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Characterization of bacteriocin ABC transporter ATP-binding protein produced by a newly isolated *Enterococcus casseliflavus* MI001 strain

Indira Mikkili[1], Venkateswarulu TC[1*], Abraham Peele Karlapudi[1], Vidya Prabhakar Kodali[2] and Krupanidhi Srirama[1*]

Abstract

Background: ATP-binding cassette (ABC) transporters constitute one of the largest transporter protein families and play a role in diverse biological processes.

Results: In the present study, bacteriocin isolated from the *Enterococcus casseliflavus* MI001 strain was identified as an ABC transporter ATP-binding protein. The optimal conditions for the production of bacteriocin were found to be at 35 °C, a pH 5.5, and an incubation time of 24 h. Purification was performed using ammonium sulphate precipitation, gel filtration, and DEAE ion exchange chromatography. The bacteriocin was purified with an eightfold purification scheme resulting with a specific activity of 15,000 AU/mg. The NMR spectrum of purified bacteriocin revealed the presence of amino acids, namely lysine, methionine, cysteine, proline, threonine, tryptophan, and histidine. Further, the bacteriocin ABC transporter showed antimicrobial activity against food spoilage microorganisms.

Conclusions: The ABC transporter ATP-binding protein could be used as a potential alternative for food preservation, and it may be considered as a bio-preservative agent in food processing industries.

Keywords: Bacteriocin, *Enterococcus casseliflavus* MI001 strain, ABC transporter, Three-step purification, NMR spectrum

1 Introduction

Living organisms depend on various means of transport for the uptake of external nutrients and sequestration of waste products into the surrounding environment [3]. ATP-binding cassette (ABC) transporters are one of the largest super families of transport proteins present in all forms of life. They play diverse roles in both prokaryotes and eukaryotes. These transporters are the primary transporters functioning as both importers and exporters. Importers mediate the uptake of essential nutrients, vitamins, and trace metals from the surrounding environment, whereas exporters export substrates to the surrounding environment. Based on the type of substrate exported, they are categorized into various types. One such substrate is bacteriocin, and the transporter proteins exporting bacteriocin are called bacteriocin ABC transporters. They export bacteriocin across the cell membrane via a proteolytic function. The proteolytic domain resides in the N-terminal region, the ABC transporter domain is in the C-terminal and central multi-pass transmembrane region. Bacteriocins synthesized as propeptides are processed through this ABC transporter system and exported from cells as mature peptides [7]. Bacteriocins are secreted by either a double glycine leader in the N-terminal part of the pre-bacteriocin or secreted by a *sec*-system [12]. Due to the presence of a glycine leader on the N-

* Correspondence: venki_biotech327@yahoo.com; krupanidhijuly2012@gmail.com
[1]Department of Bio-Technology, Vignan's Foundation for Science, Technology & Research, Vadlamudi, Andhra Pradesh 522213, India
Full list of author information is available at the end of the article

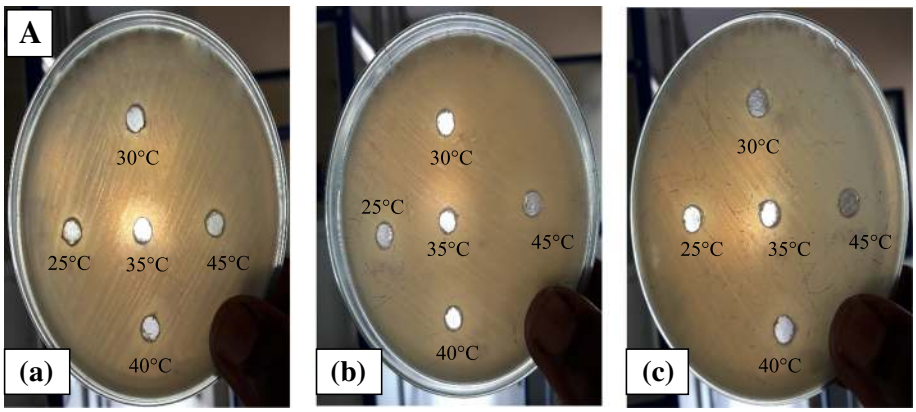

Fig. 1 A Effect of temperature on bacteriocin production after 24 h against (a) *P. aeruginosa*, (b) *S. aureus*, and (c) *E.coli*

terminal side, these transporters remove the N-terminal leader peptide from its bacteriocin precursor by cleavage at a Gly-Gly bond and transport the mature bacteriocin across the cytoplasmic membrane. The transporter acts as an efflux system, which helps the secretion of bacteriocins, proteins, polysaccharides, toxic compounds, and enzymes. The importance of these transporters in the multiple cellular functions and biosynthetic pathways of bacteria represents a novel strategy for the secretion of bacterial proteins and points out potential drug targets [10]. ABC transporters were found to play a key role in virulence and also identified as suitable targets for the development of antibacterial vaccines [1, 4]. However, an improved understanding of the structure, function, and action mechanism of these transporters have enabled us to develop new approaches for investigating lead molecules in terms of treating various diseases caused by problematic organisms [8]. An attempt has been made to characterize the bacteriocin ABC transporter ATP-binding protein from the *Enterococcus casseliflavus* MI001 strain.

2 Methods

2.1 Bacterial strains and culture medium

The indicator organisms were procured from the MTCC at the Institute of Microbial Technology, Chandigarh. The cultures were revived and stored in refrigerated conditions for further work. A nutrient broth medium (Hi-Media Chemicals, India) was used for maintenance and growing the indicator organisms for bacteriocin activity. The de Man, Rogosa, and Sharpe (MRS) broth medium (Hi-Media Chemicals, India) was used for production of the bacteriocin. Finally, the Muller–Hinton agar (Hi-Media Chemicals, India) medium was used for bacteriocin activity.

2.2 Optimization of culture conditions for bacteriocin production

2.2.1 Effect of incubation temperature and pH on bacteriocin production

Optimum conditions were determined by growing the test organism at a wide range of temperatures and pH values. The MRS broth was inoculated with *E. casseliflavus* MI001 and incubated at different temperatures

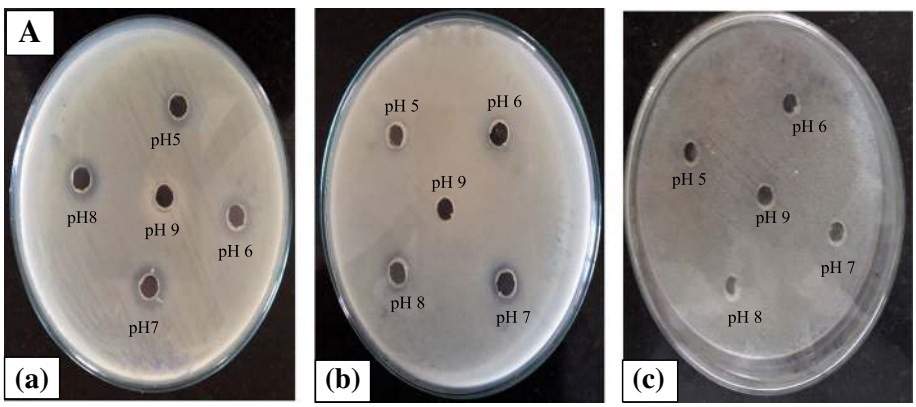

Fig. 2 A Effect of pH on bacteriocin production after 24 h against (a) *P. aeruginosa*, (b) *S. aureus*, and (c) *E.coli* at 37 °C

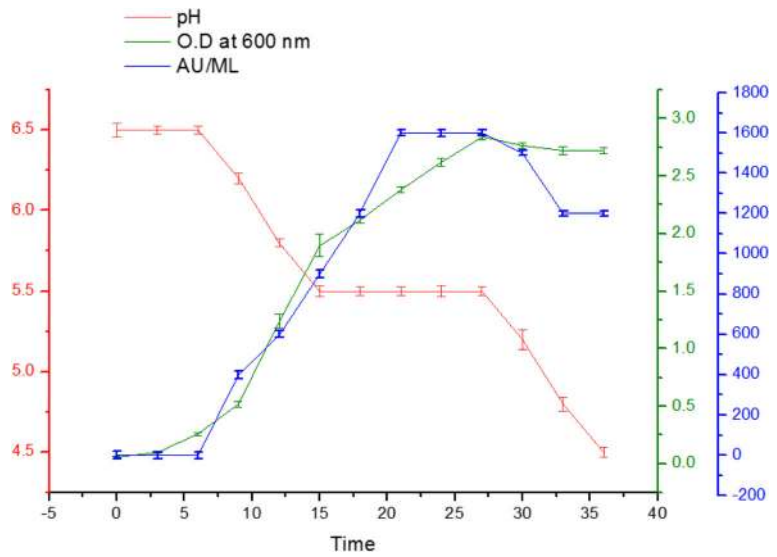

Fig. 3 Growth curve and bacteriocin production by *Enterococcus casseliflavus* MI001 strain

(25 °C, 30 °C, 35 °C, 40 °C, and 45 °C) and pH values ranging from 5 to 9 for 48 h. The samples were centrifuged at 10,000 rpm for 15 min. The supernatant collected was assayed for bacteriocin activity via the agar well diffusion method [15, 16].

2.2.2 Growth curve and production of bacteriocin

For growth curve measurement and production of bacteriocin, MRS broth (dextrose—20 g, peptone—10 g, beef extract—8 g, yeast extract—4 g, dipotassium phosphate—2 g, triammonium citrate—2 g, sodium acetate—5 g, magnesium sulphate—0.2 g, manganese sulphate—0.05 g, Tween 80—1 g, distilled water—1000 ml) with a pH of 6.5–7.0 was inoculated with 1% v/v of overnight-grown *E. casseliflavus* MI001, then incubated at 35 °C for 36 h. For every 3-h time interval, the optical density at 600 nm and pH were determined. Bacteriocin activity was determined every 3 h by agar well diffusion assay. Bacteriocin activity was measured using the formula $AU/mL = 1000/V \times D$ (where AU stands for arbitrary unit, V is the volume of the cell-free supernatant, and D is the dilution factor). Specific activity was measured as bacteriocin activity (AU)/total protein (mg) [17].

2.2.3 Purification of bacteriocin

Cell-free supernatant was saturated using 70% ammonium sulphate at ice-cold conditions (4 °C) with continuous stirring (300 rpm) and incubated overnight to precipitate the proteins. The precipitate was centrifuged at 6000 rpm for 10 min and dialyzed overnight at 4 °C using a 5 mM Tris buffer (pH 7.0). The dialysis sample was subjected to gel filtration chromatography using Sephadex G25 (13 mm radius × 150 mm length) pre-equilibrated with 100 mM phosphate buffer (pH 7.0), and the flow rate was set to 1 ml/5 min using the same buffer. The active fractions collected from the gel filtration chromatography were subjected

Table 1 Summary of purification profile of bacteriocin ABC transporter ATP-binding protein produced by *Enterococcus casseliflavus* MI001 strain

Type of sample	Volume collected (ml)	Total protein (mg)	Total activity (AU)	Specific activity (AU/mg)	Fold purification
Crude	150	129	240,000	1860	1
Ammonium sulphate precipitation and dialysis	10	3.6	18,000	5000	2.688
Gel filtration chromatography (SephadexG-75)	5	1.2	11,000	9166	4.927
Ion exchange chromatography (DEAE cellulose)	2	0.32	4800	15,000	8.064

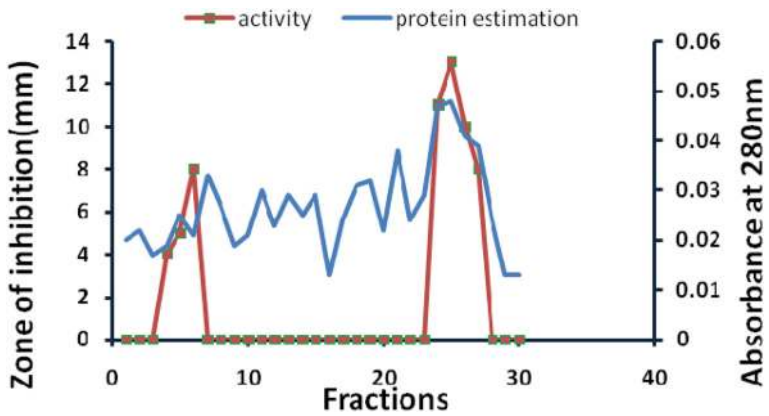

Fig. 4 Purification of bacteriocin by gel filtration chromatography. The blue line shows the protein content in terms of absorbance values at 280 nm, and the brown line shows the peak pertaining to bacteriocin activity

to ion exchange chromatography using a DEAE cellulose column (13 mm radius × 150 mm length) equilibrated with 25 mM Tris-HCl and 25–150 mM NaCl buffers (pH 7.0) [16]. The protein concentration was determined for all fractions, and an antimicrobial assay was performed against *Staphylococcus aureus*.

2.2.4 FTIR spectroscopy and NMR analysis

The purified fraction collected was subjected to FTIR analysis, and the spectrum was recorded in the frequency range of 4000 to 400 cm^{-1} using Agilent Technologies. The NMR spectrum was recorded for a purified sample using a Bruker 400-MHz spectrophotometer at 295 K. The purified bacteriocin sample was dissolved in methanol and analysed for ^{1}H and ^{13}C NMR spectra. The chemical shifts were recorded using tetramethylsilane (TMS) as an internal reference [9].

3 Results and discussion

3.1 Optimization of culture conditions for bacteriocin activity

3.1.1 Effect of temperature

Temperature influences the growth and production of bacteriocin, and it is one of the physical factors that play an important role in the metabolic activities of bacteria. From Fig. 1A (a, b, c), it was found that the

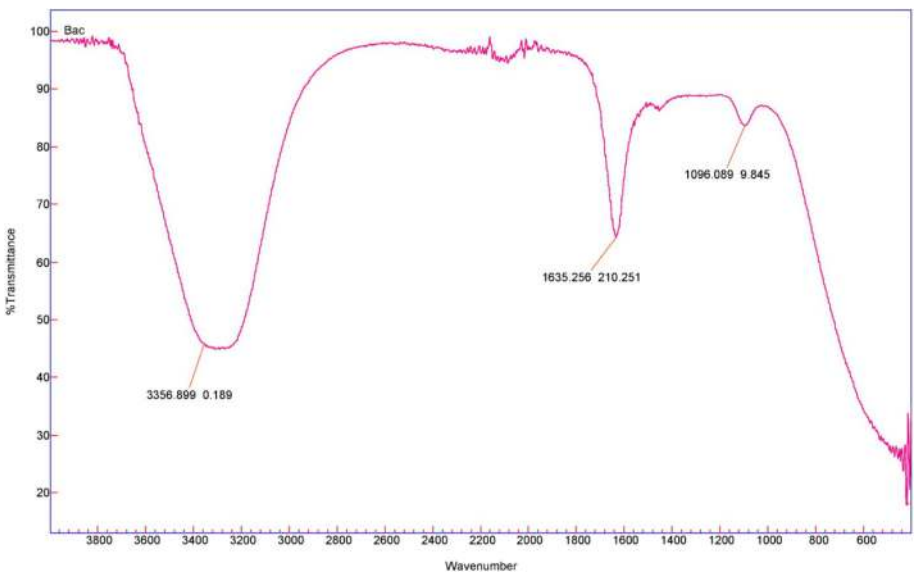

Fig. 5 FT-IR spectral analysis of purified bacteriocin from *Enterococcus casseliflavus* MI001 strain

Table 2 Wave number represents the presence of bonds

Wave number cm^{-1}	Possible bonds	Intensity
3367.186	Amine stretch (N–H)	Medium
1635.441	Alkene (C=C)	Variable
1096.238	C–N	Medium, weak

E. casseliflavus MI001 grown in MRS broth at 35 °C showed high antimicrobial activity against the indicator organisms *Pseudomonas aeruginosa*, *S. aureus*, and *Escherichia coli* after 24 h of incubation, which disappeared after 48 h of incubation. After an increase in temperature up to 40 °C for 24 h of incubation, bacteriocin production was at a minimum, as it showed a reduction in antimicrobial activity. Bacteriocin activity was not observed at temperatures of 25 °C, 30 °C, and 45 °C when incubated for 24 and 48 h. Zotta et al. [18] also reported that the cultivation of *Lactobacillus plantarum* at 25 °C reduced the growth compared to the optimal temperature of 35 °C. Our results suggest that the bacterium is surviving in human body conditions; this feature helps in that this organism could be used as a probiotic. In consonance, a recent study by Phumisantiphong et al. [13] reported that the bacteriocin production from *Enterococcus faecalis* 478 was found to be high at 37 °C.

3.2 Effect of pH

The synthesis of metabolites and metabolic activities of bacteria are usually affected by varying the pH. It was found that *E. casseliflavus* MI001 incubated for 24 h at pH 5.0–8.0 supported the growth and production of bacteriocin with good antimicrobial activity, as represented in Fig. 2A (a, b, c). The bacteriocin activity was not observed at acidic pH values and above a pH of 9.0. This result suggests that the bacteriocin activity is not due to organic acid production by bacteria. Iyapparaj et al. [6] reported that high bacteriocin production was recorded above a pH of 5.0, and reduced activity was observed at a pH of 9.0.

3.3 Growth curve and production of bacteriocin

Growth of the bacterium under various physical conditions, along with medium components, is important for the production of bacteriocin. MRS is a complex synthetic medium which supports bacteriocin production. Higher bacteriocin production was observed at a temperature of 35 °C, a pH of 5.5, and 27 h of incubation time, as represented in Fig. 3. Bacteriocin production was high during the exponential phase and was found to be 1500 AU/ml. A reduction in bacteriocin production was observed at temperatures above 35 °C, pH values below 4.5, and incubation times in excess of 30 h. In a previous report by Todorov et al. [17], it was found that the production of bacteriocin was high at 37 °C and pH 5.5. According to Fabricio et al. [2], bacteriocin production was high at 37 °C, pH 5.0, and an incubation time of 8 h during the exponential growth phase.

3.4 Purification of bacteriocin

The crude extract produced by *Enterococcus casseliflavus* MI001 was purified by a three-step procedure, as represented in Table 1. The crude extract precipitated with 70% ammonium sulphate increased the specific activity about 2.6-fold. The active sample purified by gel filtration chromatography showed two peaks, the specific activity increased about 4.9-fold, and the fractions from 24 to 28 showed high antimicrobial activity against the *S. aureus* (15 mm) (Fig. 4). In anion exchange chromatography, an 8.0-fold increase in specific activity at 15,000 AU/mg was observed. During each purification step, a considerable loss in protein concentration and a marked increase in the specific activity of the bacteriocin were observed. The purified fraction containing bacteriocin revealed the molecular weight of a 22-kDa protein via

Table 3 ^{13}C NMR spectral data. Various groups of carbon being detected in purified bacteriocin ABC transporter isolated from *E. casseliflavus* MI001 strain

^{13}C NMR spectra standard values		Purified bacteriocin sample NMR spectra (predicted values)
Carbon environment	Chemical shift (ppm)	Chemical shift (ppm)
C=O (in ketones)	205–220	217.51, 207.26
C in aromatic rings	125–150	146.65
C=C (in alkenes)	115–140	115.46, 114.12, 112.90
C=C (in alkynes)	90–100	96.57
RCH_2O–	50–90	80.02, 61.16, 58.24
RCH_2Cl	30–60	49.86, 49.64, 49.43, 49.21, 49.01, 48.79, 48.57
RCH_2NH_2	30–65	

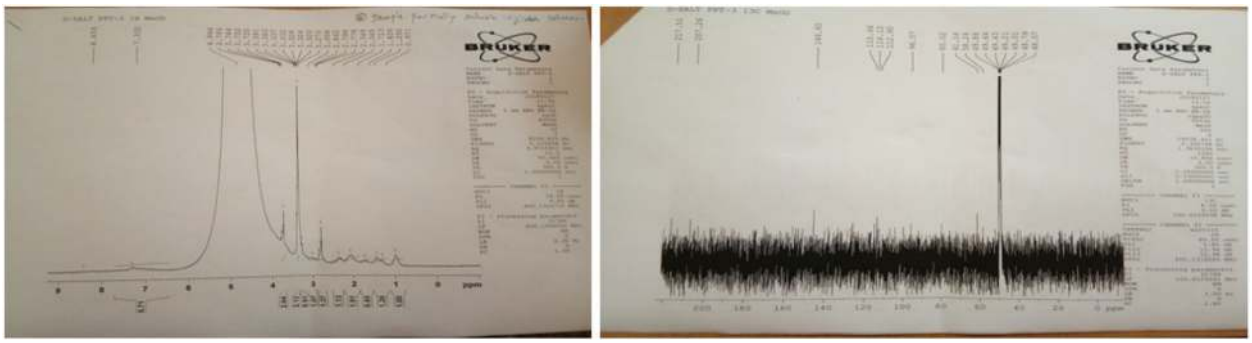

Fig. 6 ^{13}C NMR spectra (Left) and ^{1}H spectra (Right) of purified bacteriocin isolated from *E. casseliflavus* MI001 strain

SDS-PAGE [5]. In a recent study reported by Qianwen et al. [14], it was found that the bacteriocin produced by *Enterococcus faecalis* TG2 has a molecular weight of 6.33 kDa protein using a three-step purification procedure.

3.5 FTIR spectrum and NMR spectral analysis

The FTIR spectral analysis showed the presence of a C=C stretch and amine group coupled with a C–N group (Fig. 5). The correlation of various peaks is represented in Table 2. A previous study by Kadirvelu et al. [9] reported the presence of an amide and hydroxyl group coupled with C–H. Further, ^{13}C NMR and ^{1}H NMR analyses were carried out to discover the different amino acids and carbon compounds forming the molecular structure of the bacteriocin of *Enterococcus casseliflavus* MI001. The NMR spectra obtained were compared with the standard ^{13}C NMR spectrum presented in Table 3. From Fig. 6, it can be seen that the ^{13}C NMR showed the presence of 17 carbons, of which 2 are in C=O (ketones), 1 is in an aromatic ring, 3 are in C=C (alkenes), 1 is in C=C (alkyne), 3 are in R-CH_2O, and 7 are in the carbon environment R-CH_2Cl and RCH_2NH_2. The ^{1}H NMR spectrum was compared with the peaks of a standard spectrum of 20 amino acids. Of the 20 amino acids, seven, viz., lysine, methionine, cysteine, proline, threonine, tryptophan, and histidine, were involved in forming the peptide structure of the bacteriocin of *E. casseliflavus* MI001, as represented in both Fig. 6b (Right) and Table 4. In a previous study by Neha et al. [11], it was reported that the purified bacteriocin of *Lactobacillus brevis* UN examined by a ^{1}H NMR spectrum exhibited the following amino acids: proline, glutamic acid, arginine, leucine, isoleucine, and serine.

4 Conclusions

In this study, the optimized conditions for the bacteriocin production and three-step procedure for purification yielded a bacteriocin peptide molecule with antimicrobial activity. The purified bacteriocin molecular weight was found to be 22 kDa, which matched the results from MALDI-TOF. The ^{13}C NMR and ^{1}H NMR analyses revealed that the carbon environment and combinations of the unique amino acids in purified bacteriocin result in antibacterial activity, which has been reported for the first time through the present study. The antimicrobial activity of this ABC transporter bacteriocin has attracted much attention due to the antimicrobial resistance of the bacteria, which can be used as a next-generation antimicrobial in various fields.

Table 4 ^{1}H NMR spectral data, delta (ppm) of various amino acids detected in purified bacteriocin of *E. casseliflavus* MI001

Name of the amino acid	Chemical shift (ppm)—standard values	Chemical shift (ppm)—predicted values
Lysine	1.7	1.713
Methionine	2.0	2.065
Cysteine	3.2	3.270
Proline	2.1–3.6	3.320–3.391
Threonine	4–5	4.866
Tryptophan	6.5–7.8	7.332
Histidine	6.5–8.5	8.433

Abbreviations
ABC: ATP-binding cassette; MTCC: Microbial Type Culture Collection; NMR: Nuclear magnetic resonance

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Authors' contributions
MI carried out the experimental studies and drafted the manuscript. SK and KV participated in the design of the study. KA and TCV participated in optimization studies. KA and TCV conceived of the study, participated in its design and coordination, and helped to draft the manuscript. All authors read and approved the final manuscript.

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Not applicable.

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Competing interests
The authors declare that they have no competing interests.

Author details
[1]Department of Bio-Technology, Vignan's Foundation for Science, Technology & Research, Vadlamudi, Andhra Pradesh 522213, India. [2]Department of Bio-Technology, Vikrama Simhapuri University, Nellore, Andhra Pradesh 524001, India.

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