blue dextran was eluted through the column in order to obtain the void volume. Urease (hexamer, 545 KDa), Urease (trimer, 270 KDa), Alcohol dehydrogenase (150 KDa), Bovine serum albumin (66 KDa), Egg albumin (45 KDa), Carbonicanhydrase (29 KDa), lacto albumin (14KDa) and lacto albumin (14KDa) were used as standard protein for calibration of the gel column.

The column was eluted under the same conditions that were used in above gel filtration step.

Properties of methyl transferase

Effect of pH on the activity of methyl transferase

Enzyme was incubated at different pH values ranging from pH 6.5 –7.9 and the activity was assayed for methyl transferase under the condition given in standard enzyme assay.

Effect of incubation temperature on the activity of methyl transferase

Enzyme activity was assayed at different incubation temperatures ranging from 25°C to 50°C under the conditions given in standard enzyme assay.

Thermal stability of methyl transferase

The enzyme was pre-incubated at 45°C, 62°C, 70°C, 80°C, 90°C and 100°C in a thermostat water bath for 30 min. The samples were immediately cooled in ice water and then residual enzyme activity was assayed under the conditions given in standard enzyme assay.

Determination of Km

Km was determined by assaying activity of the purified enzyme with dihydro-isocoumarin as substrate at concentration ranging 0.2 mM from 2 mM. Assays were done under the conditions given in standard enzyme assay.

RESULTS AND DISCUSSION

Detection of metabolites under the given HPLC conditions shows that retention times of dihydro isocoumarin and that of monocerin 23.25 min. and 27.56 min. respectively.

Kinetics of the Enzyme activity with the growth of *D. monoceras* was studied to determine the ideal time to harvest the mycelia. As shown in Fig.1 optimum activity for methyl transferase was observed at 10 V day of inoculation and no activity was found after 14th day. Crude enzyme extract which prepared at this stage was fractionated with different saturation of ammonium sulphate. The result shows that most of the methyl transferase precipitates at 60-90% saturation of ammonium sulphate.

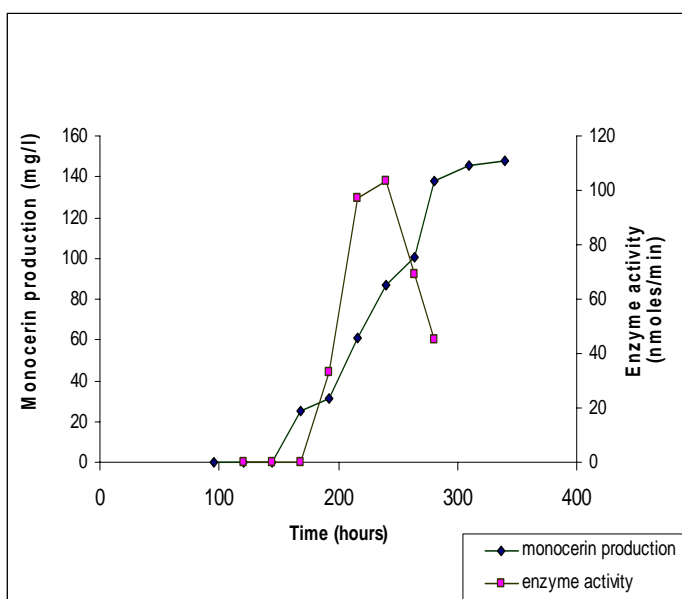


Figure 1. Kinetics of methyl transferase activity with the culture growth

Ultrafiltration of the enzyme solution through 10 kDa membrane aids to eliminate most of the small molecules (e.g. proteins such as proteases and cofactors), present in the extracts. This step enhanced the enzyme activity, providing a higher degree of purification.

Initial Q sepharose chromatography trial showed that the enzyme eluted before the gradient. Hence purifying the protein through SP sepharose chromatography instead of Q sepharose was decided. In the SP sepharose chromatography enzyme was eluted at 300-320 mM NaCl over three fractions (fraction no. 23-27). Highest activity was found in fraction no. 24-26 (Fig. 2). Degree of purification was satisfactory.

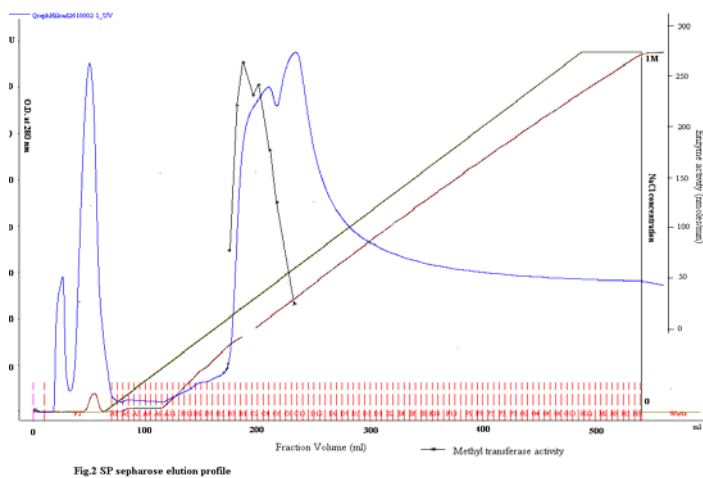


Fig.2 SP sepharose elution profile

Figure 2. SP sepharose elution profile

To achieve further purification active fractions obtained from SP sepharose step were chromatographed through Superdex TM 200 gel filtration chromatography. Methyl transferase was eluted as a single peak at fraction no.9-10 (Fig. 3).

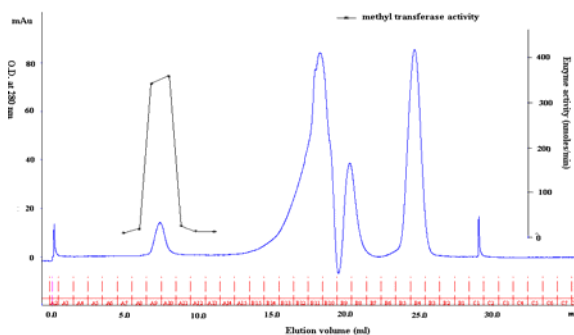


Fig. 3 Superdex TM 200 gel filtration chromatography profile

Figure 3. Superdex TM 200 gel filtration chromatography profile

SDS-PAGE (10%) gel electrophoresis of fraction no. 9 and 10 showed a single band which appear around 55 KDa (Fig.4) indicating that enzyme has been purified to near homogeneity with 11.1% recovery (Table 1).

Table 1. Purification Table for methyl transferase from *D. monoceras*

Step	Total protein (mg)	Total activity	Specific activity ($\mu\text{moles mg}^{-1} \text{min}^{-1}$)	% recovery	Purification fold
Crude	813.71	675.37	0.83	100	1
Ultrafiltration	118.23	558.14	4.731	82.1	5.69
SP Sepharose	40.12	468.9	11.70	69.3	14.0
Gel filtration	2.11	79.65	37.93	11.1	45.6

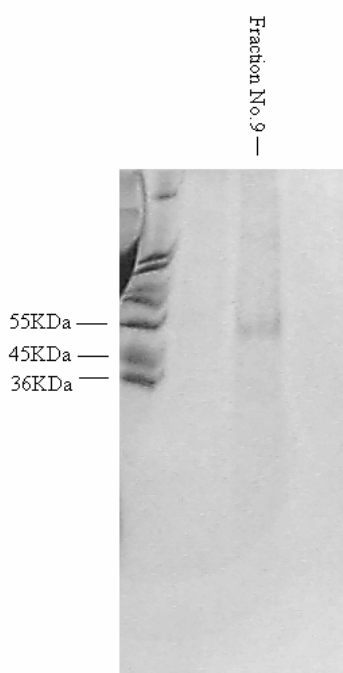


Figure 4. SDS-PAGE (10%) after Superdex TM 200 gel filtration chromatography

Molecular weight of the purified enzyme as determined by Superdex™ 200 gel filtration chromatography is ~ 165 KDa (Fig. 5). This value falls in the molecular mass range that reported for methyl transferase of *Saccharomyces cerevisiae*. These results indicate that methyl transferase could be a trimer with anative molecular mass of ~ 165 kDa.

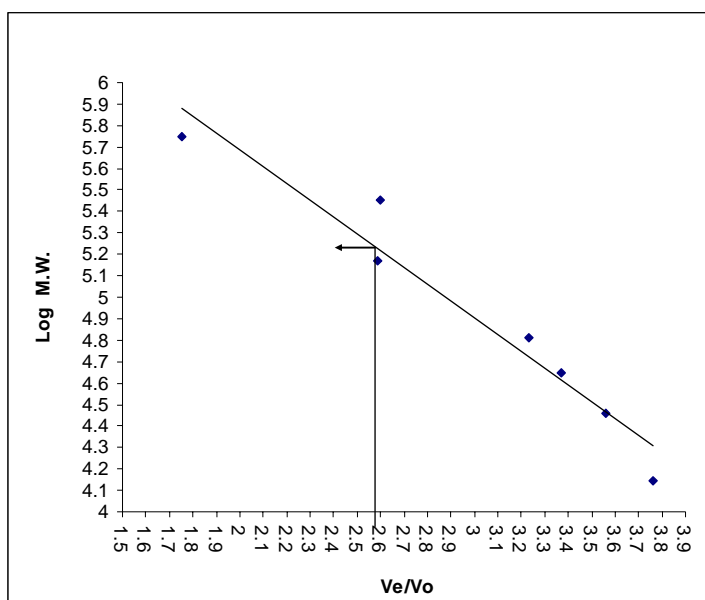


Figure 5. Determination M.W. of methyltransferase

However, methyl transferase seems to be a highly labile enzyme. Hence, maintaining minimum number of freezing and thawing as much as possible would help to minimise the risk of denaturation.

At this purification stage properties of the enzyme were studied. Under this effect of pH, effect of incubation temperature on enzyme activity, thermal stability and Km of the enzyme were investigated.

Optimum pH and optimum assay temperature for the purified enzyme were found to be around pH 7.5-7.66 (Fig.6) and 35-37°C (Fig.7) respectively.

These values were very close to that reported for O- methyl transferase of *Streptomyces fradiae* (Bauer et al. 1988 ; Seno & Baltz 1981).

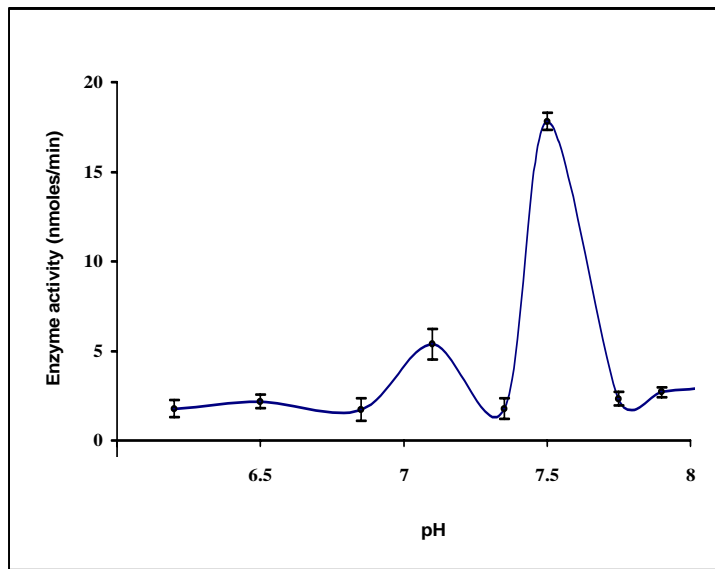


Figure 6. Effect of pH on methyl transferase

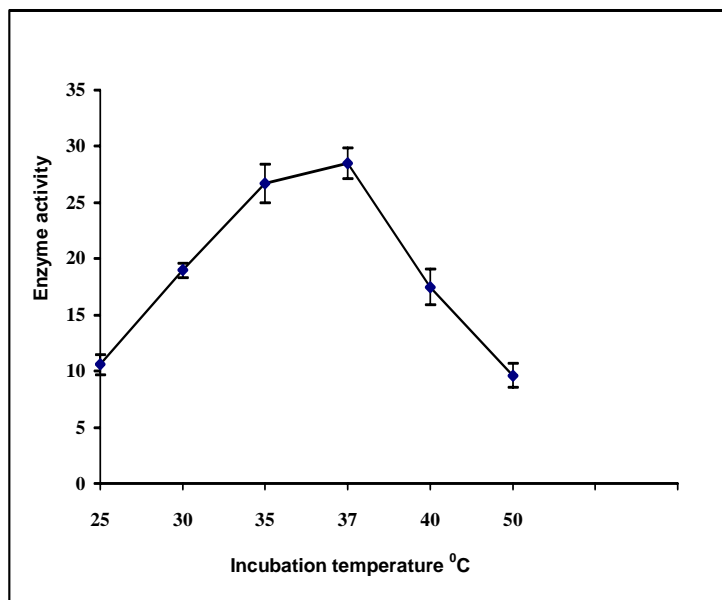


Figure 7. Effect of incubation temperature on methyl transferase activity

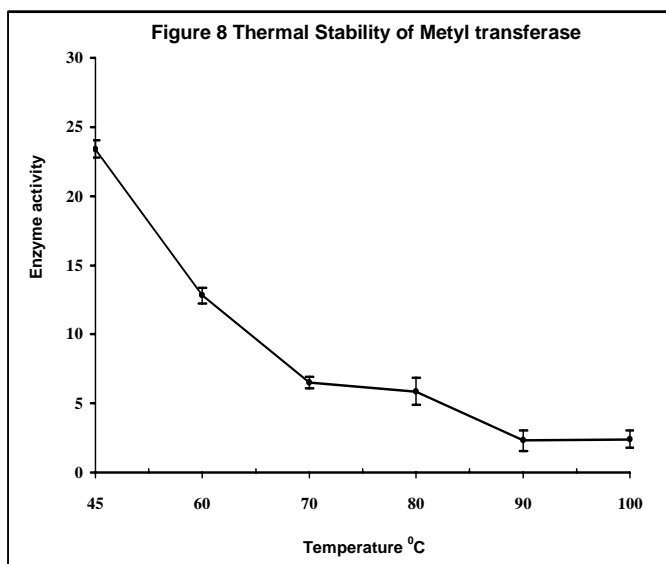


Figure 8. Thermal stability of methyl transferase

Studies on the thermal stability of the enzyme showed that enzyme is stable up to 45°C above this temperature enzyme activity slowly declined and was inactivated at 70°C. The K_m value of the enzyme is found to be ~ 0.083 mM (Fig.9). These values are comparable with that reported for O- methyl transferase of *Streptomyces fradiae*.

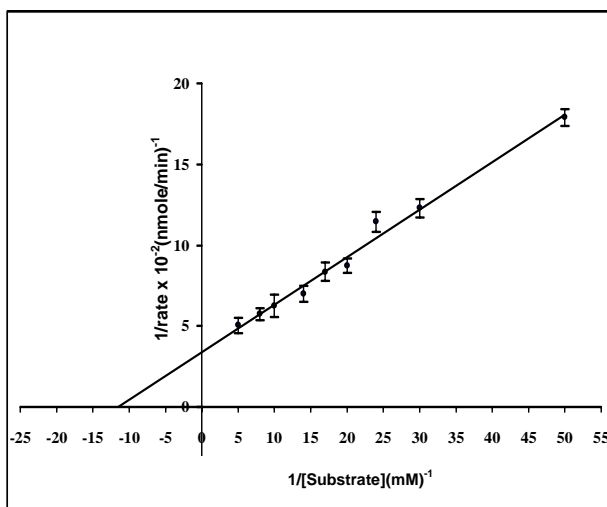


Figure 9. Determination of K_m of methyl transferase Line Weaver Burk Plot

ACKNOWLEDGEMENT

European Physical Science Research Council is acknowledged for funding.

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