Influence of Cytosolic Malic Enzyme in Oleaginous Yeast Rhodotorula mucilaginosa IIPL32 for Lipid Biosynthesis

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Abstract

A cytosolic NADPH+ dependent malic enzyme has been purified and characterized from an oleaginous yeast Rhodotorula mucilaginosa IIPL32 to investigate its role in lipid biosynthesis. The enzyme has selective and high affinity for NADP+ and L-malate. Sesamol, a nonoil component of sesame seed oil, has been used during nitrogen depleted condition to evaluate inhibition of malic enzyme and lipid production. Sesamol did not inhibit purified malic enzyme. Sesamol was converted into catechol like metabolite to impart enzyme inhibition and thus lipid production by decreased supply of NADPH+.

Keywords: Malic enzyme; Oleaginous yeast; Purification; Lipid; Sesamol

Introduction

Microbial single cell oil has been visualized as an alternative to plant based natural fatty oils as feedstocks for biofuels or oleochemicals, owing to its short generation time, low space requirement and product uniformity independent of climate and geography. Oleaginous yeasts are advantageous over oil bearing microalgae, as the former could be harvested for lipid synthesis using cheap carbon sources under controlled conditions with higher productivity, against the latter, which depends on atmospheric CO2, as carbon source with less productivity [1]. Yeast lipid is generally quantified in two ways; first, percentage lipid with respect to dry cell mass and second, amount of lipid generated by conversion of sugar. Latter represents more realistic real time features, based on yield and economics. Theoretically, sugar to lipid conversion is maximum 33% however; practically ~20-22% conversion has been based on yield and economics. Theoretically, sugar to lipid conversion is maximum 33% however; practically ~20-22% conversion has been based on yield and economics. Theoretically, sugar to lipid conversion is maximum 33% however; practically ~20-22% conversion has been based on yield and economics. Theoretical, sugar to lipid conversion is maximum 33% however; practically ~20-22% conversion has been based on yield and economics.

Rhodotorula mucilaginosa IIPL32, isolated from soil of oil dumping shed in CSIR-Indian Institute of Petroleum, Dehradun and optimally grown at 32°C, was used as oleaginous yeast for this experimental study. Cell biomass generation, lipid accumulation and ME activity were conducted in a designed medium (composition in g/L; xylose rich lignocellulosic biomass hydrolysate, adjusted to 20.0; (NH4)2SO4, 1.98; KH2PO4, 1.26; NaHPO4, 0.748; MgSO4, 0.05; MnSO4, 0.005; H3BO3, 0.005; CoNO3, 0.005; NH4MoO4, 0.005; yeast extract 1.0; pH 4.5-5.0). Taxonomic characterization of the yeast was done based on partial nucleotide sequence of 18S rDNA gene, amplified using forward (5’ ACCCGCTGAACCTTAAAGC3’) and reverse (5’CCGTGTATTCAGACGGG3’) primers with genomic DNA as template. Amplified DNA was sequenced via paired end (PE) DNA sequencing method and aligned (BLASTN) with submitted sequences available in NCBI database to classify the isolate as R. mucilaginosa (Gen Bank accession number KF313359, MTCC 25056) (Figure 1).

Quantitative estimation of lipid and ME

Cell biomass generation, lipid accumulation and ME production were accomplished in 15 L fermenter (BioSac, India; working volume of 12 L equipped with automated control and SCADA) at 32°C and pH 4.5. An initial growth phase of 24 h (consumption of 90% pentose sugar) was followed by maturation phase where the carbon-to-nitrogen (C/N) ratio in the broth was increased by pumping of concentrated pentose feedstock derived pentose and hexose sugars. In this paper, we have evaluated the presence and role of cytosolic ME in Rhodotorula mucilaginosa IIPL32, as a supplier of NADPH+ for lipid biosynthesis. The enzyme was purified and characterized to confirm its affinity towards L-malate and NADPH+. Effects of selective inhibitors were also correlated to enzyme activity and lipid production.

Experimental Methodologies

Materials and microorganism

Lignocellulosic hydrolysate (xylose rich) was generated in Biotechnology Conversion Area (CSIR-IIP) as per our earlier report [7]. Standards for lower carbon chain fatty acids (C6:0 to C14:0) palmitic acid (C16:0), palmitoleic acid (C16:1), stearic acid (C18:0), oleic acid (C18:1), linoleic acid (C18:2), linolenic acid (C18:3) and Sesamol, catechol and other inhibitors were procured from Sigma and were of analytical grade. All other chemicals and reagents were bought locally and were either of analytical or commercial grades.

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sugar solution. During maturation phase, cell biomass was harvested after 80% consumption of sugar to recover lipid and ME from *R. mucilaginosa* (henceforth designated as RMME).

Lipid rich cells were collected by gravity settling and oven dried (60°C). Lipid was gravimetrically extracted with chloroform (CHCl₃) and methanol (CH₃OH) in a 2:1 ratio as solvent system. Fatty acid compositions were profiled in multidimensional GC (Agilent, USA) after transesterification with BF₃ as described by Patel et al. with certain modifications [8].

To determine RMME activity, cell pellet was washed with deionized water and resuspended in lysis buffer (50 mM triethanolamine-HCl, pH 7.4, 1 mM EDTA, 20% w/v glycerol, 1 mM dithiothreitol) for disruption in high pressure homogenizer (Panda Plus 2000, Gea) with continuous recycling for 30 min. RMME activities were estimated as per Qiguo et al. with minor modifications using a U2900 UV-Vis spectrophotometer (Hitachi, Japan) for 5 min at 340 nm after 1 min delay time to nullify endogenous reduction of 0.3 mM NADP [9]. NADPH (extinction coefficient 6.22 × 10³ L/M/cm) generation was measured as variance in absorbance for a defined time span. Reaction mix contained 66.6 mM triethanolamine HCl buffer, 5 mM MnCl₂, 3.33 mM malate, and 0.33 mM NADP apart from enzyme solution. Each activity determination was made in triplicate sets with standard deviations not exceeding 5%. Enzyme activity was also determined by replacing NADP with NAD to confirm its cytosolic presence. One unit of ME activity was defined as the amount of enzyme required to catalyze the formation of 1µmol of NADPH⁺ per min under specified conditions. Total protein was determined by the method of Bradford with bovine serum albumin as standard [10].

**Effect of sesamol on malic enzyme and lipid production**

Sesamol, a nonoil component of sesame seed oil was reported to inhibit ME activity and affect lipid production. After cell biomass generation in fermenter, sesamol (0 mM, 5 mM and 10 mM) and catechol (10 mM) were separately added along with concentrated sugar solution (biomass prehydrolysate to enhance C/N ratio for lipid accumulation). Batches were terminated after 24 h and in between intracellular lipid contents and ME activity were measured in every 6 h interval as described.

**Purification of malic enzyme**

RMME was purified to homogeneity in two steps. Entire purification procedure was carried out at 4°C unless indicated otherwise. Crude enzyme extract was loaded in a MacroSep High S (Bio-Rad, USA) cation exchange column (20 × 1.85 cm) pre-equilibrated with 10 mM Tris-HCl buffer containing 2 mM β-mercaptoethanol and 10% glycerol (pH 7.4). The column was eluted (1 ml/min) with same buffer until absorbance (λ₂₈₀ nm) became zero. Each fraction of 2 ml was collected and those with high specific activity were pooled and lyophilized. Lyophilized powder was re-dissolved in minimum volume of same buffer solution and was passed through pre-equilibrated adenosine 2',5'-bisphosphate agarose (Sigma–Aldrich, USA) column (5.0 × 0.70 cm). Column was then washed until λ₂₈₀ nm of effluent reached zero. Bound proteins were eluted by NADP gradient (0–100 μM) in same
buffer. Fractions were collected at a flow rate of 20 mL/h and active fractions were pooled [11]. Purified protein migrated as single band in gel electrophoresis and was activity stained against 5% acetic acid after 2 h immersion into a solution of 55 mM triethanolamine-HCl buffer (pH 7.4), 0.47 mM NADP, 17.2 mM L-malate, 2.75 mM MnCl2, 0.55 mg/ml nitroblue tetrazolium and 0.097 mg/ml phenazinemethosulfate [11].

Enzyme characterization

$RM_{ME}$ was characterized to identify its kinetics parameters, role of divalent cations and effect of inhibitors to correlate its role in lipid accumulation. In vitro characterizations were performed under optimum pH and temperature.

Influence of pH on $RM_{ME}$ activity was determined within pH ranges of 4.0–9.0 by adjusting reaction mixture with 66.6 mM of various buffer solutions, viz. N-methyl piperazine (pH 4.0–5.5), piperazine (pH 6.0–6.5), and triethanolamine-HCl (pH 7.0–9.0). Enzyme activity was quantified as mentioned earlier. Thermostability of $RM_{ME}$ was investigated by pre-incubating enzyme solutions at 15–55°C for 90 min. After 15 min interval, residual activities were quantified as per protocol mentioned and compared with non-pre-incubated sample. Each enzyme activity was quantified in triplicates and further characterizations were performed under optimized pH and temperature.

Initial reaction rate for NADP reduction was calculated by estimating $RM_{ME}$ activity at different malate (0.25 to 5.0 mM) and NADP (0.025 to 0.5 mM) concentrations. Michaelis Menten constant ($K_v$) and rate of reaction ($V_{max}$) were determined by Lineweaver and Burk plot [12]. Effect of various metal ions was investigated by quantifying $RM_{ME}$ activity with 5 mM metal salts of Mn$^{2+}$, Ca$^{2+}$, Fe$^{3+}$, Co$^{2+}$, Cu$^{2+}$, Mg$^{2+}$, Zn$^{2+}$, and Ni$^{2+}$. Enzyme activity quantified in absence of metal salt was considered as 100% and residual activity was determined accordingly. To study the effect of inhibitors, $RM_{ME}$ was incubated for 15 min in 66.6 mM triethanolamine-HCl buffer (pH 7.4) with inhibitors (EDTA, idoacamide, tosylphenylalanyl chloromethyl ketone (TPCK), 4-chloromercuribenzoic acid, Phenyl Methane Sulfonyl Fluoride (PMSF), phenyglyoxal hydrate and peptatin A, sesamol and catechol at a final concentration of 1 mM. Residual enzyme activity was determined in same way as stated before and activity without any inhibitor was considered 100%.

Analytical techniques

Residual xylose in biomass hydrolysate during yeast growth and lipid maturation were quantitatively analyzed with HPLC (UFLC, Shimadzu, Japan) with PL Hiplex-H acid 8 μm column (100 × 7.7 mm diameter, by PL Polymer laboratory, UK) using a Refractive Index (RI) detector. Column was eluted with a mobile phase of 1 mM sulfuric acid at a flow rate of 0.7 mL/min and oven temperature was maintained at 70°C.

Total nitrogen was analysed in TN 3000 Total Nitrogen Analyzer (Thermo Fisher, USA) as per ASTM D 4629.

Dry cell mass was determined with known quantity of cellular broth (1 ml) by hot air drying of cell pellets in microfuges. Average of triplicate data was considered for dry cell mass determination.

Qualitative multi-dimensional gas chromatograph analyses (GC × GC) were performed for transesterified lipid with Agilent 7890B GC (California, United States) fitted with a thermal modulator assembly. FID and three different capillary columns (PAC Analytical, Canada) (1st dimension non-polar, 30 m × 250 μm × 0.25 μm; 2nd dimension mid-polar, 10 m × 320 μm × 0.25 μm and bleed column, 4.7 m × 100 μm × 0.25 μm) connected serially with thermal modulator. Oven temperature was programmed from 40°C (7 min holdup) to 270°C (20 min holdup) with different ramping rates. Helium was used as a carrier gas under constant flow (0.8 ml/min) mode. All samples were analyzed in splitless mode (100:1) at an injection temperature of 250°C. 0.4 μL injection of samples was performed with a 10 μL micro syringe.

Results and Discussion

Cytosolic ME convert L-malate into pyruvate with reduction of NADP$^+$ into NADPH$^+$, and generated NADPH$^+$ is used in lipogenesis along with PPP's NADPH$^+$ pool. In the following sections, we have discussed the confirmation of NADP$^+$ dependent cytosolic malic enzyme, present in RMIIPL32 and correlated its role in lipid production.

Effect of sesamol for production of lipid and malic enzyme

RMIIPL32 produced 8.3 g/L yeast biomass during growth, with Yx/S~0.415 and an overall lipid content of 0.30 g/L in 24 h. Increase in C/N ratio after growth phase, resulted in total non-polar lipid accumulation of 3 g/L within next 28 h with ~80% consumption of sugar. Lipid accumulation improved with increase in $RM_{ME}$ activity over the entire fermentation cycle. Lipid yield during maturation phase was 0.21 g/g of dry cell biomass (Figure 2) corresponding to maximum $RM_{ME}$ activity of 0.012 U/mg-1. Fatty acid profiles depicted presence of higher monounsaturated fatty acids (MUFA) namely palmitoleate and oleate along with highest content of Saturated Fatty Acids (SFA) namely palmitate and stearate and absence of Poly Unsaturated Fatty Acids (PUFA). Such composition would lead to a fuel with better cetane number, high Cold Filter Plugging Point (CFPP) and oxidative stability (Table 1) [13]. Sesamol affected cytosolic ME activity and inhibit cell growth and lipid biosynthesis in RMIIPL32. Sesamol was added at the onset of lipid maturation phase, along with concentrated sugar solution to determine its effect in $RM_{ME}$ activity and lipid yield under nitrogen depleted condition. Overall lipid content was reduced by 64.32%, with inclusion of the inhibitor even at low concentration (5 mM) with ~50% attenuation in $RM_{ME}$ activity. Sesamol was proposed to produce catechol like compound by undergoing the removal of its methylene carbon [14]. This imparted its inhibitory effect to ME and thereby decreased fatty acid accumulation through limited NADPH supply. Apart from drastic change in lipid quantity, fatty acid profiles were also affected. Absence of MUFA downgraded the fuel properties. Further increase in sesamol concentration completely abolished $RM_{ME}$ activity with minimum lipid production (1 to 2 % w/w cell lipid) that ensured cell survival. Increase in yeast biomass during the maturation phase was primarily attributed to cell biomass generation. Similar profile was depicted in case of catechol based lipid maturation, where negligible fatty acids were reflected in GC × GC. Inhibitions in lipid production were reported in R. toruloides, C. utilis and S. cerevisiae, when sesamol was used to determine ME activity as well as lipid production in bioreactor [15].

Characterization of $RM_{ME}$

$RM_{ME}$ was purified and characterized in detail. Approximately 23.25-fold purification of crude enzyme was achieved with ~13.48% recovery and specific activity of 0.279 U/mg proteins (Table 2). Purified enzyme migrated as a single band in gel-electrophoresis (Mr~200 kD), suggesting its homogeneity (Figure 3). Evans and Ratledge reported ME from Rhodosporidium toruloides CBS14 with a molecular size of


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The molecular weight is in accordance with literature reported purified ME from other microbial origins [17,18]. Song et al. identified 4 isoforms of ME and reported possible role of isoforms III and IV (~90 kD each) in lipid biosynthesis [19].

RMME showed optimal activity at pH 7.0 (Figure 4a) and 15°C temperature. The pH profile is similar to the other reported MEs from human breast cancer cell (7.2), and little lower than that of M. alpine (7.5) [17,20]. Between pH 6.0 and 8.0, RMME retained over 50% of its activity and beyond that a sharp loss in activity was noticed. This might be attributed to changes in protonation state of its active site residues which catalysed the oxidative decarboxylation. Activity of RMME was reduced with lowering of pH, whereas maximum enzyme activity related to lipid production in fermenter was observed at pH 4.5. Temperature optimum for RMIIPL32 was found to be 32°C for maximum cell biomass generation as well as lipid maturation (data not given). However, in vitro, RMME was found to be thermolabile. It was most active at 15°C upto 90 min, and at 25°C it remained stable upto 45 min. Elevation in temperature beyond 25°C resulted in marked performance decrease, with no oxidative activity at 55°C (Figure 4b). Temperature profiling under different incubation periods demonstrated a typical activity pattern of RMME. Protein half-life of 45, 15 and 5 min were observed with increased incubation temperature of 35, 45 and 55°C respectively.
Cofactor specificity and Kinetic determinations

RM<sub>me</sub> activity was observed with only NADP<sup>+</sup> as cofactor. A negligible change in absorbance (with L-malate as substrate) was detected when NADP<sup>+</sup> was replaced by NAD<sup>+</sup> (Figure 5) and confirmed the preference of NADP<sup>+</sup> over NAD<sup>+</sup> for oxidatively decarboxylation of L-malate. K<sub>m</sub> and V<sub>max</sub> values were determined to be 0.500 mM and 0.503 (IU/mg) respectively for L-malate (Figure 6) and 0.37 mM and 1.13 (IU/mg) respectively for NADP (Figure 6). K<sub>m</sub> for L-malate was observed to be in between that of M. circinelloides (0.4 mM) and R. toruloides (0.7 mM) [16]. RM<sub>me</sub> K<sub>m</sub> for NADP was comparable to that of Mortierella alpina (0.38 mM) and higher than that of Mucor circinelloides (0.32 mM) [18,19]. Low K<sub>m</sub> value of RM<sub>me</sub> for both L-malate (0.5 mM) and NADP (0.37 mM) confirmed high affinity for the substrate as well as coenzyme, leading to faster NADPH<sup>+</sup> generation for lipid biosynthesis.

Effects of metal ions

RM<sub>me</sub> activity was studied on different metal ions as shown in Table 3. Loss of over 50% activity was depicted with Zn<sup>2+</sup>, Cu<sup>2+</sup>, Fe<sup>3+</sup> and Ni<sup>2+</sup>, whereas almost complete activity were retained with Mg<sup>2+</sup> and Co<sup>2+</sup>. Mn<sup>2+</sup> and Ca<sup>2+</sup> enhanced enzyme activity which acted by stabilizing its quaternary structural integrity during catalysis [21]. Pry and Hsu have reported the order of events for pigeon liver ME wherein Mn<sup>2+</sup> binds first, followed by NADP and malate [22]. Metal ion presumably act by polarizing the carboxyl group of the oxaloacetate intermediates during decarboxylation, in agreement with the accepted theory of metal assisted decarboxylation of β-keto carboxylic acids [23]. Zn<sup>2+</sup> strongly suppressed~65% RM<sub>me</sub> activity similar to the inhibition reported in Escherichia coli [24]. Divalent Cu<sup>2+</sup> has been known to inhibit ME activity and RM<sub>me</sub> activity was decreased by more than 50%. Cu<sup>2+</sup> ions have been purposely, used in Aspergillus niger, where with addition of Cu<sup>2+</sup> at the early stage of fermentation to abolish ME activity, was an effective strategy to decrease total lipid [25].

Effects of inhibitors

Decrease in RM<sub>me</sub> activity in presence of various inhibitors suggested the possible presence of these amino acids in enzyme's active site. Inhibitors effects were investigated on activity of RM<sub>me</sub> to ascertain the string of functional amino acid residues in the catalytic site. Maximum inhibition was obtained with 4-chloromercuribenzoic acid (Figure 7).
acid (22.06%) which was a circumstantial evidence for the presence of cysteine in the enzyme's active site. The result was in accordance with for duck liver ME, where cysteine played important role in binding of L-malate and divalent metal ions. Pepstatin A (64.15%), PMSF (66.98%), iodoacetamide (71.29%), EDTA (89.11%), TPCK (85.85%) had inhibitory effect for RM_ME activity, while phenylglyoxal hydrate did not inhibit RM_ME [26]. PMSF acts as covalent modifier of serine in proteases. It was concluded that serine might be present in the RM_ME active site and could play a key role in binding of either NADP or the L-malate moiety. TPCK and Pepstatin A were selective for histidine and arginine/aspartate respectively [27,28]. Importance of thiol groups for RM_ME activity was confirmed by the inhibitory effect of iodoacetamide. In vitro RM_ME was not inhibited by sesamol; rather catechol resulted in ~90% activity inhibition. Sesamol might be assimilated by RMIIPL32 to yield catechol or similar compounds, which might impart activity inhibition by irreversible binding with RM_ME [14]. This also supported the inhibitory role of sesamol for RM_ME activity and lipid production (Table 4).

Conclusion

_Rhodotorula_ sp., being non model oleaginous yeast, lack of available genetic information prompted us to investigate the presence and role of cytosolic ME for lipid biosynthesis. Purification and characterization of RM_ME confirmed its cofactor specificity and high affinity for NADP+. Correspondingly, RM_ME activity inhibition by sesamol and catechol established its role in lipid biosynthesis by supplying NADPH+ in RMIIPL32. By this study, we conclude that supply of reductants for lipid biosynthesis could be sufficed by RM_ME apart from pentose phosphate pathway in RMIIPL32. Based on fatty acid profile, RMIIPL32 lipid can be transesterified to generate high quality biodiesel.

Table 3: Effects of metal ions on the activity of RM

<table>
<thead>
<tr>
<th>Metal ions</th>
<th>Specific activity</th>
<th>Relative activity</th>
</tr>
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<tbody>
<tr>
<td>Control</td>
<td>0.202</td>
<td>100.00</td>
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<tr>
<td>Mn2+</td>
<td>0.279</td>
<td>138.13</td>
</tr>
<tr>
<td>Mg2+</td>
<td>0.193</td>
<td>95.68</td>
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<tr>
<td>Ca2+</td>
<td>0.235</td>
<td>116.55</td>
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<td>Co2+</td>
<td>0.192</td>
<td>94.96</td>
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<tr>
<td>Fe2+</td>
<td>0.129</td>
<td>64.03</td>
</tr>
<tr>
<td>Zn2+</td>
<td>0.070</td>
<td>34.53</td>
</tr>
<tr>
<td>Cu2+</td>
<td>0.087</td>
<td>43.17</td>
</tr>
<tr>
<td>Ni2+</td>
<td>0.067</td>
<td>33.09</td>
</tr>
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</table>

Table 4: Effects of inhibitors on the activity of RM

<table>
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<tr>
<th>Inhibitors</th>
<th>Specific activity</th>
<th>Relative activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.279</td>
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</tr>
<tr>
<td>Phenyl glyoxal hydrate</td>
<td>0.279</td>
<td>100.00</td>
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<td>EDTA</td>
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<td>Iodoacetamide</td>
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<td>TPCK</td>
<td>0.240</td>
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<td>PMSF</td>
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<tr>
<td>Pepstatin A</td>
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<td>64.15</td>
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<td>4-chloromercuribenzoic acid</td>
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<td>Sesamol</td>
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<td>96.05</td>
</tr>
<tr>
<td>Catechol</td>
<td>0.044</td>
<td>15.81</td>
</tr>
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</table>

Authors’ Contributions

SB, TS, DA, DG and DDG designed overall research plan, study oversight and conducted hands-on experiments with data collection along with drafting of the manuscript; SB and DG had primary responsibility of data representation and final shaping of the manuscript; SB carried Experimental work with TS and DA, DDG and DKA supervised overall work and participated in result interpretation with SB, TS and DDG; DDG is also the corresponding author. All authors read and approved the final manuscript.

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