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Characterization of Lipase from Nyamplung (*Calophyllum inophyllum* L.) Seeds

Lalu Rudyat Telly Savalas[a*)], Mukhtar Haris[a], Eka Junaidi[a], Saprizal Hadisaputra[a], Yunita Arian Sani Anwar[a], and Lalu Anugrah Dimas Juniarly[a]

[a)] *Department of Chemistry Education, Faculty of Teacher Training and Education, University of Mataram, Jl. Majapahit No. 62 Mataram, 83125, Indonesia*
[*)] *Corresponding Author: telly@unram.ac.id*

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ABSTRACT

Lipases are biocatalysts widely used in industrial and biotechnological processes for their ability to catalyze ester bond hydrolysis and synthesis. The search for novel sources of lipase from plant materials, particularly for sustainable and eco-friendly enzyme production, continues to attract attention. This study aimed to isolate and characterize lipase from nyamplung (*Calophyllum inophyllum* L.) seeds. The enzyme was extracted from germinated seeds and subjected to activity assays under varying temperatures, pH, and germination length conditions. The lipase activity assay was based on the titrimetric determination of free fatty acid released after hydrolysis. The highest lipase activity of the crude extract against the VCO substrate was observed at 35 °C and pH 7.5, with an activity of 0.92 U/mL. The maximum activity was observed after 15 days of imbibition, reaching 1.06 U/mL. Protein characterization through SDS-PAGE revealed multiple bands with molecular weights of ~28, ~21, and ~19 kDa, indicating the presence of multiple protein bands associated with lipase activity. These results suggest that Nyamplung seed lipase exhibits suitable catalytic properties for biotechnological applications. The moderate temperature and neutral pH of the enzyme make it a potential candidate for use in food and oleochemical industries.

Keywords: biocatalyst, *Calophyllum inophyllum*, enzyme characterization, lipase, SDS-PAGE

1. Introduction

Lipases (EC 3.1.1.3) constitute one of the most versatile classes of hydrolases, catalyzing the hydrolysis and synthesis of ester bonds in triglycerides. Their dual functionality in aqueous and nonaqueous systems makes them indispensable in industries ranging from food and pharmaceuticals to oleochemicals, detergents, and biodiesel production [1], [2]. Over the past decade, the global drive toward sustainable catalysis has re-emphasized the importance of biocatalysts that operate under mild conditions while maintaining high selectivity and turnover. Recent advances in enzyme technology—particularly immobilized, nano-supported, and solvent-tolerant lipases—have demonstrated their potential to replace conventional chemical catalysts in transesterification and esterification reactions [3], [4], [5]. This green-chemistry transition aligns with international goals for carbon-neutral industrial processes and circular bioeconomy models [6].

Although microbial lipases dominate the commercial market, plant-derived lipases have regained interest owing to their intrinsic safety, low immunogenicity, environmental compatibility, substrate specificity, operational stability under mild conditions [7] , and low downstream purification costs [8]. Germinating oil seeds are abundant natural sources of lipases that mobilize stored lipids during early development. From 2020 onward, studies have reported novel lipase isoforms from *Jatropha curcas*, *Aleurites moluccanus*, and *Ricinus communis*, revealing diverse catalytic efficiencies and stability profiles [9], [10], [11], [12]. These enzymes often exhibit moderate optimal temperatures (30 °C – 40 °C) and neutral pH values, which are advantageous for eco-industrial applications. Furthermore, omics-based analyses have identified gene clusters encoding seed

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lipases with unique lid-domain conformations that influence interfacial activation [13], [14]. These findings underscore the biochemical and evolutionary diversity of plant lipases as untapped reservoirs of biocatalysts.

Among tropical oil-bearing plants, *Calophyllum inophyllum* L. (Nyamplung) stands out for its high-yielding, nonedible seed oil, rich in long-chain fatty acids such as oleic, linoleic, and calophyllic acids. Nyamplung oil has been traditionally used in folk medicine and, more recently, for biodiesel production [15]. However, research on its endogenous enzymatic systems remains limited. A few reports have identified hydrolytic enzymes within Nyamplung seeds that facilitate lipid mobilization during germination [16]. Despite the increasing interest in *Calophyllum inophyllum* as a biodiesel feedstock, most studies have focused on its oil composition and conversion processes, while the enzymatic systems involved in lipid mobilization remain poorly understood. Information on the catalytic properties of lipases in Nyamplung seeds remains limited. Characterization of this enzyme is important because plant seed lipases can serve as sustainable biocatalysts for oleochemical transformations and other green bioprocesses. Therefore, the systematic characterization of Nyamplung lipase is necessary to evaluate its catalytic potential and support future enzyme-based applications.

Lipase activity is sensitive to multiple environmental factors, such as temperature, pH, ionic strength, and the physiological state of the enzyme source and preparation [17], [18], [19]. Temperature governs enzyme kinetics by affecting molecular collision frequency and protein conformational flexibility; however, excessive heat leads to irreversible denaturation. Similarly, pH influences the ionization of active-site residues, thereby determining substrate affinity at the binding pocket [20]. Since 2020, studies have reported improved thermostability, pH tolerance, and sustainable use through protein engineering and immobilization on biopolymers such as chitosan and cellulose nanofibers [21], [22]. Further engineering by modifying the disulfide bond might also be a valuable approach to improving thermostability [23]. These developments reinforce the relevance of using natural lipases with inherent stability as industrial optimization templates under moderate conditions.

Therefore, this study aimed to isolate and characterize the lipase enzyme from *C. inophyllum* L. seeds, focusing on its physicochemical and catalytic properties. Specific objectives include determining the optimum temperature, pH, and germination stage influencing enzyme activity, followed by molecular weight assessment via SDS-PAGE. By elucidating these properties, this study seeks to expand the current knowledge of plant-derived lipases and establish a foundation for their application in green bioprocesses. The work also aligns with recent trends emphasizing bio-resource valorization, enzyme-based catalysis, and sustainable industrial biotechnology [24], [25].

2. Methods

Nyamplung kernels from mature seeds (3-4 months old) were collected from Nyamplung trees in Lombok, Indonesia. The materials and equipment used in this study are described in the following subsections. Except otherwise stated, all materials are p.a. or molecular biology grade.

2.1. Equipment/Tool/Material

All chemicals used were of analytical grade. The buffer components for crude extract preparation included NaOH, Na_2HPO_4, and NaH_2PO_4 (Merck, Darmstadt, Germany). Lombok VCO lipase substrate was obtained from a local vendor. The endpoint indicator for titration was phenolphthalein (Sigma-Aldrich, Missouri, USA). Ingredients for Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) were acrylamide, bis-acrylamide, sodium dodecyl sulfate, 10% ammonium persulfate, Dithiothreitol (DTT), glycine (all from Bio Basic, Toronto Canada), and N,N-dimethylformamide, N,N,N,N-Tetramethyl ethylenediamine (TEMED), glycerol, β-mercaptoethanol, bromophenol blue, Coomassie brilliant blue (Sigma-Aldrich, Missouri USA). The broad-range protein marker was obtained from Himedia. SDS-PAGE units were obtained from Wix (China). Cold centrifugation was performed using an Allegra(R) X-30R Benchtop (Beckman Coulter, USA). The data for the graph were processed using Prism 7.0 (GraphPad Software Inc., California, USA).

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2.2. Experiment

2.2.1. Enzyme Extraction and Isolation

Lipase was isolated from germinated Nyamplung seeds following a cold phosphate buffer extraction (0.1 M, pH 7.0). The nyamplung flesh (Figure 1B) was collected, crushed using a juice maker, and homogenized in phosphate buffer. The homogenate was centrifuged at 10,000 rpm for 30 min at 4 °C. The upper creamy layer was carefully removed from the 50 mL conical tube. The clear phase in the middle of the centrifuge tube containing crude extract (Nyamplung lipase) was collected and stored in -20 ºC before analysis.

2.2.2. SDS-PAGE

Protein profiling was performed using standard SDS-PAGE (12%) according to the classic Laemmli method as described previously in our laboratory [26]. Briefly, a 20 µg protein sample was loaded into an individual well of a 4.5% stacking gel. The samples were focused on the gel border at 110 V. Separation was performed in the resolving gel at 150 V until the front dye reached the bottom of the gel. The gel was washed and stained with Coomassie Brilliant Blue dye upon completion. The staining contrast was achieved after repeated washing with the destaining solution.

2.2.3. Effect of Temperature and pH

The optimum temperature and pH were evaluated by performing VCO digestion activity assays using Nyamplung crude enzyme at different temperatures and pH values. Lipase activity was measured using the standard assay described in Section 2.2.5. Preliminary screening of the effect of temperature on Nyamplung lipase activity was first conducted at 25 °C, 30 °C, 35 °C, 40 °C, and 45 °C. The preliminary experiment showed that the highest activity was obtained at 35 °C. Therefore, a second experiment was conducted to more precisely determine the optimum temperature by examining a narrower range around this value, namely 32 °C, 34 °C, 35 °C, 36 °C, 37 °C, and 40 °C. The temperature that produced the highest activity in the second experiment was considered the optimum temperature. Preliminary screening of the effect of pH on Nyamplung lipase activity was initially performed using 100 mM phosphate buffer at pH 5.0, 6.0, 7.0, 8.0, and 9.0. The preliminary experiment indicated that the optimum activity occurred within the neutral-to-slightly alkaline range, particularly around pH 7.0–8.0. Therefore, a second experiment was performed using a narrower pH interval consisting of pH 5.0, 6.0, 7.0, 7.5, 8.0, 8.5, and 9.0 in order to determine the optimum pH more precisely. Lipase activity was measured at the optimum temperature obtained from the temperature experiment (35 °C). The pH value giving the highest activity in the second experiment was considered the optimum pH.

2.2.4. Effect of Imbibition Time

Nyamplung seeds were germinated in humid media (wet cotton). During this imbibition, the kenels were kept humid by daily watering. Because Nyamplung seeds germinated asynchronously, samples were grouped according to sprout height rather than fixed daily intervals. Seeds with sprout heights of 0, 2, 4, 6, 8, 10, 12, and 14 cm were collected. These stages corresponded approximately to 0, 9, 11, 13, 15, 18, 20, and 23 days after imbibition (DAI), respectively. Additional samples without visible sprout emergence were collected at 5 and 7 DAI. At 7 DAI, the seed coat had started to crack, indicating the onset of germination, although no visible sprout had yet emerged. Lipase activity of each sample group was determined according to the procedure in 2.2.5.

Table 1. Sampling stages used during Nyamplung seed germination

Days after imbibition	Sprout height (cm)	Germination stage
0	0	Fresh mature seeds
5	0	Imbibed seed, no visible sprout
7	0	Seed kernel crack, no visible sprout
9	2	Early sprout emergence
11	4	Early germination
13	6	Intermediate germination
15	8	Advanced germination
18	10	Late germination
20	12	Late germination
23	14	Advanced seedling stage

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2.2.5. Lipase Activity Assay

Lipase activity of crude enzyme was measured using a modified method that we previously used for coconut lipase [26]. Briefly, 2.5 mL n-hexane, 5 mL of 100 mM phosphate buffer pH 7.5, and 1 mL of crude enzyme were incubated for 45 minutes at 37 °C in a water bath shaker. Following incubation, 25 mL of acetone-ethanol (1:1, v/v) and a few drops of the phenolphthalein indicator were added to the mixture. After that, 0.01 M NaOH was used to titrate the mixture. In the blank treatment, no lipase was added. The lipase activity was calculated according to Eq. 1.

$$\text{Lipase activity} = \frac{(\text{Vsample} - \text{Vblank})\text{x}[\text{NaOH}]\text{x}1000}{Venzyme\ x\ t} \quad \text{U/mL}$$

where:
V_{sample} : titrant volume for sample
V_{blank} : titrant volume for blank
V_{enzyme} : Nyamplung lipase volume
[NaOH] : Sodium hydroxide concentration
t : time (in minutes)

All experiments were conducted in triplicate (n = 3), and results are expressed as mean ± standard deviation.

3. Results and Discussion

Nyamplung seeds were processed by removing the outer shell to obtain the kernel, which serves as the primary source of lipase. The kernel was further subjected to extraction followed by high-speed centrifugation to achieve phase separation of the crude extract. This fractionation step is critical for isolating enzyme-rich fractions based on their physicochemical properties. The resulting layers indicate differential distribution of biomolecules, with the middle fraction hypothesized to contain lipase. The morphology of the seed, kernel, and the fractionation outcome are presented in Figure 1.

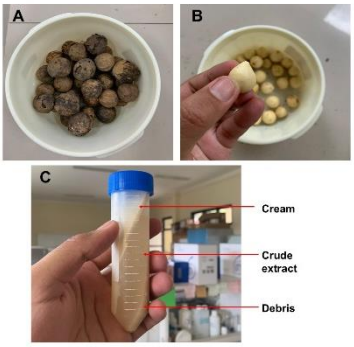

Figure 1. Ripe Nyamplung seed (A) and kernel flesh (B). Fraction of Nyamplung seed after high-speed centrifugation. Crude extract in the middle contains lipase (C)

3.1. Extraction and Isolation

Extraction of Nyamplung seed extract was accomplished by grinding the mature seed in phosphate buffer using a juice maker. The resulting suspension was centrifuged at high speed. The suspension was separated into three phases. The creamy phase floating on the top of the conical centrifuge tube contains lipid material. The aqueous phase in the middle contains crude lipase extract. Debris of large solid objects was accumulated at the bottom of the tube (Figure 1C).

The crude extract was further subjected to SDS-PAGE analysis, lipase characterization, and activity assay.

3.2. SDS-PAGE

Electrophoretic profiles revealed several bands (~28, ~21, and ~19 kDa) under SDS-PAGE (Figure 2). Several bands were detected that may be related to lipolytic activity, but further confirmation is needed. Similar molecular weight distributions (20–40 kDa) have been reported for other plant lipases, such as *Brassica napus* and *Avena sativa* [27]. This aligns with our previous finding that multiple isoforms in plant seed lipases correlate with varied substrate specificity [26], [28]. The presence of low-molecular-weight fractions (~19 kDa) may correspond to catalytic subunits or degradation products, reflecting active participation in early lipid metabolism.

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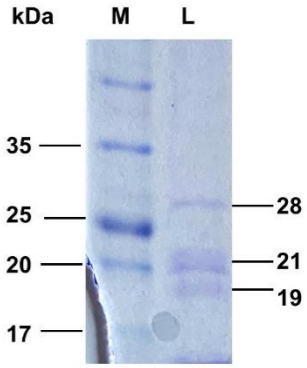

Figure 2. SDS PAGE of Nyamplung crude extract shows several bands from which lipolytic activity is foreseen. M: Himedia broadband prestained protein ladder. L: lipase extract from Nyamplung seed flesh

3.3. Effect of Temperature and pH on Lipase Activity

3.3.1. Effect of temperature

Lipase activity of crude enzyme from *C. inophyllum* seeds was assayed against VCO as substrate. VCO oil was chosen as it has a comprehensive composition of fatty acid esters from various chain lengths, in comparison, for example, with olive oil [26]. The activity increased gradually with temperature, reaching a maximum of 0.92±0.11 U/mL at 35 °C, then declined sharply to 0.41±0.11 U/mL at 40 °C (Figure 3). This optimum indicates a mesophilic enzyme profile similar to lipase from rape seed, Barbados nut, and wheat seed, showing optima around 35–37 °C [2]. Lipases belong to the α-β hydrolase superfamily whose catalytic triad (Ser–His–Asp) is surrounded by the α-helix. Upon elevated temperature, the intake structure is partially denatured, reducing substrate binding efficiency [29]. A similar thermal behavior in plant esterases from oleaginous seeds was also evident [28], [26], reinforcing that structural stability in lipases is affected by the hydrogen bonding around the active site [30]. Thus, the *C. inophyllum* lipase is suitable for biocatalysis under mild conditions, conserving energy and preserving labile substrates.

The observed optimum at 35 °C suggests that Nyamplung lipase is adapted to mesophilic conditions typical of tropical plant metabolism. During seed germination, enzymatic processes involved in lipid mobilization occur at moderate temperatures compatible with cellular stability. The rapid decrease in activity above 35 °C likely reflects thermal destabilization of the catalytic triad environment and disruption of hydrogen bonding networks that maintain the active conformation of the enzyme. Such thermal sensitivity is typical of plant lipases that function primarily in intracellular metabolic pathways rather than in extreme environmental conditions.

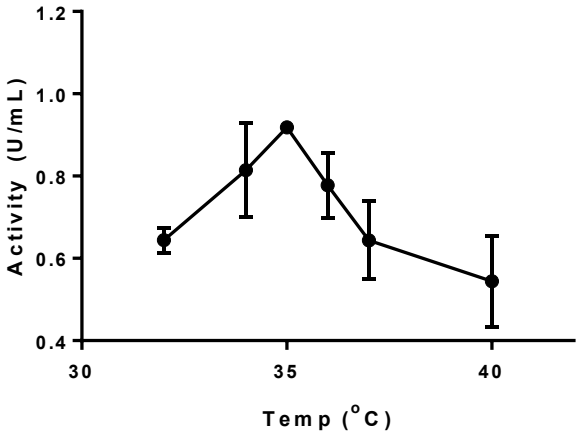

Figure 3. Effect of temperature on the Nyamplung seed lipase activity. Preliminary screening at 25–45 °C indicated that the highest activity occurred at 35 °C; therefore, a more detailed analysis was conducted at 32, 34, 35, 36, 37, and 40 °C. Optimum activity is reached at 35 °C with an activity of 0.92±0.013 U/mL. Data was obtained from triplicate

3.3.2. Effect of pH

The enzyme exhibited maximal activity at pH 7.5, with 0.92±0.01 U/mL, while activity declined at both acidic and alkaline extremes (Figure 4). Deviations from neutrality likely caused protonation or deprotonation of amino acid residues near the active site, disrupting enzyme–substrate interactions [31]. This trend mirrors reports on *A moluccana* and *Hevea brasiliensis* seed lipases, whose neutral pH optima reflect cytosolic enzyme environments [10], [27]. We have previously reported a similar pH profile in esterase systems from coconut endosperm, noting pH-induced conformational rearrangements affecting catalytic stability [26]. Consequently, *C. inophyllum* lipase is classified as a neutral lipase, aligning with applications in food and detergent formulations that operate near physiological pH.

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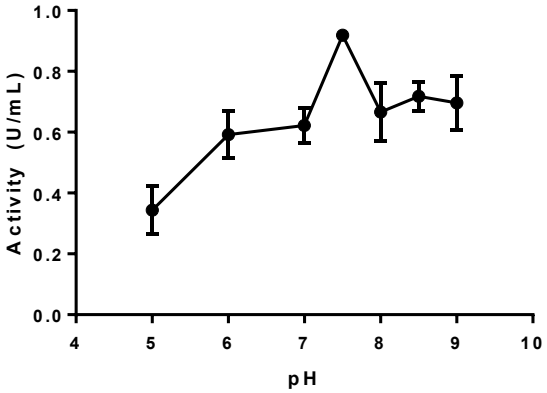

Figure 4. Effect of pH on the activity of Nyamplung lipase. Preliminary screening at pH 5.0–9.0 indicated that the optimum activity occurred between pH 7.0 and 8.0; therefore, additional measurements at pH 7.5 and 8.5 were performed to more precisely determine the optimum pH. Only the second-stage data are shown. Data was obtained from triplicate.

The neutral pH optimum observed for Nyamplung lipase is consistent with the intracellular environment of germinating seeds, where cytosolic enzymes typically operate near neutral conditions. Changes in pH influence the ionization state of amino acid residues forming the catalytic triad and the substrate-binding pocket. Deviation from the optimal pH may alter the charge distribution within the active site, thereby reducing catalytic efficiency.

3.4. Effect of Imbibition Time on Lipase Activity

Table 1 summarizes the relationship between sprout height and DAI used in this study. Lipase activity increased during imbibition even before visible sprout emergence. Seeds at 5 and 7 DAI still had 0 cm sprout height, but exhibited higher activity than fresh seeds, indicating that water uptake during imbibition had already initiated lipid mobilization.

Visible sprout emergence began at approximately 9 DAI (2 cm sprout height), and lipase activity continued to increase with sprout height, reaching a maximum at 15 DAI when the sprout height was approximately 8 cm with crude lipase activity of 1.06 ±0.09 U/mL before declining (Figure 5). During germination, triacylglycerols are hydrolyzed to provide carbon and energy for embryonic growth through the rapid conversion of lipid storage into fatty acids and glycerol that enter β-oxidation and glyoxylate pathway. The decline beyond 15 days of imbibition corresponds to depletion of lipid reserves. This pattern is consistent with physiological up-regulation, peaking, and followed by down-regulation of lipid metabolism observed, for example, in coffee beans [32] and in rice bran [33]. Comparable kinetics were observed in castor bean and sunflower seeds, where peak activity occurred mid-germination [8]. The high catalytic turnover during early development underscores lipase's role in mobilizing stored oils—a finding that agrees with other reports [33].

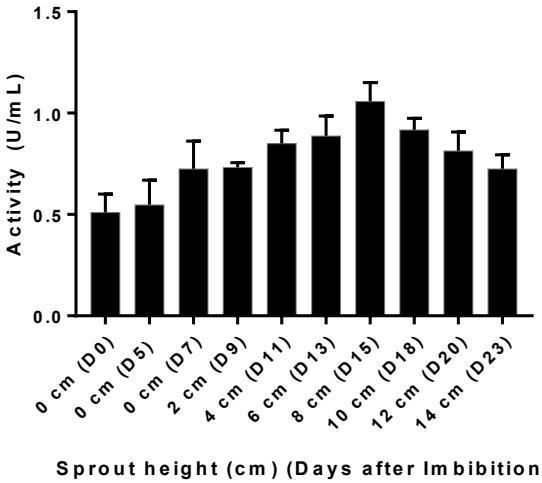

Figure 5. Effect of imbibition time on Nyamplung seed lipase activity. Samples were grouped according to sprout height, while the corresponding days after imbibition (DAI) are indicated on the x-axis. Lipase activity increased during imbibition before visible sprout emergence and reached a maximum at approximately 8 cm sprout height (15 DAI) with lipase activity of 1.06 ±0.09 U/mL. Data was obtained from triplicate.

3.5. Lipase Activity

Calculation of the lipase of Nyamplung crude enzyme activity was performed at optimized temperature and pH. The result shows that the Nyamplung crude lipase has an activity of 0.92±0.01 U/mL. Additionally, an optimized imbibition period results in an activity of 1.06±0.09 U/mL. In the setting of crude enzyme, the relatively activity is a common finding for seed lipase, for example, those form *Jatropha curcas* have an activity of 0.8-1.3 U/mL [9] and those from coconut flesh have an activity of 0.9-1.5 U/mL [26]. Moreover, the lipase activity from seeds may also vary depending on the substrate used.

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For example, an artificial substrate such as *para*-Nitrophenyl palmitate will give a higher value for lipase from *Ricinus communis* (2-5 U/mL) [12]. A purified enzyme with standard substrate will provide a distinct evaluation of lipase activity.

Taken together, the physicochemical characteristics observed in this study indicate that Nyamplung seed lipase behaves as a typical mesophilic plant lipase adapted to intracellular lipid mobilization during germination, while still possessing catalytic properties that may be useful for moderate-temperature biocatalytic applications.

4. Conclusion

Lipase enzyme from Nyamplung seeds exhibits optimum activity at 35 °C and pH 7.5, with the highest activity observed after 15 days of imbibition. SDS-PAGE analyses revealed isoenzymes in the 19–28 kDa range. The enzyme's characteristics make it a promising biocatalyst for moderate-temperature industrial processes.

CRediT authorship contribution statement

LRTS: Conceptualization, Methodology, Writing – Original Draft. MH: Funding acquisition, Review & Editing. EJ: Funding acquisition, Review & Editing. SH: Project administration, Review & Editing. YASA: Methodology, Validation, Resources. LADJ: Data Curation, Visualization.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available upon reasonable request.

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