A spectrophotometric trimethylamine monooxygenase assay

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Abstract

Trimethylamine monooxygenase (**Tmm**, EC-1.14.13.148) belongs to the family of flavin-containing monooxygenases (FMOs) that oxidize trimethylamine into trimethylamine-N-oxide (TMAO). Conventional methods for assaying **Tmm** are accurate over a narrow range of substrate/ product concentrations. Here we report a TMAO-specific enzymatic assay for **Tmm** using polyallylamine hydrochloride (PAHCl)-capped MnO $_2$ nanoparticles (PAHCl@MnO $_2$). We achieved TMAO specificity using iodoacetonitrile to remove interfering trimethylamine. The change in the concentration of TMAO is measured by observing the difference in the absorbance of 3,3´,5,5´-tetramethylbenzidine (TMB) at 652 nm. The assay is tolerant to several interfering metal ions and other compounds. This method is more reliable and easier than currently known methods. The limit of detection (LOD) and limit of quantitation (LOQ) are 1 μM and 10 μM, respectively, for direct TMAO measurement.

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The supplementary file details the trimethylamine monooxygenase cloning protocol and the nanoparticle characterization with supporting evidence.

File name: SI_PS_SM

Abstract:

Trimethylamine monooxygenase (**Tmm** , EC-1.14.13.148) belongs to the family of flavin-containing monooxygenases (FMOs) that oxidize trimethylamine into trimethylamine-N-oxide (TMAO). Conventional methods for assaying **Tmm** are accurate over a narrow range of substrate/ product concentrations. Here we report a TMAO-specific enzymatic assay for **Tmm** using polyallylamine hydrochloride (PAHCl)-capped MnO_2 nanoparticles (PAHCl@MnO_2). We achieved TMAO specificity using iodoacetonitrile to remove interfering trimethylamine. The change in the concentration of TMAO is measured by observing the difference in the absorbance of 3,3´,5,5´-tetramethylbenzidine (TMB) at 652 nm. The assay is tolerant to several interfering metal ions and other compounds. This method is more reliable and easier than currently known

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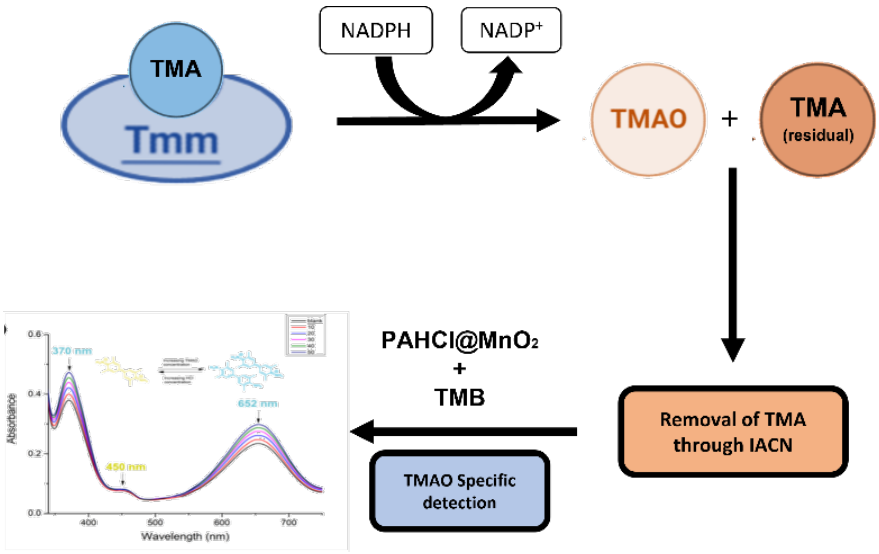

Figure I : Graphical Abstract

Keywords

Flavin monooxygenases, MnO_2 nanoparticles,*Paracoccus sp* . DMF, Trimethylamine-N-oxide detection, 3,3´,5,5´-tetramethylbenzidine.

Abbreviations

FMO: Flavin-containing monooxygenase; IACN: Iodoacetonitrile; PAHCl: Polyallylamine hydrochloride; PSS: Polystyrene sulfonate; TFA: Trifluoroacetic acid; TMB: 3,3´5,5´-Tetramethyl benzidine; **Tmm** : Trimethylamine Monooxygenase.

Introduction

Trimethylamine is ubiquitous in all major kingdoms of life, performing a notable physiological and pathophysiological role (Loo, Chan, Nicholson, & Holmes, 2022). Trimethylamine plasma concentrations directly correlate to several human diseases, such as chronic kidney disease (CKD), cardiovascular diseases, etc. (Yang*et al.* , 2019). The primary source of trimethylamine and TMAO in humans are various precursors such as carnitine, choline, betaine, etc. (Koeth *et al.* , 2013). In the human gut, microbes convert dietary precursors into trimethylamine. This trimethylamine gets converted into TMAO by FMOs (Falony, Vieira-Silva, & Raes, 2015) in the liver for excretion. Trimethylaminuria is an accumulation of trimethylamine that causes a fish-like odor among people who lack a functional FMO.

Trimethylamine and TMAO are significant carbon and nitrogen sources for methylotrophs (Colby & Zatman, 1973; Loo *et al.* , 2022). Methylotrophic bacteria are primarily found in the marine environment and possess FMOs. The FMO, trimethylamine monooxygenase (**Tmm**) that converts trimethylamine into TMAO (Li*et al.* , 2017) is an NADPH-dependent mono-oxygenase (Chen, Patel, Crombie, Scrivens, & Murrell, 2011). This enzyme is assayed by monitoring NADPH concentration. Most of the reported assays monitor NADPH conversion into $NADP^+$. Even techniques like the isothermal calorimetry (Catucci, Sadeghi, & Gilardi, 2019) and detection of NADPH (Dixit & Roche, 1984) have limitations. Although sensitive, these methods can't

be used with crude samples because other enzymes also use NADPH (Spaans, Weusthuis, van der Oost, & Kengen, 2015). Hence it is normal to subtract background signals to obtain enzymatic activity measurements. The stability of NADPH is also sensitive to low pH and high temperatures (Wu, Wu, & Knight, 1986). These limitations make NADPH-dependent assays inaccurate.

Quantifying TMAO and trimethylamine directly in a sample where their concentrations change is quite challenging. Given that TMAO and trimethylamine are simultaneously found in clinical samples (Gatarek & Kałużna-Czaplińska, 2021a), a precise quantification of TMAO requires trimethylamine separation (Awwad, Geisel, & Obeid, 2016a). Existing methodologies take long durations to quantify TMAO precisely as extraction and separation techniques result in sample loss. The amount of TMAO or trimethylamine present in biological samples like urine and blood plasma usually lies in the range of nM - μM (Gatarek & Kałużna-Czaplińska, 2021b). GC-MS detection conditions often convert TMAO to trimethylamine (daCosta, Vrbanac, & Zeisel, 1990). Sample pre-treatment is, therefore, almost always needed to eliminate trimethylamine. Such procedures require column derivatization in which solvent plays a significant role as these reactions do not proceed in an aqueous media (Wang *et al.* , 2014). Various other methods use NMR (He et al., 2021a), UHPLC (Awwad, Geisel, & Obeid, 2016b; Ocque, Stubbs, & Nolin, 2015), LCMS/MS (Hefni, Bergström, Lennqvist, Fagerström, & Witthöft, 2021), and HPLC (Lang et al., 1998), but these are not cost-effective.

Technique	Time for detection (minutes)	LOD/LOQ (μM)	Advantages	Disadvantages	Reference
NMR	˜45	1.00/3.02	Less sample volume	TMA interference Specialised instrumentation	(He *et al.*, 2021b)
LCMS/MS	˜30	0.055/0.182	TMAO Specific	Specialized column and instrumentation	(Hefni *et al.*, 2021)
UHPLC/MS	˜15	5.32/13.31	Less sample volume Aqueous samples	Specialized column and instrumentation	(Ocque *et al.*, 2015)
Cyclic Voltammetry	˜20	1.5/1.5	TMAO specific	Specialized instrumentation	(Lakshmi *et al.*, 2021)
Present method	˜20	1.0/10.0	TMAO Specific	Volume set by available UV spectrometer instrumentation	This work

Table 1 : Comparison between the reported literature on TMAO detection.

Here we report a method for assaying the enzyme trimethylamine monooxygenase in which the TMAO formed is directly quantified. The presence of trimethylamine interferes with the previously reported methods for TMAO detection (Chang *et al.* , 2021a). We overcome this issue by reacting residual trimethylamine using a derivatizing reagent iodoacetonitrile (IACN), that does not interfere with the assay (Hefni et al., 2021). Thus, our detection method becomes TMAO-specific in the enzymatic assay.

Results and discussions

2.1 TMAO Assay Optimization

Figure 1 shows that the absorbance at 370 nm and 652 nm decreases significantly at pH 1.0. The method is susceptible to the change in pH of the solution. The change in the proton concentration affects the amount of TMAO being detected. Thus, determining a pH range where TMAO detection is quantitative is imperative. A pH range where the change in absorbance is more sensitive is a better choice for detection because that will ensure a high sensitivity of the method. Therefore, a pH range of 2.0 to 2.5 was chosen for TMAO detection.

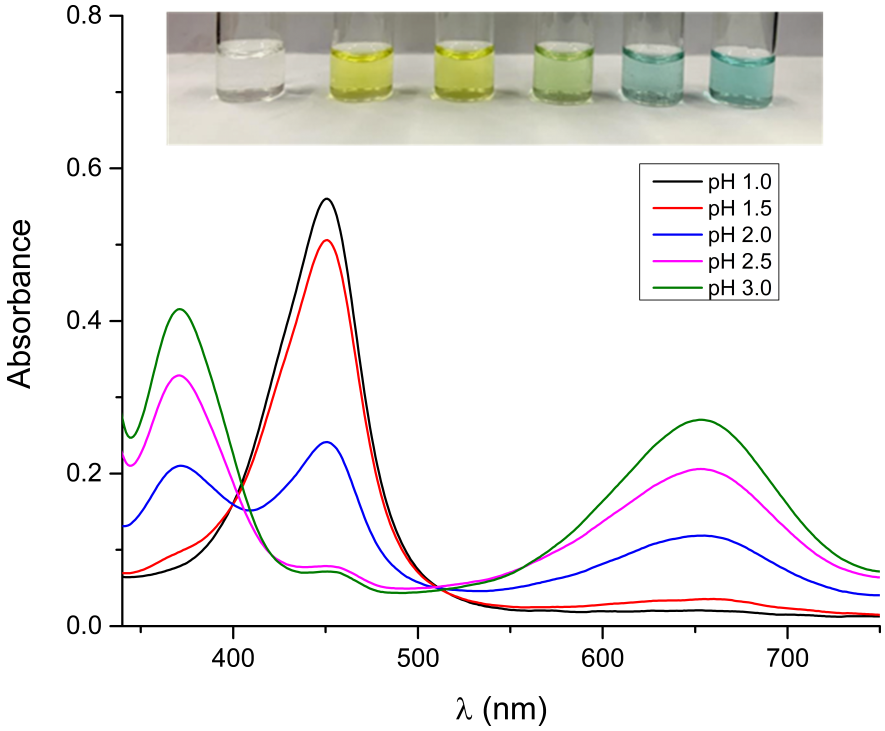

Figure 1 : The absorbance at 450 nm increases as we increase the concentration of HCl; the solution also turns yellow. As we can see from the image on the left, the colour of the solution turns from blue to yellow, meaning that as the concentration of HCl keeps increasing, the charge transfer complex dissociates according to scheme 2.

2.2 Principle: Colorimetric TMAO detection

As reported in the literature (Marquez & Dunford, 1997), PAHCl@MnO_2 catalyses the formation of the charge transfer complex of TMB, which is blue and absorbs at **370 nm**and **652 nm** . In the presence of HCl, TMB gives yellow colour due to the formation of **2**(Scheme 1).

Scheme 1 : Reaction of TMB with PAHCl@MnO_2 to give off a blue colour. At a pH pf 2 – 2.5 the reaction between the blue charge transfer complex and **2**is driven towards the **1** because of TMAO, which is where the detection happens.

Calibration plot : To trimethylamine-containing buffer, NH_4OH (5 mM) and IACN (5 mM) were added and incubated for 15 minutes, then HCl (10 mM), followed by PAHCl@$MnO_2$and TMB. The absorbance of the solutions was recorded, increasing the concentration of TMAO along with control (figure 2). The lower limit of quantification was determined to be 10 μM.

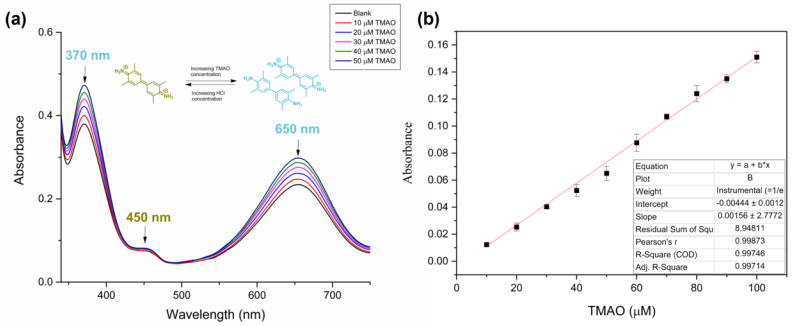

Figure 2 : **(a)**Decrease in the absorbance of TMB at 450 nm and an increase in the absorbance at 652/370 nm indicates the formation of a charge transfer complex due to TMAO. Inset of the figure is the equilibrium shift from the yellow structure to the charge transfer complex in blue.**(b)** Calibration plot.

Interference study

1. **Trimethylamine interference**

TMAO detection depends on the formation of the charge transfer complex (Scheme 2, **1**). Amines are well-known quenchers and interfere with forming charge transfer complexes (Young, Martin, Feriozi, Brewer, & Kayser, 1973). Thus, trimethylamine may interfere with forming the charge transfer complex necessary for TMAO detection.

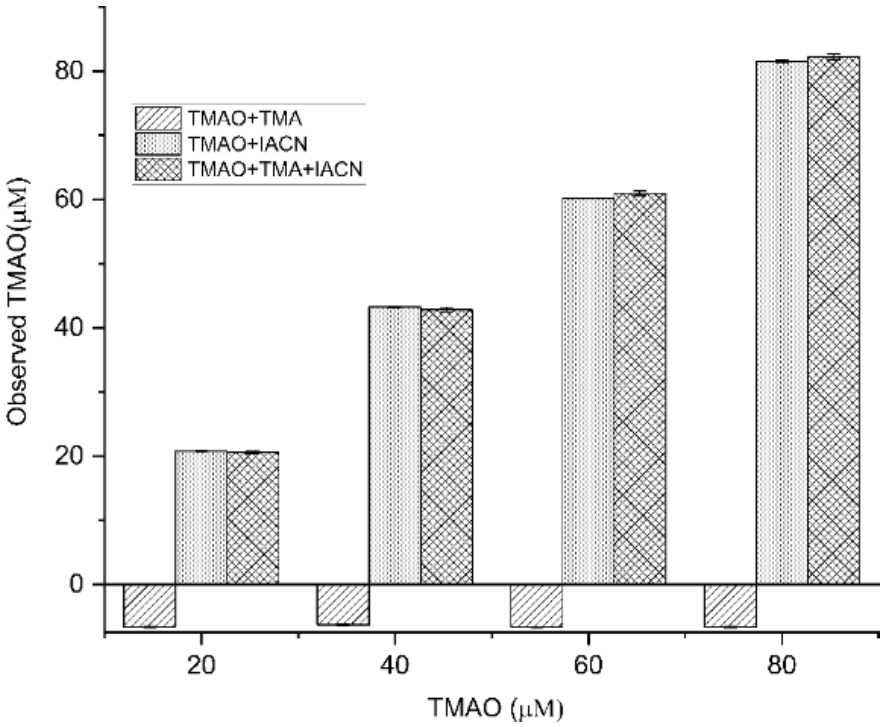

Figure 3 : Interference in the TMAO quantification due to the presence of trimethylamine.

Metal ions

Different metal ions usually found in biological systems often interfere with TMA assays; hence we checked if these metal ions were potent interfering agents causing any significant changes in our assay. As shown in Table 1, no interference is demonstrated by Mg and Mn ions. Except for Co, all the other metal ions showed mild deviation during the assay with a percentage error well within 5%.

Table 1: Interference of different metal ions in TMAO detection

S. No.	Metal ion (50 μM)	Relative error
1	Mn	0.88 ± 0.002
2	Fe	2.68 ± 0.019
3	Co	5.45 ± 0.047
4	Ni	2.09 ± 0.008
5	Cu	3.42 ± 0.021
6	Zn	1.6 ± 0.006
7	Ca	3.27 ± 0.028
8	Mg	0.37 ± 0.001

Kinetics of Tmm

2.4.1 Measurement with crude Tmm

The reaction mixture was incubated for 15 min with 0.1 mM NADPH and 0.025 mM FMN and 20 μl of **Tmm** crude enzyme mix in the Tris-NaCl buffer. The reaction starts after adding trimethylamine and is quenched after 30 min by adding 0.1% TFA.

The amount of NADPH consumed from the reaction mix is calculated by monitoring the absorbance at 340 nm. And the amount of trimethylamine consumed was monitored by taking a 500 µl aliquot from the quenched reaction using trimethylamine-picrate formation. In another 500 µl aliquot, the amount of TMAO formed is calculated. From Table 2, the observed activity in the crude sample is higher in the case of $NADPH^+$ assay. On checking the activity after each purification step, the activity values become comparable, proving that $NADPH^+$-dependent assays are unreliable for crude samples.

Table 2 : **Tmm** enzyme assay and product quantification

Purification Steps	Total Protein (mg)	Total Activity (U)	Total Activity (U)	Specific Activity (U/mg)	Specific Activity (U/mg)
		$NADPH^+$	**TMAO**	**$NADPH^+$**	**TMAO**
Crude	87	2.98	2.45	0.03	0.02
$(NH_4)_2SO_4$ cut (10-40%)	55	2.85	2.32	0.05	0.04
Anion Exchange	1.5	1.57	1.65	1.04	1.10
Gel Filtration	0.6	1.45	1.49	2.41	2.48

2.4.2 Measurement with pure Tmm

The amount of NADPH consumed and TMAO formed is assayed using the previously reported methods. The Michaelis-Menten equation is used for calculating K_m and V_{max} for**Tmm** . The values for K_m and $V_{max}$are calculated as 0.13 µM and 1.631 µM, respectively, when considering NADPH consumed in the reaction. The values for K_m and V_{max} are calculated as 0.08 µM and 0.85 µM, respectively, when the amount of TMAO formed in the reaction is monitored. Whereas activity measured through the current reported method is lower (figure 4). The $K_m$values indicate the substrate and enzyme interaction, and a lower value of K_m suggests a better binding of **Tmm** with its native substrate, trimethylamine. Thus, our method is more accurate compared to NADPH-dependent assay.

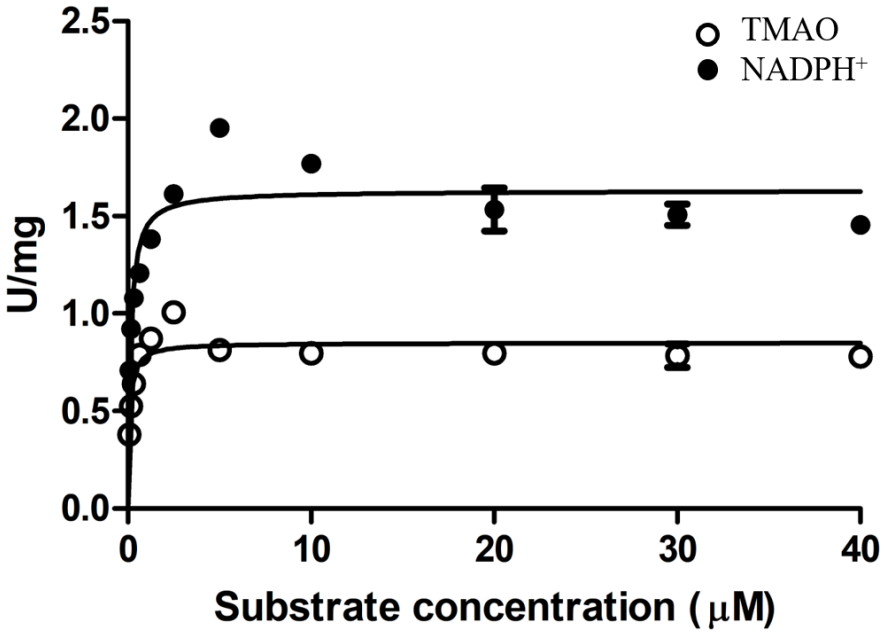

Figure 4 : The graphs represent the Michaelis Menten curve of the enzyme **Tmm** for substrate (trimethylamine) concentrations when (a) NADPH and (b) when TMAO is assayed.

Conclusion

From the experimental evidence, we can conclude that NADPH coupled assay can be used for studying the FMO's kinetics, but these always give some measurement errors. The amount of NADPH consumed in the reaction is not directly proportional to the amount of substrate consumed, possibly because NADPH is unstable and a common cofactor in biological systems. We set out to find a specific method for our enzyme. Despite numerous available methods for monitoring TMAO, most are unreliable for enzymatic assay without using any sophisticated instruments. The technique reported here can be widely applied to measure the amount of TMAO independent of trimethylamine interference, which has not been the case previously except by (Hefni *et al.* , 2021).

This method can also be applied for testing urine samples for TMAO concentrations in a healthy individual within a normal range of urea and uric acid concentrations. Our current reported method, which explicitly monitors TMAO using UV spectrophotometry, is thus versatile and gives reproducible results. TMAO formed in the human body is excreted via urine. In a healthy individual, the TMAO concentration is within 10 µM (Hefni *et al.* , 2021), and hence detection is possible using our method, and the interference is within ± 5% in the presence of urea and uric acid. This study is awaiting ethical clearance.

Material and method

4.1 Chemicals and Materials

All the reagents used were of analytical grade. Poly acrylamide hydrochloride (PAHCl) was obtained from Otto Chemie Pvt. Ltd., PSS was obtained from Thermo Fisher Scientific, $KMnO_4$ was obtained from Sisco research laboratories Pvt. Ltd., TMAO, and TMB were from TCI, Japan, IACN from Alfa-Aeser, trimethylamine was obtained from Spectrochem Pvt. Ltd. NH_4OH was obtained from Finar Limited; Tris-HCl and NaCl were obtained from Thermo Fisher Scientific. Luria-Bertani Medium was obtained from HiMedia, and the DNA ladder and protein Marker were from Banglore-GeNei.

Preparation of reagents and stock solutions: TMB stock solution was prepared fresh in 50% glacial acetic acid. IACN solution was always prepared fresh in ethanol; these solutions were kept in amber tubes and stored in a cold cabinet. In deionized water, stock solutions of NH_4OH (100 mM), HCl (5 M), TMAO (100 mM), and Tris-HCl buffer of pH 7.2 were prepared. A stock solution of PAHCl (1g/L) and PSS (1g/L) was prepared in 0.1 M NaCl.

4.2 Instruments

Spectrophotometric analysis was performed on Evolution^{TM} 300 UV-Vis spectrophotometer (Thermo Fisher Scientific), while Zeta and DLS were carried out on the Malvern Zetasizer (Malvern). Scanning electron microscopy was performed on the FEI Nova SEM 450.

4.3 PAHCl@MnO_2nanoparticle synthesis and characterization

PAHCl@MnO_2 nanoparticles were synthesized as described by (Chang *et al.* , 2021b) with certain modifications. To $KMnO_4$, PAHCl solution was added and stirred vigorously at 800 rpm for 20 minutes; a colour change was observed from dark pink to brown (inset, Fig. S2(a), colour change from left to the one on the right). The formation of MnO_2 was confirmed using UV absorbance [Fig. S2(a)]. These nanoparticles were added to the PSS solution dropwise while stirring and then left for 15 minutes. The solution was centrifuged at 12000 rpm for 15 minutes to remove unreacted starting materials, resuspended in PAHCl solution, and stirred for 30 minutes, followed by washing three times with water. The capping can be monitored by measuring each layer's zeta potential change [Fig. S2(b)]. Layer 1 shows PAHCl capping, layer 2 shows PSS caping and layer 3 is PAHCl. Hence, the final product is PAHCl@MnO_2nanoparticles. The SEM images show that the MnO_2nanoparticles are spherical [Fig. S2(c)].

Isolation and overexpression of the Tmm

Tmm gene was amplified from the bacterial strain,*Paracoccus sp* . DMF (Swaroop, Sughosh, & Ramanathan, 2009). The whole genome sequence of *Paracoccus sp.* DMF has been deposited at DDBJ/ENA/GenBank under the accession number SOKV00000000.2, BioProject number PRJNA528176 and BioSample number SAMN11175380. The gene was amplified using the PCR program described in Table ST1.

In a PCR reaction volume of 100µl in which 10µl buffer (10 X 15 mM $MgCl_2$ buffer), 4 µl template (genomic DNA), 10 µl forward primer (100 µM), 10 µl reverse primer (100 µM), 10 µl dNTPs (10 mM), 0.8 µl Taq Polymerase (5 U/µl) and remaining nuclease-free water was used.

The amplified gene was cloned in a pet22b (+) vector within NdeI and Hind III restriction sites with ampicillin-resistant markers. The positive clone was transformed into BL21(DE3) competent cell, spread on LB agar (1.8%) + ampicillin (50 µg/ml) plate, and incubated at 37 °C overnight. A single colony was inoculated into LB broth containing ampicillin and incubated overnight at 37 °C. A 1% v/v inoculum was taken from the previous and inoculated fresh 10 ml LB media. When the O.D. reached 0.6 - 0.65, 1 ml of culture was aliquoted as uninduced, and the rest of the culture was induced with 0.5 mM IPTG and incubated at 37 °C for 4 hours. Further, the uninduced and induced fractions were checked for over-expression on 12% SDS gel.

Cell Growth

The cells were grown by inoculating 1% v/v culture to terrific broth media + ampicillin (50 µg/ml) and incubated at 37 °C till OD reached 0.7 - 0.8, after which the culture was induced with 0.5 mM IPTG and incubated at 16°C for 18 hours. The bacterial cells were harvested at 4000*g, and pellets were stored at -20 °C till further use.

4.6 TMAO Assay Optimization

pH optimization : The detection of TMAO using TMB is possible in an acidic medium. The reaction is carried out at different pH ranges, varying from 1-3, to obtain an optimum range for the detection.

The derivatization of trimethylamine : To remove trimethylamine from the reaction mixture, iodoacetonitrile (IACN) was used. (Hefni*et al.* , 2021) A slightly excess amount of IACN and ammonium hydroxide was constantly added to the analyte solution and incubated at room temperature for 15 minutes. After the trimethylamine derivatization, TMAO analysis was carried out.

Scheme 2: Reaction of IACN with trimethylamine to form the derivative **3.**

Trimethylamine derivatization using IACN was confirmed through ESI-MS. After incubating it with IACN for 15-20 minutes, the reaction detected no trimethylamine. (Figure S3)

TMAO detection protocol : Colorimetric detection of TMAO was performed by adding the NH_4OH solution (5 mM) to a reaction buffer containing trimethylamine, then IACN solution, and incubation for 15 minutes. After the incubation period, 10 mM HCl was added, followed by 8 µL PAHCl@MnO_2, five mM TMB, and another incubation for 3 minutes before taking UV of the sample. TMAO was added to the reaction, and a change in the UV spectra was observed.

4.7 Interference Study

4.7.1 Interference due to trimethylamine : Three different reactions were set up with the spiked concentration of TMAO. The concentrations ranged from 20 µM to 80 µM of TMAO while keeping the trimethylamine constant at 50 µM. The high concentration of trimethylamine was chosen because it is the substrate of our enzyme. The controls did not have IACN to remove the trimethylamine.

4.7.2 Metal ion: The method developed was checked for metal ion interference; 50 µM of metal ion solutions were used in the reaction mixture with one mM TMAO. As mentioned in section 2.5, the reaction was performed and studied for metal ion interference.

4.8 Tmm enzyme assay

Cell Lysis: The cells were grown in LB media at 16 °C and induced with 0.5 mM IPTG when the OD reached 0.6. The cells were harvested after overnight growth, resuspended into the lysis buffer, and left for 45 minutes under continuous stirring at 4 °C for homogenisation. The composition of the lysis buffer was 50 mM Tris-HCl buffer (pH 7.5), 250 mM NaCl, 10% Glycerol, 0.1% Triton X-100, and one mM PMSF. After incubation, the cells were sonicated. The cell lysate was centrifuged at 12000 rpm for 1 hour to separate the cell debris.

The **Tmm** assay mixture contains 1mM trimethylamine, 0.1 mM NADPH, 0.025 mM FMN, 20 µl of crude enzyme in 25 mM tris, and 50 mM NaCl. This assay mixture was incubated for 15 min at 28 °C, and trimethylamine was added to initiate the reaction. **Tmm** activity was assayed in three ways: the amount of NADPH consumed at 340 nm, trimethylamine consumed, and TMAO product formed in the reaction mixture.

The amount of NADPH consumed was measured at 340 nm (Dixit & Roche, 1984), the amount of trimethylamine consumed was measured by the protocol as reported by Pena-Pereira, Lavilla, & Bendicho, 2010, and the amount of TMAO formed was measured by the method reported in this manuscript.

Credits

Shiwangi Maurya: conceptualisation, data curation, investigation, methodology, visualisation, writing – original draft, revision, and review

Abhishek Singh: data curation, visualization, writing – original draft, revision, and review.

Gurunath Ramanathan: Conceptualization, formal analysis, review, and supervision.

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Conflict of Interest

The authors declare no conflict of interest.

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