



Microencapsulation of reuterin to enhance long-term efficacy against food-borne pathogen *Listeria monocytogenes*

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Received: 21 April 2017 / Accepted: 4 December 2017 / Published online: 18 December 2017
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Abstract

The aim of this study was to microencapsulate the reuterin produced by *Lactobacillus reuteri* BPL-36 strain for its long-term efficacy against food-borne pathogen *Listeria monocytogenes*. *Lactobacillus reuteri* BPL-36 strain previously isolated from a human infant fecal sample in lab was selected for the present study based on its ability to produce reuterin. The organism displayed a broad-spectrum antimicrobial activity. Reuterin concentration of 89.63 mM was obtained in the MRS–glycerol medium after 16 h incubation at 37 °C. The reuterin concentration required to inhibit the growth of *Pseudomonas aeruginosa*, *Escherichia coli* O157: H7, *Salmonella typhi*, *Staphylococcus aureus*, and *Listeria monocytogenes* was found to be 1.0, 2.0, 2.0, 4.0, and 10.0 AU/mL, respectively. Microencapsulation of reuterin to enhance long-term efficacy against food-borne pathogens was done. Results in this study indicated that the release characteristics of reuterin from the encapsulated particles were pH dependent. The release characteristics were unaffected by the storage of encapsulated reuterin at 4 °C for 2 weeks. The anti-listerial efficacy of the encapsulated reuterin was tested against *L. monocytogenes* in the BHI medium adjusted to pH 5.0 with a reuterin content equivalent to 16 mM, similar to un-encapsulated (free) reuterin. Encapsulated reuterin demonstrated enhanced efficacy against *L. monocytogenes* for longer duration of time when compared with un-encapsulated (free) reuterin. The present work demonstrated a novel antimicrobial delivery system that ensured much better capability of inhibiting the growth of *L. monocytogenes* throughout 24 h incubation at 37 °C.

Keywords *Lactobacillus reuteri* · Reuterin · Microencapsulation · Pathogens · Safety

Introduction

Globally, contaminated food and drinking water cause deaths of one million people annually (Maurice 1994). Although tremendous efforts have been made by governments and food industry to reduce food-borne illnesses, there are sporadic outbreaks due to post-production contamination by food-borne pathogens (Wirjantoro et al. 2001; Cumming et al. 2008). Use of antimicrobial food preservatives in many food products has been followed to prevent post-processing contamination, extend shelf life, and maintain food quality

while saving energy costs during production and transportation (Sofos et al. 1998).

Reuterin is a small antimicrobial compound that is produced as an intermediate metabolite during anaerobic fermentation of glycerol (Talarico et al. 1988). It consists of an equilibrium mixture of monomeric, hydrated monomeric, and cyclic dimeric forms of β -hydroxypropionaldehyde (Talarico and Dobrogosz 1989). Reuterin, produced by some strains of *Lactobacillus reuteri*, has shown antimicrobial activity against a range of food-borne pathogens and spoilage organisms, including Gram-positive and Gram-negative bacteria, yeasts, moulds, and protozoa (Axelsson et al. 1989; Mishra et al. 2012; Ortiz-Rivera et al. 2017). The inhibitory effect of reuterin is related to its action on DNA synthesis (Talarico and Dobrogosz 1989). Bioprotective effect of reuterin alone and in combination with nisin against various food-borne pathogens has been investigated in milk (Mishra et al. 2011).

Antimicrobials often have reduced efficacy in foods as compared to liquid solutions or microbiological growth

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media. Reactions between antimicrobial molecules and food components such as proteins, lipids, enzymes, ions, and surfactants may reduce antimicrobial effectiveness (Thomas 2005). A carrier material is used to encapsulate drugs in a designed structure, so that the drug is gradually released from the carrier–drug composite matrix by mass transfer, or released abruptly due to collapse of a carrier matrix triggered by environmental changes (Juliano 1978). Therefore, many researchers have developed novel delivery systems for antimicrobials to obtain sustained release so as to minimize interference of food components. These delivery systems include particulates (Salmaso et al. 2004; Jin et al. 2009; Narsaiah et al. 2014) and films (Lungu and Johnson 2005; Cao-Hoang et al. 2010; Nostro et al. 2010). Particulates can be used in food matrices, while films can be used to wrap solid foods to inhibit the growth of microorganisms on surfaces. Several studies have found that delivery systems can improve the efficiency of food antimicrobials by protecting antimicrobials from adverse environmental or processing effects and interactions with food components. Nisin encapsulated in liposomes demonstrated a 2-log greater inhibition of *Listeria monocytogenes* than free nisin (Were et al. 2004).

The first objective of this study was to microencapsulate the reuterin produced by *L. reuteri* BPL-36 strain for determination of its release pattern at different pH levels. The second objective was to compare its long-term efficacy with un-encapsulated (free) reuterin against food-borne pathogen *L. monocytogenes*.

Materials and methods

Bacterial strains and culture conditions

Lactobacillus reuteri BPL-36 strain was previously isolated by our team from human infant fecal sample (Mishra et al. 2012), and was maintained and propagated in MRS broth (Hi-Media labs, Mumbai, India). *Escherichia coli* K12 (NCDC 135) was used as the indicator organism for determination of minimum inhibitory concentration (MIC). *Listeria monocytogenes* (ATCC 15303) and *Staphylococcus aureus* (NCDC 237), *Pseudomonas aeruginosa* (NCDC 105), and *Salmonella typhi* (NCDC 113), procured from National Collection of Dairy Cultures (NCDC), ICAR-NDRI, Karnal, India, were used as test organisms. *Escherichia coli* O157:H7 (ATCC 43888) was procured from American-Type Culture Collection (ATCC), USA. All the pathogenic strains were handled in class II type A2 biological safety cabinet and grown in Brain Heart Infusion broth (BHI; Hi-Media labs, Mumbai, India) at 37 °C for 18 h. Bacterial strains were maintained as stock cultures at – 80 °C in BHI broth supplemented with 15% glycerol. The organisms were propagated twice before use in the experiments. The purity of all the

bacterial cultures was always ascertained by Gram staining prior to use for any experiment.

Production of reuterin

Over-night grown cultures of *L. reuteri* BPL-36 (10^{10} CFU/mL) were inoculated in MRS broth tubes supplemented with glycerol (360 mM) at 37 °C for 24 h in anaerobic conditions. After each 4 h interval, the cells from single tube were removed by centrifugation, cell-free supernatant was filter sterilized, and the presence of reuterin in it was detected by the colorimetric method of Circle et al. (1945). This method was based on the colorimetric determination of the acrolein formed from the 3-HPA produced from glycerol by the glycerol dehydratase. The concentration of reuterin in the BPL-36 cell-free supernatant was determined by comparing the spectral extinction values from the assay to those of a standard curve of acrolein (2-propenal).

Reuterin activity units

Reuterin activity was quantified by the MIC method using microtiter plates as described by Chung et al. (1989). From the over-night grown culture of *E. coli* K-12 (indicator organism), cells were harvested, washed twice with phosphate buffer (pH 7.2, 50 mM), suspended in the same buffer, and diluted to A_{420} of 0.2 measured using spectrophotometer. This suspension was diluted 1/10, which corresponds to about 1×10^6 CFU/mL. The diluted suspension (0.1 mL) was used to inoculate 0.2 mL of serial dilutions of reuterin diluted in Muller-Hinton medium (Himedia, India Laboratories). The microplate was incubated at 37 °C and growth was examined after 48 h. Reuterin concentration was defined as the reciprocal of the highest dilution that did not permit visible growth of the indicator strain and expressed as Arbitrary Units (AU) with 1 AU of reuterin being defined as the reciprocal of the highest dilution that did not permit visible growth of the indicator strain (El-Ziney et al. 1998).

Determination of minimum inhibitory concentration of reuterin to inhibit other food-borne test organisms

Listeria monocytogenes and *E. coli* O157:H7 were grown in Brain Heart Infusion broth (BHI) at 37 °C for 20 h. *Salmonella typhi*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa* were grown at 37 °C for 20 h in Nutrient broth (Merck, Germany). Inocula of the cultures were then prepared (1×10^6 CFU/mL) as mentioned above and used (0.1 mL) to inoculate aliquots of the diluted reuterin (0.2 mL) in 96 wells microplates. Quantified reuterin was serially diluted in Muller-Hinton medium to obtain dilutions with 0.0, 2.0, 4.0, 6.0, 8.0, 10.0, and 12.0 AU/mL. The

microplates were incubated at 37 °C and visible growth was checked after 48 h.

Encapsulation of reuterin

Reuterin was encapsulated (20:80 v/v) by mixing with 2% (w/v) sodium alginate and 0.4% (w/v) guar gum. Concentric two fluid glass nozzle with air atomization method (developed at CIPHET, Ludhiana, Punjab) was used for preparation of calcium alginate microcapsules incorporated with guar gum. Sodium alginate and guar-gum solution is prepared by dissolving polymers in distilled water using magnetic stirrer (Labco Instruments, New Delhi, India). The aqueous solution of sodium alginate–guar gum was infused from a peristaltic pump (Ravel Hiteks Pvt. Ltd., Chennai, India) at controlled flow rate into an air atomizing nozzle and sprayed under pressurized air into a reaction vessel containing 3 L of 0.2 M calcium chloride solution with continuous magnetic stirring at 1000 rpm for 30 min. The distance between the nozzle tip and the liquid level in reaction vessel was fixed at 12 in. The size of the orifice of inner nozzle (carrying guar gum and sodium alginate solution/matrix fluid) was 1 mm. It has 1.3 mm annular space (carrying pressurized air) between inner and outer nozzle. The pressurized air was used to break up the jet of matrix fluid. The kinetic energy of high-pressure air or inert air is used for break-up of matrix fluid jet. The small droplets produced fall in reaction vessel. The reaction vessel contains that the divalent calcium ions cross-link with sodium alginate on contact to form calcium alginate–guar-gum microcapsules by ionotropic gelification yielding microcapsules. The prepared microcapsules was sieved through buchner funnel and washed twice with sterile distill water and selected for further studies.

Evaluation of encapsulation properties

Various encapsulation properties given below were further studied:

Encapsulation efficiency

Reuterin content of supernatant was estimated. Reuterin content of polymer solution of alginate + guar gum before spraying was also estimated. Encapsulation efficiency was calculated using following formula:

$$\text{Encapsulation efficiency (\%)} = \frac{\text{Total reuterin concentration in the capsule}}{\text{Total reuterin concentration in polymer solution}} \times 100.$$

Mass yield

Mass yield was calculated using following formula:

$$\text{Mass yield (\%)} = \frac{\text{Non-solvent mass in the capsule}}{\text{Mass of collected capsule}} \times 100.$$

pH-dependent release characteristics of encapsulated reuterin

Release characteristics of encapsulated reuterin were checked by the method described by Oh et al. (2006) with slight modification. One gram of encapsulated reuterin was suspended in 100 mL of 50 mM sodium citrate buffer at pH levels ranging from 2 to 7. Changes in reuterin concentration after every hour of incubation at 37 °C were evaluated by the method prescribed by Circle et al. (1945). Similarly, changes in reuterin concentration up to 15 days of storage at 4 °C were evaluated.

Anti-listerial assay

Selected food-borne pathogen *L. monocytogenes* was incubated at 37 °C with two consecutive transfer to 9.31 logs CFU/mL. BPL-36 cell-free supernatant and encapsulated reuterin were added to 9 mL BHI broth (supplemented with 0.6% yeast extract) medium pre-adjusted to pH 6.0. Medium was then mixed with 1 mL different test organisms (10⁶ CFU/mL) by vortexing. Overall reuterin concentration was used at 16 mM in all treatments. After incubation at 37 °C for 0, 1, 2, 4, 6, 8, 10, 12, 16, 20, and 24 h, the surviving bacterial population was determined by the plate count on BHI agar. Media without reuterin were used as positive control for all treatments.

Determination of mean and standard error of the mean (SEM)

The experimental data, as and when necessary, were presented as the mean and standard error of the mean (SEM) of different parameters studied in the present investigation. The mean and SEM were determined running Microsoft Excel 2000 Software Package, Microsoft Corporation, USA.

Results and discussion

Production of reuterin from *L. reuteri* BPL-36 strain

The reuterin production kinetics by BPL-36 resting cells in MRS–glycerol medium was studied. A sigmoidal increase in reuterin production was observed. The reuterin concentration increased markedly between 4 and 16 h and decreased

after 16 h. Maximum reuterin concentration of 89.63 mM was obtained in the medium after 16 h of incubation at 37 °C (Fig. 1). Reuterin concentration was, however, declined on further incubation. This is probably because of the conversion of reuterin into 1,3-propanediol, the end-product of glycerol fermentation, by enzymation reaction of an NAD⁺-dependent oxidoreductase (Bieble et al. 1999; Daniel et al. 1999; Zeng and Bieble 2002), whose expression could possibly be triggered by the accumulation of reuterin.

Determination of minimum inhibitory concentration of reuterin to inhibit other food-borne test organisms

The reuterin concentrations required to inhibit the growth of *E. coli* K12, *P. aeruginosa*, *E. coli* O157: H7, *S. typhi*, *S. aureus*, and *L. monocytogenes* were 1.0, 1.0, 2.0, 2.0, 4.0, and 10.0 AU/mL, respectively (Table 1). It is evident from the above results that the strain *L. monocytogenes* was relatively resistant to reuterin as the higher concentration of reuterin is required to inhibit its growth in contrast to the other pathogens used in this study. Chung et al. (1989) reported the MIC of reuterin for *Pseudomonas aeruginosa*,

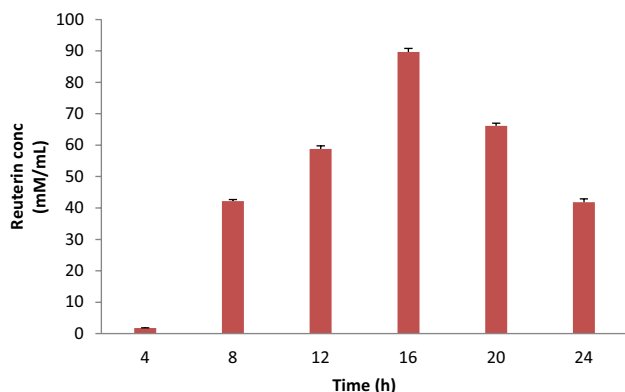


Fig. 1 Colorimetric quantification of reuterin produced by *L. reuteri* BPL-36 at different time intervals after incubation at 37 °C. Data points are means of triplicate readings with SEMs represented by vertical bars

Table 1 Determination of minimum inhibitory concentration of reuterin to inhibit other food-borne test organisms

Organisms	MIC (AU/mL)
<i>Escherichia coli</i> K12	1.0
<i>Pseudomonas aeruginosa</i>	1.0
<i>Escherichia coli</i> O157: H7	2.0
<i>Salmonella typhi</i>	2.0
<i>Staphylococcus aureus</i>	4.0
<i>Listeria monocytogenes</i>	10.0

Escherichia coli, *Salmonella typhimurium*, *Staphylococcus aureus*, and *Listeria monocytogenes*, respectively, as 2, 4, 4, 2, and 4–8 AU/mL which agrees to our results in some aspects.

Evaluation of encapsulation properties

Summary of encapsulation performance of encapsulated reuterin is given in Table 2. Maximum encapsulation efficiency of 37.97% was observed with mass yield 71% at optimized controlled condition. Morphologically capsules were spherical in shape, were shiny and whitish in colour (Fig. 2). Surface of the capsules was not very smooth with small pits on its walls.

Effect of pH on release kinetics of encapsulated reuterin from alginate capsules

Release kinetics of encapsulated reuterin from alginate capsules are shown in Figs. 3 and 4. When encapsulated reuterin was suspended in sodium citrate buffer at pH level ranging from 2 to 7, 100% release of reuterin was observed during storage at 37 °C after 24 h. Reuterin activity was fully

Table 2 Summary of encapsulation performance of encapsulated reuterin

Sample	Mass yield (%)	Encapsulation efficiency (%)
A	74.36 ^a	36.35 ^c
B	74.55 ^a	37 ^b
C	71 ^b	37.55 ^a
D	71 ^b	37.97 ^a

Values containing the same superscript are not significant ($P > 0.5$)

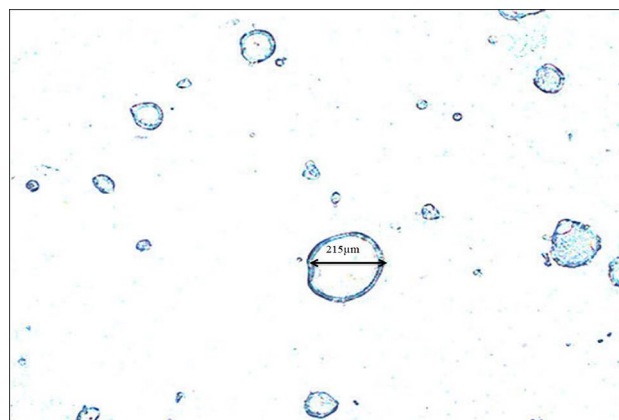


Fig. 2 Morphology of microcapsules at 10× magnification

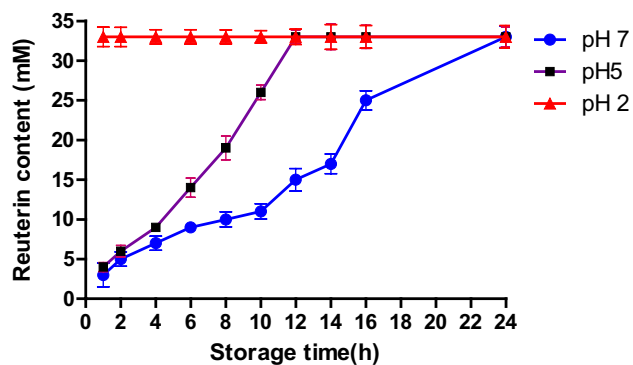


Fig. 3 pH-dependent release pattern of encapsulated reuterin during storage at 37 °C. Data points are means of triplicate OD₆₂₀ readings with SEMs represented by vertical bars

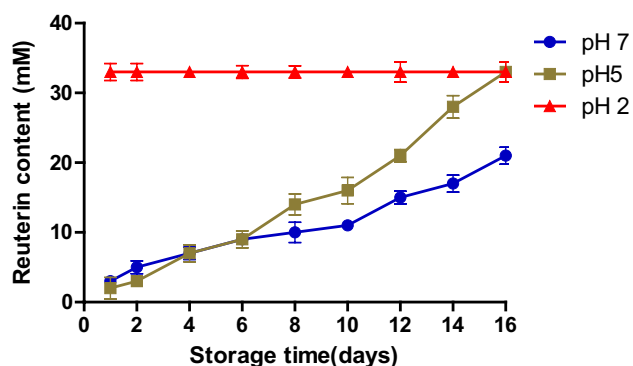


Fig. 4 pH-dependent release pattern of encapsulated reuterin during storage at 4 °C. Data points are means of triplicate OD₆₂₀ readings with SEMs represented by vertical bars

manifested at pH 2 and 5. All samples had complete release of reuterin in 30 min at pH 2.0. At pH 5, complete release of reuterin was observed only after 10 h, while at pH 7, complete release of reuterin was observed only after 24 h incubation at 37 °C (Fig. 3).

These release characteristics were unaffected by the storage of encapsulated reuterin at 4 °C for 2 weeks (Fig. 4), except at pH 7, where only about 64.24% of total encapsulated reuterin was released during storage at 4 °C. At pH 5, it took almost 15 days for the complete release of encapsulated reuterin in the buffer medium. These results indicate that the release characteristics of reuterin from the encapsulated particles are pH dependent. Our results are also in agreement with the published report of Oh et al. (2006), who had also encapsulated bacteriocin from *Lactobacillus plantarum* KU 107 in acid soluble coating polymer, Eudragit EPO in a hybridization system. He found no bacteriocin activity above pH 6 and at pH 6 only 25% of the total activity was detected. Stability of reuterin in aqueous solutions at pH 2.0–7.0 also is to be considered because Rollema et al. (1995) showed significant losses of nisin activity during incubation at

different temperatures. As given in Figs. 3 and 4, the release of reuterin after 8 h incubation at 37 °C at pH 5 was found to be ~ 55%. Similar results were given by Degnan and Luchansky (1992) using un-encapsulated pediocin AcH in slurries of heated beef muscle and tallow.

Anti-listerial properties of reuterin delivery system

The anti-listerial efficacy of the encapsulated reuterin was tested against *L. monocytogenes* in the BHI medium adjusted to pH 5.0 with a reuterin amount equivalent to 16 mM, similar to un-encapsulated (free) reuterin. Free reuterin initially deactivated *L. monocytogenes* to undetectable level in < 7 h and the effectiveness against the growth of *L. monocytogenes* was observed for 8 h (Fig. 5). After 12 h, however, *L. monocytogenes* appeared to have recovered from the antimicrobial action and grew to a population no different from the initial log count of 0 h control sample (without antimicrobial treatment) after 24 h.

For encapsulated reuterin, the initial bactericidal and later inhibitory functions were lesser relatively as compared to the than free reuterin. When comparing to the release profile of encapsulated reuterin as given in Figs. 3 and 4, the release of reuterin after 8 h incubation at 37 °C at pH 5 was ~ 55% that may have accounted for less effectiveness than the free reuterin. Furthermore, sustained release of reuterin only lasted 11–12 h, corresponding to gradual recovery of *L. monocytogenes* population before reaching a level well below to the initial log count of 0 h control sample (without antimicrobial treatment) after 24 h. As observed from Fig. 5, it may be inferred that sustained release of reuterin to a sufficient concentration is needed to receive long-term efficacy to inhibit the growth of pathogenic bacteria in food systems. The similar conclusion was made by Chi-Zhang et al. (2004) who observed that effectiveness of nisin strongly depends on its mode of delivery. He also observed that *L. monocytogenes* cells developed resistance to higher concentration of nisin, compared to cells treated with slowly added nisin at the same total amount of antimicrobial which was similar to our finding with reuterin. Narsaiah et al. (2014) have also used the

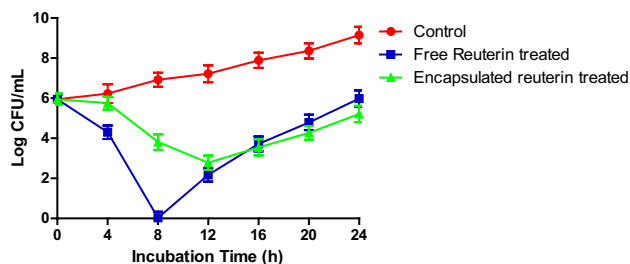


Fig. 5 Log counts (CFU/mL, mean ± SD) of pathogens *Listeria monocytogenes* in BHI medium without or with free reuterin, encapsulated reuterin during incubation at 37 °C (reuterin 16 mM)

same encapsulator for encapsulation of nisin using sodium alginate and guar gum. Nisin encapsulated in liposomes produced a 2-log greater inhibition of *L. monocytogenes* than free nisin (Were et al. 2004). In our study sustained release of reuterin, only lasted 11–12 h, corresponding to gradual recovery of *L. monocytogenes* population before reaching a level well below to the initial log count of 0 h control sample (without antimicrobial treatment) after 24 h, better than the overall inhibition shown by free reuterin.

In conclusion, our work demonstrated a novel antimicrobial delivery system that had much better capability of inhibiting the growth of *L. monocytogenes* during long-term storage at 37 °C. The effectiveness might be longer if tests were performed at lower temperatures. Characterization of release profiles of reuterin unveiled that sustained release of reuterin was required to improve long-term efficacy of reuterin against the potent pathogen. The release profiles established in this work can be used as guidelines to identify applicability of a particular reuterin delivery system in appropriate food products. Because food products have long shelf lives and our delivery system is based on GRAS ingredients produced using commercially feasible processes, our work may be developed into practical intervention systems to enhance microbial safety of various food products.

Acknowledgements Mr. Santosh Kumar Mishra is the recipient of scholarship from National Dairy Research Institute, Karnal, Haryana, India and duly acknowledges the financial assistance received. Funds received from NICRA project for carrying out this work are also acknowledged. He also acknowledges the help received from Dr. K. Narsaiah, Senior Scientist, CIPHET, Ludhiana for his generous help while using micro-encapsulator for encapsulation of reuterin.

Compliance with ethical standards

Ethical approval This article does not contain any studies with human or animal subjects.

Informed consent Additional informed consents were obtained from all the parents of infants from whom fecal samples were collected for isolation of lactic acid bacteria.

Conflict of interest Santosh Kumar Mishra, R. K. Malik, Harsh Panwar, and Amit Kumar Barui declare that they have no conflict of interest.

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