Research Journal of

Microbiology

ISSN 1816-4935

Academic
Journals Inc.

www.academicjournals.com

OPEN ACCESS Research Journal of Microbiology

ISSN 1816-4935
DOI: 10.3923/jm.2020.1.8

Research Article
Determination of Probiotic Bacteria from Intestine of *Sparisoma viride* and Bioencapsulation of *Artemia salina* with Probionts

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Abstract
Background and Objective: Now a days, the need for remarkable disease resistance, growth of aquatic organisms and feed competence has brought about the employ of probiotics in aquaculture field. The objective of the present study was to isolate probiotic bacterial strains from fish intestines and screen them by *in vitro* testing of their antagonism to pathogens and bioencapsulation efficacy. **Materials and Methods:** Six bacteria were isolated from marine fish gut and identified by biochemical test. All six isolates were assessed *in vitro* for its inhibitory activity against common fish pathogens of *Aeromonas hydrophila* and *Vibrio harvey* by well diffusion assay. Based on the antimicrobial activity, the *Enterococcus, Streptococcus* and *Bacillus* were selected for assaying probiotic properties of acid tolerance, bile salt concentration and auto aggregation assay. The 24 h old *Artemia salina* nauplii were selected for probiont encapsulation study. **Results:** The isolates *Enterococcus, Streptococcus* and *Bacillus* were confirmed as probionts by above mentioned three methods. The research of encapsulation studies exposed that the gut loading and evacuation time of *Artemia* nauplii differed according to the oils and probiotics used for the trials. Probiotics take less time for gut loading and retention when compared to oils. **Conclusion:** The findings of this study indicated that the indigenous fish gut bacteria provided benefit to the culture fishes in terms of pathogen protection and increasing growth potential and thus fulfilled the major requirements of being effective probiotics

Key words: Aquaculture, gut bacteria, *Sparisoma viride*, antagonistic activity, probiotics, bioencapsulation, *Artemia salina*

Citation: N. Yogananth, M. Akila, M. Syed Ali, M. Jayaprakashvel and R. Muthezhilan, 2020. Determination of probiotic bacteria from intestine of *Sparisoma viride* and bioencapsulation of *Artemia salina* with probionts. Res. J. Microbiol., 15: 1-8.

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Shrimp and fish culture is grown as a million dollar industry and rearing of shrimps and fish in culture system becomes popularized throughout the world especially in Southeast Asia. Worldwide, major economic losses in cultured shrimp and fish result from a relatively small number of opportunistic pathogenic bacteria[1]. *Vibrio* and *Aeromonas* are most important pathogen recognized in larval cultures, causing haemorrhagic septicaemia, ulcerative conditions, abdominal distensions, fin/tail rot and exophthalmia in freshwater fish species[2,3].

Many pathogenic bacteria become resistant to antibiotics and hence farmers resort to dump more antibiotics[4]. At the same time, use of prophylactic antibiotics is detrimental to aquatic and terrestrial environments, animal and human health[5,6]. Hence, the scientific communities have proposed friendly alternatives such as vaccines[7], antibiotic substitutes[8] or use of probiotics[9].

During, last few decades probiotics have been used in a number of trials to improve human health and to reduce pathogenic problems in intestinal tracts[10,11]. Most of the results are promising and gave confidence about the use of probiotics for improving human health. The research on probiotics for aquatic animals is thus increasing with the demand for environment-friendly aquaculture and it is expected that it can assure the nutritional security in the next millennium[12,13]. The beneficial effect of probiotics have been attributed to their ability to promote the immunological and non- immunological defense barriers in the gut, normalization of increase in intestinal permeability, exogenous production of digestive enzymes and altered gut microflora. They have also been shown to enhance humoral response and consequently to promote intestine's barriers. Probiotics have also been shown to stimulate non-specific host resistance to microbial pathogen and thereby, aid in immune elimination of pathogen[14].

Different kinds of live prey have been used to feed larvae of diverse marine organisms. These include copepods, nematodes, rotifers and *Artemia* nauplii. Until now, brine shrimp nauplii was considered the best diet for feeding zoophagous organisms and was provided as a live food to over 85% of the aquaculture species[15].

Even though enrichment of *Artemia* is widely used in aquaculture, it has to date been unclear as to what proportion of the enrichment is incorporated into body tissue and how much remains resident in the gut. The gut loading and evacuation trial demonstrated that the gut loading and retention time of *Artemia* nauplii (2 day old) bioencapsulated with vegetable oils and probiotics. Information regarding the gut loading and unloading time of *A. parthenogenetica* nauplii is scarce. Immanuel *et al.*[16] conferred a study on delivery of highly unsaturated fatty acid, probionts and biomedicine through bioencapsulated *Artemia* and further its application was done on *Penaeus monodon* as growth and survival. They had reported that in products tested with probiont *Lactobacillus*, the *Artemia* reached full gut condition in 38$\pm$1 min.

The present study was undertaken to isolate probiotic bacterial strains from fish intestines and screen them by *in vitro* testing of their antagonism to pathogens and bioencapsulation efficacy.

MATERIALS AND METHODS

Collection and processing of sample: A total of 10 healthy parrot fishes (*Sparisoma viride*) with average weight of 50-75 g and average length of 10-13 cm were collected in year of December, 2018, from fish farm in Besant Nagar, Chennai, Tamil Nadu and transferred to the PG laboratory, PG and Research Department of Biotechnology, Mohammed Sathak College of Arts and Science, Sholinganallur, Chennai.

The surface of the fish is sterilized using 70% ethanol and sterile water. By using the surgical knife, cut the skin from the anal fin along the belly of the fish to the operculum and the fish gut was dissected out under the sterile condition.

The gut was removed by aseptic dissection box and homogenized in a mortar and pestle by using 5 mL sterile 0.9% NaCl solution for 24 h at 30°C followed by incubation at 45°C for 10 min in a convection oven to activate the sporulation process. Ethanol (50% v/v) was added to a volume of 20 mL to each of the flasks which were incubated at 20°C for 1 h. The contents were centrifuged at 10,000 g the supernatants decanted and the resultant pellets incubated at 105°C in a convection oven for 5 min. The dry pellets were reconstituted into 20 mL of sterile physiological saline and serially diluted to 10^{-4} in 10^{-1} increments. The 0.1 mL of the diluted sample were taken from 10^{-3} and 10^{-4} dilutions and spreaded in nutrient agar plates and incubated at 37°C for 12 h. After incubation, the each colony appeared on the plates were streak individually in fresh plate for further biochemical and antimicrobial characterization

Antagonistic activity: Nearly 6 strains were isolated at first phase which were designated as B1, B2, B3, B4, B5 and B6 undergoes agar well-diffusion method to detect the anti-microbial activity against the fish pathogens like *Aeromonos hydrophilia* and *Vibrio harveii*.

Screening for probiotics properties

Acid tolerance test: The B1, B2, B5, isolates were grown overnight 37°C for 12 h and were centrifuge at 7000 rpm for 10 min. The cell pellet were washed with twice with PBS (phosphate buffer saline) of pH 7.3 and resuspend in PBS and final volume 1 mL the strains were diluted in PBS 1:100 dilution at pH 1, 2, 3 and 4. The mixture of each strain was then incubated at 37°C for 12 h. the viability of bacterial cell were determined by plating the cells in nutrient agar plates at different time interval 0, 60, 180 min growth of the bacteria were expressed in colony forming units/mL (log10 CFU mL^{-1}) and the survival percentage of the 6 isolates to different pH values were calculated.

Bile salt concentration: The probiotics cultures were grown at 37°C for 12 h in nutrient broth without bile. Then 1 mL of the culture broth was poured onto the nutrient broth with varying bile salt concentration 0.15, 0.25, 0.5, 0.75 and 1.0%. The ability of the isolates was measured by absorbance at 595 nm using ELISA reader after different periods of incubation (0, 2, 4 and 6 h).

Auto aggregation assay: The probiotic culture was centrifuge and discard the supernatant in the pellet add twice the amount of PBS and dissolve the pellet in the PBS. The OD value was taken at various time intervals 0 min, 2, 6, 12, 24 and 48 h.

Bioencapsulation potential of probiotics

Collection site: The cysts of *Artemia salina* were obtained from the Department of marine Biotechnology, Amet University, Kanathur, Chennai and were purified and stored according to Krishnakumar[17].

Selection of experimental sources: The vegetable oil such as coconut oil, sunflower oil, cod liver oil and Gingelly oil were purchased commercially. In the present study probiotics such as B1, B2 and B5 strains were cultured in the nutrient agar slant.

Culturing of probiotic bacteria: The slant culture of the probiotic bacteria is transferred to the broth culture under aseptic techniques. B1, B2 and B5 strains were cultured in nutrient broth. After 48 h at 37°C incubation, the both bacterium has sub cultured again in same medium composition (50 mL of broth culture were prepared). Pure culture of probiotics was centrifuged at 5000 rpm for 30 min. After centrifugation, the pellets were washed twice with saline. After washing probiotics were aseptically enriched with 24 h old *Artemia salina* nauplii according to Divya *et al.*[18].

Experimental setup: Purified cysts were hatched out in 35 ppt sea water. The brine shrimp *Artemia salina* nauplii were collected on a sieve from the stock, rinsed with filtered seawater and transferred to enrichment containers (2 L capacity) at a density of 100 individuals/mL of seawater (35 ppt) at room temperature (28±1°C). The enrichment diets were then standardized and added at a rate of 0.5 mL L^{-1} (oil emulsions) and 6×10^{10} CFU mL^{-1} (probiotics) to the respective containers. Completely randomized experimental design was followed with 3 replicates. Strong aeration was provided to the rearing containers to maintain an optimum oxygen level at 5 ppm and to increase the enrichment rate[16]. To ensure the encapsulation of the diets, the enriched *Artemia* nauplii were examined under the microscope for assessing the time taken for 100% gut loading and unloading. The enriched *Artemia*, were kept in water free from the enrichment particle, to calculate *Artemia* nauplii gut unloading time. As the nauplii were transparent, the presence of the emulsion could readily be assessed by the yellowish gut. The time required for filling the gut with emulsions in the nauplii was observed periodically under light microscope.

RESULTS

Total 6 different isolates from fish guts were obtained on nutrient agar plates for their morphological and biochemical characterization. Mixed cultures of organisms in the nutrient agar plates with smooth round colony and large dull white irregular colonies and small pin point colonies were screened as individual isolates and given no B1, B2, B3, B4, B5 and B6 and identified to their species level. The description of the organisms is given in the Table 1. The strains 3 (2 Gram+ve Cocci and 1 Gram-ve rod) out of 6 were selected on the basis of specific morphological and antagonistic properties for further studies.

All 6 isolates from the gastrointestinal tract of *S. viride* was assessed *in vitro* for its inhibitory activity against common fish pathogen *A. hydrophila* and *V. harvey* by well diffusion assay. Out of the 6 strains, the B1, B2 and B5 strains registered the antagonistic activity and remaining 3 strains gave no zone of inhibition against the 2 fish pathogenic bacteria. The size of zone of inhibition was measured using centimeter scale and found between 2-5 mm (Table 2).

Screening for probiotics properties

Resistance to low pH: The selected isolates, *Enterococcus* sp., *Streptococcus* sp. and *Bacillus* sp. recorded maximum tolerance to pH 3 even after an exposure of 24 h. However, with pH 4, the viability was constant in all isolates even after 24 h. Table 3, 4 and 5 show the pH resistance.

Table 1: Identification of bacterial isolates by morphological and cultural characteristics

	Morphological characterization					
Strains	B1	B2	B3	B4	B5	B6
Gram staining	+ve	+ve	+ve	+ve	+ve	-ve
Motility	Motile	Motile	Motile	Motile	Motile	Motile
Indole test	-ve	-ve	-ve	-ve	+ve	-ve
Methyl red	-ve	-ve	-ve	-ve	-ve	-ve
Voges proskauer	+ve	+ve	+ve	+ve	-ve	-ve
Citrate utilization	+ve	-ve	-ve	-ve	-ve	-ve
Catalase	-ve	-ve	+ve	+ve	+ve	-ve
Starch hydrolysis	+ve	+ve	+ve	+ve	-ve	-ve
Urease test	-ve	-ve	-ve	-ve	-ve	-ve
Oxidative	-ve	-ve	-ve	-ve	-ve	+ve
Nitrate reduction	-ve	-ve	-ve	-ve	-ve	-ve
Glucose	+ve	+ve	+ve	+ve	+ve	+ve
Fructose	+ve	+ve	+ve	+ve	+ve	+ve
Sucrose	+ve	+ve	+ve	+ve	+ve	+ve
Maltose	+ve	+ve	+ve	+ve	+ve	+ve

Table 2: Antagonistic activity of gut microflora

Strains	B1	B2	B3	B4	B5	B6
Aeromonas hydrophila	2.0 mm	2.5 mm	-	-	3 mm	-
Vibiro harvey	2.5 mm	3.0 mm	-	-	5 mm	-

Table 3: Low pH resistance of strain 1-*Enterococcus* sp.

pH	0 h	1 h	2 h	3 h	6 h	12 h	24 h
7.3	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC
1	-	-	-	-	-	-	-
2	-	-	-	-	-	-	-
3	90	144	TNTC	TNTC	TNTC	TNTC	TNTC
4	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC

TNTC: Too numerous to count

Table 4: Low pH resistance of strain 2-*Streptococcus* sp.

pH	0 h	1 h	2 h	3 h	6 h	12 h	24 h
7.3	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC
1	-	-	-	-	-	-	-
2	-	-	-	-	-	-	-
3	140	186	TNTC	TNTC	TNTC	TNTC	TNTC
4	210	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC

TNTC: Too numerous to count

Table 5: Low pH resistance of strain 5-*Bacillus* sp.

pH	0 h	1 h	2 h	3 h	6 h	12 h	24 h
7.3	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC
1	-	-	-	-	-	-	-
2	-	-	-	-	-	-	-
3	170	200	TNTC	TNTC	TNTC	TNTC	TNTC
4	207	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC

TNTC: Too numerous to count

Bile salt concentration: The results showed the bile salts at 0.15, 0.25 and 0.50% concentrations did not affect the viability of *Enterococcus*, *Streptococcus* and *Bacillus* in MRS medium (above 50% of survival). The results are given in Fig. 1a-c.

Auto aggregation: The cellular autoaggregation of probiotic strains were evaluated and compared with other standard probiotic cultures (Fig. 2). The highest percentage of autoaggregation was noted in the B1 strain (86%) when compared to B2 (74%) and B5 (46%) after 6 h incubation.

Bioencapsulation potential of probiotics

Gut loading time of *Artemia salina* nauplii: Results showed that the time required for loading coconut oil, sunflower oil, cod liver oil and gingelly oil) and probiotics (*Enterococcus*, *Streptococcus* and *Bacillus*) varied significantly among themselves. Of the diets tested, the probiotics enriched *Artemia* nauplii reached a full gut condition between 30-40 min, vegetable oil emulsions enriched *Artemia* nauplii reached a 100% gut full condition ranged between 55 and 80 min (Table 6). *Streptococcus* strain enriched *Artemia*

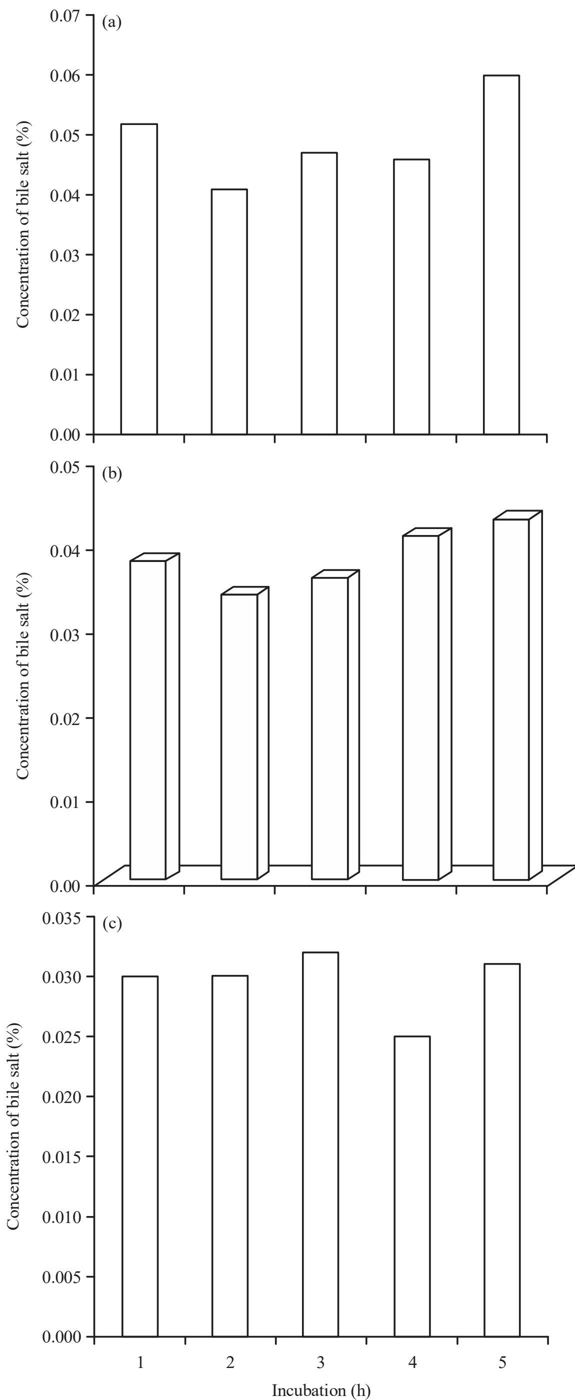

Fig. 1a-c: Bile salt tolerance of (a) *Enterococcus* B1, (b) *Streptococcus* B2 and (c) *Bacillus* B5

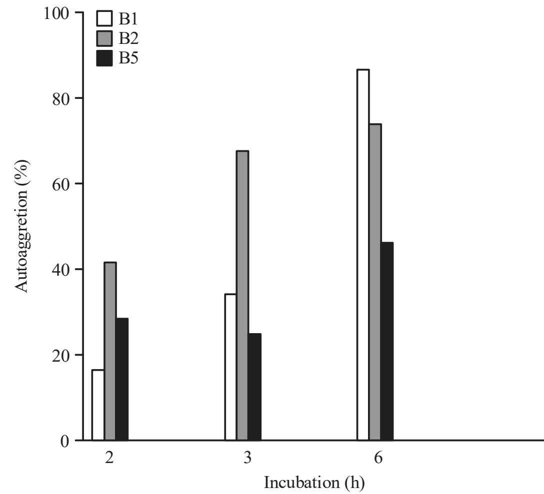

Fig. 2: Autoaggregation potential of strain B1, B2 and B5

Table 6: Gut loading time of *Artemia salina* nauplii

Experiments	Artemia gut loading time (min)
Coconut oil	80
Sunflower oil	50
Cod liver oil	70
Gingelly oil	55
Enterococcus	35
Streptococcus	30
Bacillus	40

Table 7: Evacuation time of *Artemia salina* nauplii

Experiments	Artemia gut evacuation time (min)
Coconut oil	58
Sunflower oil	35
Cod liver oil	78
Gingelly oil	55
Enterococcus	15
Streptococcus	12
Bacillus	20

nauplii reached a full gut condition at 30 min followed by *Enterococcus* enriched *Artemia* nauplii (35 min).

Evacuation time of *Artemia salina* nauplii: The gut evacuation for enriched *A. salina* with probiotics time ranged between 12-20 min, while for fish oil emulsion, it ranged between 35 and 78 min and for vegetable oil emulsion enriched *Artemia* nauplii (Table 7). The gut retention time in the *A. salina* nauplii revealed that the time required for evacuation with loaded vegetable oils and probiotics varied significantly.

DISCUSSION

The parrot fish is a famous fish for export in Chennai, India, so improvement of its growth and immune system is very important. It was shown that probiotics can improve the growth parameters and immune system of various fish species in aquaculture. Therefore, the present study was aimed to use some isolated probiotics from digestive tract of parrot fish to improve its culture condition.

In the present study, out of the 6 bacterial isolates screened for use as probionts, only 3 strains (50% of the total) viz., B1, B2 and B5 showed antagonism against *A. hydrophila* and *V. harvey*.

The fish intestine is a favorable ecological niche for microorganisms, which reach much higher numbers than in the surrounding water[19]. There are several studies have been reported for the isolation of bacterial strains with probiotic potential from different sources. Jahangiri *et al.*[20] reported the 6 probiotic bacteria were isolated from digestive tract of Guppy fish and these isolates demonstrated inhibition zones in the range of approximately 14-20 mm against the indicator strain. Also, a similar frequency of inhibitory bacteria was observed for isolates from halibut larvae[21], rainbow trout[22], turbot[23] and shrimp[24]. The reduction of pathogen growth and cell density indicate that extracellular bacteriolytic products produced by probiotic bacteria were responsible for this inhibition[13].

The present study, the selected bacteria with probiotic potential is resistance to acid and bile salts because they should tolerate low pH and bile acids. Being resistant to low pH and bile salt are the major selection criteria for probiotic strains. Since, to reach the small intestine they have to pass through from the stressful conditions of stomach[25]. Although in the stomach, pH can be as low as 1.0 in most *in vitro* assays pH 3.0 has been preferred. Due to the fact that a significant decrease in the viability of strains is often observed at pH 2.0 and below[26]. Although the bile concentration of the human gastro intestinal tract varies, the mean intestinal bile concentration is believed to be 0.3% w/v and the staying time is suggested to be 4 h[26].

Presently, the cellular autoaggregation of probiotic strains were evaluated and compared with other standard probiotic cultures. The ability of probiotic bacteria to form cellular aggregates is considered a desirable characteristic as they can potentially inhibit adherence of pathogenic bacteria to intestinal mucosa either by forming a barrier via self-aggregation or coaggregation with commensal organisms on the intestinal mucosa or by direct coaggregation with the pathogens to facilitate clearance[27,28].

For the gut loading time, of the 7 different diets tested, the probiotics enriched *Artemia* nauplii reached a full gut condition between 30-40 min than other diets tested. Similarly, Citarasu *et al.*[29] depicted the effect of feeding *Artemia* enriched with stresstol and cod liver oil on the growth and stress resistance of the *Penaeus indicus* post larvae. Full gut loading time of *Artemia* with emulsified fish oil, stresstol and oil mixed with stresstol was 127, 95 and 110 min, respectively. The results obtained in the present study were more or less similar with the above results. Smith *et al.*[30] described a method whereby the food in the gut is evacuated and replaced by inert plastic beads. The materials used for enrichment of *Artemia* nauplii are differed from the above study. So the time variations recorded for oils and probiotics are totally different from the above results.

The enrichment process in *Artemia* is generally regarded as a "bioencapsulation" process whereby the *Artemia* ingests enrichment particles until the gut is full. Feeding on large nauplii with higher energy content is advised since the target species will spend less energy capturing a smaller number of prey to fulfill its energetic requirements, unless that interferes with the feeding processes of the target species[31]. The result revealed that the gut loading and evacuation time of *Artemia* nauplii varied according to the oils and probiotics used for the experiments. Probiotics take less time for gut loading and retention when compared to oils.

CONCLUSION

It could be concluded that the indigenous fish gut bacteria provided benefit to the culture fishes in terms of pathogen protection and increasing growth potential and thus fulfilled the major requirements of being effective probiotics. Results of this experiment indicated that adult *Artemia salina* had high ability in enrichment with the probiotic bacteria viz., *Enterococcus, Streptococcus* and *Bacillus* and enrichment time had a positive ratio with attached bacteria to *Artemia* nauplii. However the principles behind their uses remain sound and their full potential needs to be explored further more and works are needed to assess the probiotic effects on growth and immune responses in fishes, including Indian major carps and other ornamental fishes.

SIGNIFICANCE STATEMENT

This study discover the antagonistic effect of probiotic against fish pathogens and their bioencapsulation potential that can be beneficial for Aquaculture. This study will help the researcher to uncover the critical areas of bioencapsulation potential by *Artimia* nauplii that many researchers were not able to explore. Thus a new theory on these gut loading and gut evacuation time of probiotics may support of growth and immune responses of fish community may be arrived at.

ACKNOWLEDGMENT

The authors are thankful to the authorities of Mohamed Sathak College of Arts and Science, Sholinganallur, Chennai, India for providing necessary facilities.

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