Novel Anticancer enzyme from Marine Bacteria to fight against neoplastic cancer-Acute lymphoblastic leukemia†

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Abstract Recently there has been an explosion of information about novel bioactive compounds that have been isolated from marine microbes in an effort to further explore the relatively untapped marine microbes and their secondary metabolites for drug discovery. New therapeutic agents are urgently needed to treat medicinal needs that are currently unmet .The biodiversity of marine microbes and the versatility of their bioactive metabolites has not been fully explored. L-asparaginase therapy, alone is finding increased success in the management of acute lymphocytic leukemia's. Large amounts of purified enzyme and increasing recognition of successful therapy will generate increased demand. The production of L-asparaginase by using bacteria has attracted great attention owing to their cost effective and eco-friendly nature. In search of efficient microorganisms potential for production of anticancer enzymes, bacteria isolated from mangrove soil of Nizampatnam Guntur district were screened for L-asparaginase .The screened isolates were biochemically characterized and identified as pseudomonas species. In the present study one of the potential *Pseudomonas* strain was selected and identified as *Pseudomonas aeruginosa* AVP17 by 16s rRNA partial sequence. Effect of pH, temperature, carbon source, salt tolerance was studied for growth optimization and maximum production of enzyme.

KEYWORDS Mangrove soil, Marine bacteria, L-Asparaginase, neoplastic cancer**,** Acute lymphoblastic leukemia, *Pseudomonas aeruginosa,* 16S rRNA partial sequence.

1. INTRODUCTION

Cancer is defined as uncontrolled division of cells. Acute lymphoblastic leukemia is cancer of white blood corpuscles (WBC) characterized by the excessive multiplication of malignant and immature WBC (lymphoblast) in bone marrow. Treatment of acute leukemia includes chemotherapy, steroids, radiation therapy and intensive combined treatments including bone marrow or stem cell transplants.

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Among these, chemotherapy is most preferred. The drugs being employed for treatment includes prednisolone, dexamethasone, vincristine, asparaginase, daunorubicin, cyclophosphamide, cytarabine, etoposide, thioguanine, mercaptopurine, hydrocortisone, methotrexate etc[1]. Although, variety of drugs is available today, their efficacy in treatment of cancers at third and fourth stage is doubtful. The side effects caused by these chemotherapeutic agents are many such as infertility, secondary neoplasm, nausea and vomiting, immunosuppression etc. The term "acute" means that the leukemia can progress quickly, and if not treated, would probably be fatal within a few months. Lymphocytic means it develops from early (immature) forms of lymphocytes, a type of white blood cell. L-asparaginase is the first enzyme with anti-leukemic activity to be intensively studied in human beings [2]. It is an enzyme drug of choice used in combination therapy for treating acute lymphoblastic leukemia in children [3,4,5,6,]. Since 1922, L-asparaginase has been considered as a therapeutic agent against malignant tumors [7,8,9]. It was further shown that growth of normal cells was not dependent on L-asparagine. L-asparaginase was introduced in the therapeutics due to the fact that in a significant number of patients with acute leukemia, particularly lymphocytic, the malignant cells are dependent on an exogenous source of L-asparagine for survival. Normal cells, however, are able to synthesize L-asparagine and thus are less affected by its rapid depletion produced by treatment with the enzyme L-asparaginase.Amino acid degrading enzymes are important chemotherapeutic agents for the cure of some types of cancers. The manufacture or processing of enzymes for use as drug is an important facet of today's pharmaceutical industry[10](Cassileth, B., 1998).
The microbes are the best sources of L-asparaginase because they can be cultured easily and the extraction and purification step is also convenient, facilitating the large scale production [11] The common side effect of this medication is [12].

Recent times, L-asparaginase emerged as potent health care agent for the treatment of acute lymphocytic leukemia [13,14,15,16] because tumor cells cannot synthesize L – asparagine and hence take L-asparagine from blood circulation or body fluid. More over L-asparaginase is biodegradable, non-toxic and can be administered at the chemotherapeutic agents which were qualitatively specific for any type of cancer cell.

2. MATERIALS AND METHODS

2.1 Chemicals

All chemicals used in this study investigation were of analytical grade and procured from Sigma (USA), Hi – media (India) and Merck (India).

2.2 Collection of Soil Sample

Soil samples were collected from mangrove soil sediment of Nizampatnam of Guntur district at a depth of 3 ft. and placed in zip locked plastic bags at 4^0 C .The soil contained 3.8% of organic matter and pH8.8.

2.3 Isolation of Bacterial Strains

1 gm. of soil was separately suspended in 9 ml of physiological saline soil in a flask and placed on an orbital shaker (at 100 rpm) at room temperature (28± 20C) for 1 hr. At the end of shaking the soil samples were serially diluted up to 10^{-6} with physiological saline. 10^4-10^6 dilutions were placed on modified nutrient agar medium containing Fluconazole (antifungal antibiotic) by pour plate technique and incubated at 28^0C .The most prominent colonies were isolated maintained on Nam slants at 4^0c for further studies.

Pure culture of AVP 17 bacterial isolate was grown until log phase achieved and genomic DNA was isolated essentially[20].The amplification of 16S rRNA gene was done by using universal bacterial primer 1492R (5´-TACGGYTACCTTGTTACGACTT-3´) and 27F (5´ AGAGTTTGATCMTGGCTC AG- 3´) as per the conditions[21].The PCR product was sequenced at Macrogen South Korea. The sequences obtained were compared with those from the GenBank using the BLAST program[22]and Phylogenetic trees reconstructions were obtained by the Neighbor joining method 1000 bootstrap replicates were performed to assess the statistical support for each branch in the tree[22,23].

2.4 Primary screening

The L-asparaginase producing strains were initially screened by rapid plate assay method, based on their capability to form a pink zone around colonies on agar plates of modified M-9 medium [17]. The medium contained Na2HPO4.2H2O, 6.0 g; KH2PO4, 3.0 g; NaCl, 0.5 g; L-Asparagine, 10.0 g; 1mol- MgSO4.7H2O, 2.0 ml; 0.1 M solution of CaCl2.2H2O, 1.0 ml; 20% glucose stock, 10.0 ml; agar 20.0 g. per liter of distilled water. The medium was supplemented with 0.005% phenol red dye (prepared in ethanol) and the pH was adjusted to 6.2 using 1N HCl. Plates were then incubated at 37°C for 24-48 hrs. A set of tubes was also run as a control without L-asparagine. The strains having potential for L-asparaginase production were selected on the basis of pink zone formation and retained for further screening.

2.5 Characterization of microorganism By Morpho Physiological and Biochemical Studies

Morphological characters such as shape and color of the colonies were examined. Grams staining and motility were also done. Isolates were biochemically analyzed for the activities of oxidase, catalase, MR-VP test, starch hydrolysis and gelatin hydrolysis, indole production, hydrogen sulphide test, nitrate reduction, sugar fermentation and citrate utilization. The results were compared with Bergey's Manual of Systematic Bacteriology. Out of 46 bacterial isolates 9 selected bacterial isolates showing excellent L-asparaginase activity were characterized on the basis of morphological and biochemical characteristics and the results were interpreted according to Bergey's Manual of Determinative Bacteriology & PIBWIN software version 19.2[18,19].

2.6 Molecular Identification

2.7 Effect of physico-chemical parameters

Effect of different temperature ranges (25^0c,37^0c,50^0c,100^0c), different incubation periods(24 hrs,48 hrs,72 hrs), pH values(7,9,10,12,14), salinity concentrations(0.5%,5%,10%,15%,20%), different carbon sources(21), different Nitrogen sources(11), different amino acids(16) and metal ions/mineral salts (51) on enzyme production was studied.

2.8 Determination of L-asparaginase activity

The rate of hydrolysis of L-Asparagine was determined by measuring the ammonia released using Nessler's reaction[24].The color reaction was allowed to develop for 10 min and the absorbance read at 480 nm with a spectrophotometer. The ammonia liberated was extrapolated from a curve derived with ammonium sulphate. One unit (U) of L-Asparaginase was defined as that amount of enzyme which liberates 1 µ mole of ammonia per minute under the assay conditions [25].

2.9 Partial purification of Enzyme

The culture filtrate was filtered through whatmann No. 1 filter paper and centrifuged at 8000 rpm for 10 min at 4^0C.

The culture filtrate (crude enzyme) was brought to 45 per cent saturation with ammonium sulphate at pH 8.4 and kept overnight in a cold room at 4^{0}C. It was thereafter subjected to centrifugation at 8000 rpm for 10 min at 4^{0}C. The precipitate was discarded, while the supernatant was brought to 70 per cent saturation with ammonium sulphate and centrifuged at 8000 rpm at 4^{0}C for 10 min. The precipitate from this step was collected and stored at 4^{0}C.

2.10 Kinetics of the L-asparaginase enzyme

To measure the kinetics of L-asparaginase, Michaelis constant (Km) and Maximal velocity (Vmax) of the partial purified enzyme was determined. They are one of the important parameters for the evaluation of the potential usefulness of the enzyme for anti-leukemic therapy. They were determined using L-asparaginase as substrate in the range of 0.01M-1M concentration. Each reported velocity I the mean of at least three measurements. The apparent Km was determined [26].

3. RESULTS AND DISCUSSION

Bacterial strains were isolated from soil samples collected from Nizampatnam of Guntur district. L-Asparaginase bacterial strains were identified by pink colored colony on agar medium with phenol red as an indicator. whereas one plate maintained as control as uninoculated (Figure 1) and another plate was inoculated with selected bacterial strain AVP 17 (Figure 2).Microbial strain AVP 17(from Nizampatnam soil sample), was selected and further characterized by morphological, physiological and biochemical studies. The isolated strain, AVP 17 was Gram-ve and rod shaped colony (Table 1) colonies with fluorescent blue colour under UV light (Figure 3). According to the data of Bergy's manual of systemic bacteriology,[27] based on morphological physiological and biochemical characteristics(Table 1) the bacterial strain AVP 17 was classified to be *Pseudomonas sp.* A 1466 bp PCR product of gene was amplified from the genomic DNA of AVP 17 .A sequence similarity showed that the 16srDNA gene sequence of AVP 17 had 99% similarity to the 16srDNA of *Pseudomonas aureginosa* strain and *Pseudomonas* species PPB2(AC:HM771657).The sequence was blast in NCBI and for analysis. Based on phylogenetic analysis revealed that AVP 17 was closely related to *Pseudomonas aureginosa* (Figure 4) and sequence was deposited in NCBI as *Pseudomonas aureginosa* AVP 17 with accession number KF527831. Optimization of physico chemical parameters like pH, temperature and salinity plays an important role in production of L-asparaginase.

Optimization revealed that AVP 17 showed maximum production (1.10 IU/ml) at 37^{0}c after 48 hrs incubation (Fig.5). Production of L-asparaginase varies with incubation period at 37^{0}c indicating optimization of incubating period also exhibit a significant role(Fig.5) Enzyme production of AVP 17 showed variation at different pH. L-asparaginase production was found to be maximum (16.19 IU/ml) at pH 9 and observed to be gradually decreased beyond pH 12(Fig. 6). Percentage of NaCl concentration also effect the L-asparaginase production of AVP 17.The production was inversely related with increasing concentration of NaCl and found to be maximum(19.11 IU/ml) at 0.5% concentration(Fig.7).Earlier literature revealed that L-asparaginase production in *Streptomonas albidoflavus* was observed to be high(11.9 IU/ml) at 40^{0}c at pH 7.5 Isolate AVP 17 showed maximum l-asparaginase production with Dextrose (166.25 IU/ml) and Urea (89.1 IU/ml), the carbon and nitrogen source respectively.(Fig.8)Amino acid Glutamine and sodium tungstate were observed to be potential inducers for L-asparaginase production of 66.07 IU/ml and 774.91IU/ml respectively(Table 2). After optimization Dextrose, Urea, Glutamine and sodium tungstate were selected as potential inducers and enhancers. An attempt was made to evaluate the extent of improvement in production of l-asparaginase in the modified formulated production media with necessary inducers and enhancers. 5 folds of enhancement in L-asparaginase production observed with modified production medium indicating highly significant improvement so far observed. Enzyme kinetics, Vmax and Km values were studied at different substrate concentrations (0.01-1 M) and at different incubation periods (24 hrs, 48 hrs and 72 hrs). At 24 hrs of incubation, Vmax value of L-asparaginase is 133.82 and Km value is 0.5. At 48 hrs , Vmax is 193 and Km value is 0.2 and at 72 hrs AVP 17 showed 27.2 Vmax value and 0.03 Km value. Enzyme kinetics study revealed that L-Asparaginase of AVP 17 showed High Vmax at 0.7 M substrate. (Fig.11). Enzymatic activity of the strain AVP17 measured at physiological temperature showed lower Km values. The Km values obtained were closer those of some mesophilic L-asparaginase of earlier studies.

4. CONCLUSION

Production of L-asparaginase using different microbial systems has attracted much attention, owing to the cost-effective and ecofriendly nature. A wide range of microorganisms including bacteria, fungi and yeast have proved to be the beneficial sources of this enzyme. L-asparaginase is an important natural product that possesses a broad spectrum of antitumor activity. It has been successfully applied to the treatment of several diseases such as lymphocyte sarcoma and leukemia. It has been proved that L-asparaginase from *Escherichia coli* and *Erwinia carotovora* has anti-neoplastic activity against cancer and is being used as anticancer drug. But, it is observed that the action of enzyme is coupled with some side effects. Moreover, the yield of enzyme was not enough to fulfill the demand of the drug. Solid state fermentation is being

adopted all over the world for the production of the enzyme as it has many advantages over submerged fermentation. In this concern the isolated bacterial strain AVP17 was potent and produces high yield of L-asparaginase under optimized conditions.So, it created the need to discover new sources and techniques to enhance the yield and decrease the side effects of the enzyme. Enzyme isolated from different sources has different optimized conditions for production and activity. Recombinant work and formulation of enzyme is also in progress, yet there is still a long way to go.

Fig. 1. Plate showing (a) control LASP
Fig. 2. Plate showing production of L-asparaginase by AVP 17

Figure 1	Figure 2
	

Fig 3. L-Asparaginase Producer AVP 17 Isolated from Mangrove Soil showing Fluorescence under UV

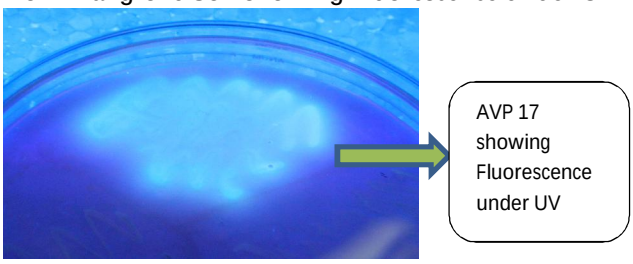


Table 1:
Morphological, Physiological and Biological
Analysis of Bacteria Isolated From Marine Environment

Isolated strain	AVP 17
Morphological analysis	
Gram staining	Negative
Shape	Short Rod
Physiological analysis	
Starch hydrolysis	Positive
Lipase	Negative
Urease	Positive
H2S Production	Positive
Protease	Positive
Gelatin hydrolysis	Negative
HCN Production	Positive
Litmus	Positive
Biochemical analysis	
Indole	Negative
Methyl red	Negative
Vogues Proskeur	Negative
Citrate	Positive
Catalase	Positive
Oxidase	Positive

Figure 4:

Phylogenetic tree of Pseudomonas aureginosa

AVP 17 based on 16S rRNA gene analysis

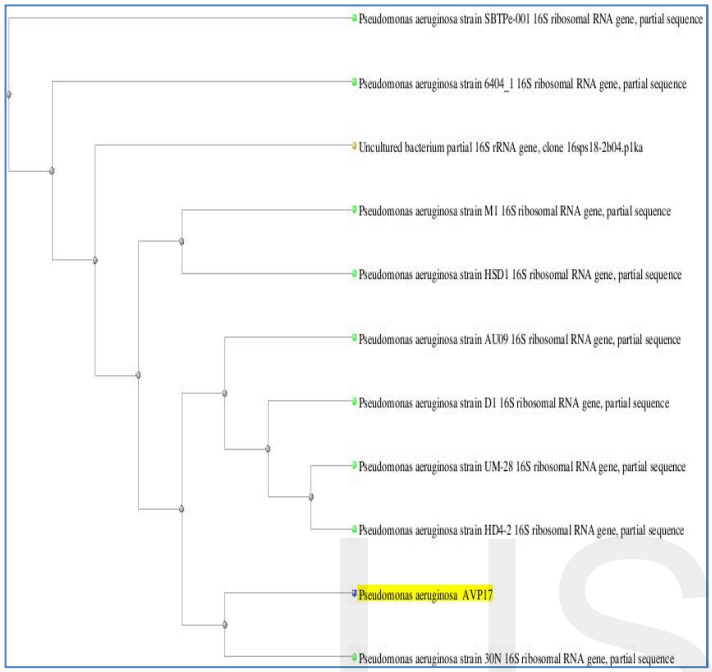


Figure 5: Influence of Temperature on enzyme activity

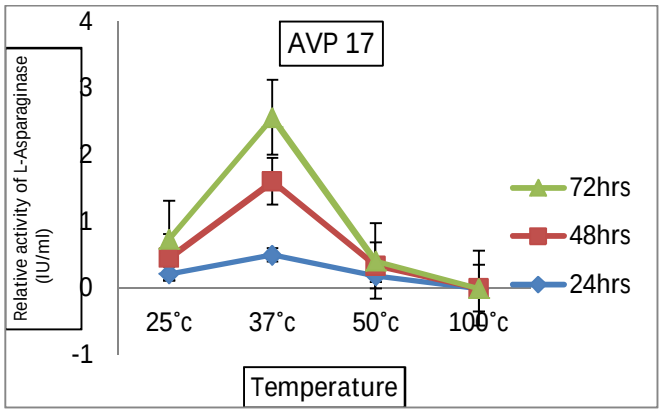


Figure 6: Influence of pH on enzyme activity

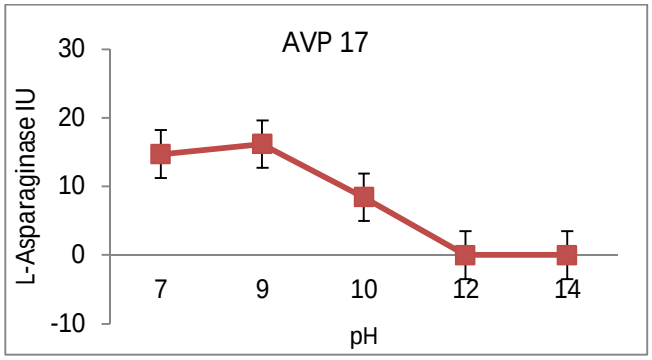


Figure 7: Influence of NaCl on enzyme activity

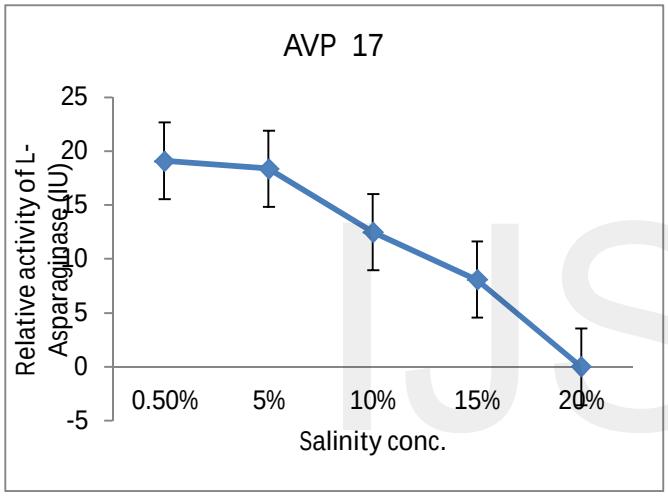


Figure 8: Influence of carbon sources on enzyme activity

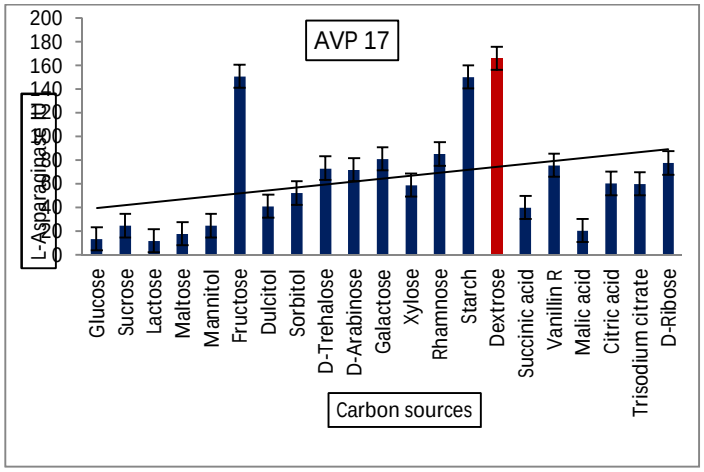


Figure 9: Influence of various Nitrogen sources on enzyme activity

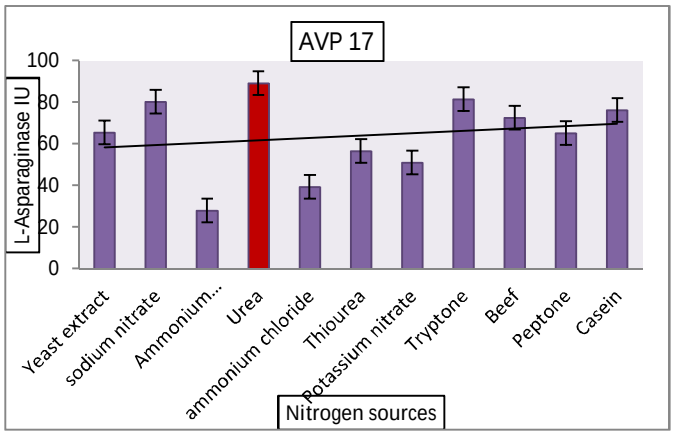
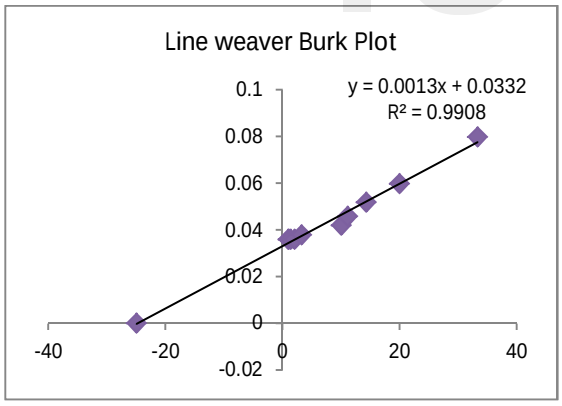


Table 2 : Influence of various Metals/Mineral Sources on enzyme activity

S.N0	Mineral salts	IU
1	calcium oxalate	50.31
2	ammonium ceric sulphate	48.3
3	ammonium sulphate	69.33
4	cupric sulphate	34.82
5	ammonium carbonate	54.41
6	ammonium oxalate	63.08
7	ammonium borate	74.46
8	Ammonium Chloride	39.28
9	Calcium chloride	45.04
10	potassium chloride	174.1
11	ammonium thiocyanate	74.46
12	cobaltous acetate	74.01
13	Manganese chloride	82.41
14	alluminium sulphate	81.83
15	alluminium nitrate	84.5
16	cupric nitrate trihydrate	84.64
17	barium bromide	66.42
18	lead acetate	79.68
19	copper-phosphate	82.67
20	Sodium chloride	69.59
21	Mercuric chloride	71.33
22	Lithium chloride	72.58
23	Magnesium sulphate	81.56
24	Creatinine	74.91
25	Manganese sulphate	57.54
26	copper sulphate	50.13
27	ferric citrate	76.2
28	Zinc	76.33
29	Silver nitrate	76.25
30	Selenium	84.06

31	Barium chloride	75.08
32	Potassium ferricyanide	65.8
33	Ferric choride	72.32
34	Ferrous sulphate	84.59
35	Potassium thiocyanate	52.5
36	Calcium carbonate	84.15
37	Sodium thiosulphate	53.52
38	Sodium tungstate	774.91
39	ammonium molybdate	79.86
40	Potassium acetate	52.94
41	ammonium ferrous sulphate	53.08
42	Lithium sulphate	63.48
43	Potassium nitrate	50.84
44	potassium carbonate	54.86
45	sodium hydroxide	40.13
46	sodium acetate	44.1
47	zinc sulphate	65.49
48	Sodium hydrogen carbonate	58.43
49	Zinc chloride	57.23
50	Trisodium citrate	60.13

Figure 11: Line weaver Burk Plot



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CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this paper.

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