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Evaluation of bagasse assisted biostimulation in coastal aquaculture through field and molecular approaches

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ABSTRACT

A zero-water exchange system is an environmentally friendly alternative to conventional aquaculture for producing high-density shrimp. We present here the development and evaluation of bagasse biostimulation technology for maintaining lower ammonia and achieving higher shrimp production under field conditions. Total thirteen field trials were conducted in zero water exchange systems cultured with tiger shrimp Penaeus monodon. Results demonstrated up to 52% ammonia removal in the biostimulated culture ponds, due to a biofilm mode of growth of nitrifying consortia onto bagasse. The autotrophic ammonia oxidizing bacterial biofilm population was quantified using real-time PCR assay targeting ammonia monooxygenase (amoA) gene, which was found to be in the range of 10⁴-10⁵ and 10⁵-10⁶ amoA gene copies/g of the substrate based on the amount of aeration and duration of the treatment with bagassebiostimulator applied in the ponds. There was no significant difference in total plate counts of heterotrophic bacteria and Vibrio sp. in soil and water of control and the treatment ponds. Higher shrimp production from 4-23% in Tamil Nadu and 23-28% in Gujarat was achieved due to periphyton formation onto bagasse serving as a natural feed for the shrimp. Bagassebiostimulation technology is advantageous due to its cost effectiveness and simple technique and can easily be adopted by aqua-farmers using locally available sugarcane bagasse thereby ensuring water remediation and good yield. © 2013 Trade Science Inc. - INDIA

INTRODUCTION

Over the last few decades, there has been the continuing decline of marine fisheries and the increased de-

KEYWORDS

Penaeus monodon; Bagasse; Ammonia; Nitrifying biofilm; *amoA*; Real-time PCR.

mand for seafood by consumers^[1], which has led to the development and enhanced production of marine aquaculture. The current worldwide growth rate of the aquaculture business (8.9 - 9.1% per year) is needed in or-

der to cope with the problem of shortage in protein food supplies^[2]. However, environmental challenges are being increasing recognized as significant constraints on aquaculture production and trade worldwide. Shrimp aquaculture has expanded rapidly worldwide especially in tropical areas, such as Southeast Asia and Latin America. One approach to improving sustainability has been the development of high intensity grow-out systems with no water discharge during the crop cycle^[3]. Zero-water exchange system is capable of producing high-density shrimp yields^[1]. However, this system also generates ammonia, which is the major end product of protein catabolism. Ammonia remains in the form of unionized ammonia (NH₃) and ionized ammonia $(NH_{4}^{+})^{[4]}$. The proportion of unionized and ionized ammonia varies with pH and temperature. Unionized ammonia is a critical water quality parameter and toxic to aquatic life, which adversely affect shrimp yield. Development of new economically feasible eco-friendly products from agricultural wastes / byproducts for coastal aquaculture is the objective of our continued research^{[5-} ^{7]}. Provision of artificial feed accounts for nearly 50– 60% of the production cost and more often is beyond the reach of poor farmers. Further, only 15-30% of nutrient input is converted into harvestable products in most feed-driven, pond production systems, the remainder being lost to the sediments, effluent water and the atmosphere^[8]. In recent years, efficient utilization of agrowastes has been increasing. Substrates provide sites for epiphytic microbial production consequently eaten by fish food organisms and fish. Fish easily exploit the sessile forms of bacteria colonized on the surface of substrates as compared to free planktonic forms. In this direction, adoption of microbial biofilm-based on agrowaste-periphyton in freshwater aquaculture system has the capacity to increase the productivity by conversion of nutrients into harvestable products^[9-15]. However, reports on integration of this technology in coastal aquaculture under field condition is not available.

In India, large amount of solid wastes are produced by agriculture-based industries, mainly sugarcane bagasse from distilleries and sugar industries. Sugarcane bagasse, the residue obtained after crushing the sugarcane to extract the broth, is the most abundant lignocellulosic residue^[16]. Although most of bagasse has been employed in the own sugarcane industry to generate energy, there is a surplus of this agro-industrial waste, and several alternatives for its utilization have been evaluated, among which bagasse as cell support in different bioprocesses^[17]. Bagasse consists of approximately 50% cellulose, remaining hemicellulose and lignin and 2.4% ash. Because of its low ash content, bagasse offers numerous advantages in comparison to other crop residues such as rice straw and wheat straw, which have 17.5% and 11.0% ash contents, respectively for usage in bioconversion processes using microbial cultures. Bagasse can be used as the source of carbon (energy), and it can also be used as an inert solid support. Over the years, a large number of micro-organisms including bacteria, yeasts and fungi have been used for cultivation on bagasse^[16]. Diego et al.^[18] has used bagasse as alternative low cost biomaterial for the immobilization of C.guilliermondii for fermentation application. We had earlier reported that bagasse has the great potential in bioremediation of ammonia and nitrite from coastal water under laboratory condition^[5-7], which has mainly been attributed to the enhancement of autotrophic nitrifying bacteria. However, we neither demonstrate this through any field trial, nor any molecular tool was applied to quantify ammonia oxidizing bacteria (AOB's) in bagasse biofilm under field condition. Whether this translates into improvements in shrimp growth and production efficiency remains to be established. AOB's are extremely slow growing organisms and resist culture. This makes them difficult to be detected in coastal environments by cultivation-dependent traditional methods, which have qualitative and quantitative biases and underestimate by several orders of magnitude, due to the slow growth rates and long incubation period, the small size of the colonies and co-contamination with fast growing heterotrophic bacteria^[19,20]. Alternatively, culture independent molecular techniques are therefore used to monitor bacterial populations, for enhancement of biological treatment, because they tend to recover the entire diversity. Molecular detection systems based on the functional genes, which do not rely on traditional cultivation methods appear promising in determining microbial populations in aquaculture systems^[21,22]. Real time PCR has shown much higher sensitivity for the detection of nitrifying bacteria^[23-25]. In this paper, for the first time, we present the successful field demonstration of bagasse as a biostimulator to control ammonia and to achieve higher shrimp production in coastal

aquaculture with major emphasis on the quantification of indigenous ammonia oxidizing bacteria onto bagasse biofilm using molecular technique targeting *amoA* genes.

MATERIALS AND METHODS

Preparation of biostimulator

The following product was prepared from bagasse: Raw bagasse was immersed in water and then autoclaved to kill the bagasse bacteria. The resultant material was then sun-dried for 12 h. Bagasse material weighing approximately 200 g each was tied in Cuddapah black limestones. Approximately 10 kg of bagasse materials were placed in one hectare pond.

Identification and participatory monitoring of shrimp farms for field trials

Shrimp farms for field trials were selected at Tamil Nadu and Gujarat where ammonia was perceived as one of the major problems affecting the productivity of shrimps. Those farmers, who continually encountered such problems of ammonia and pond bottom blackening were identified for carrying out the field trials. The farms were also selected in such a way that the performance and results of field trials could be observed by large number of neighbouring farmers in a particular locality. In addition to that farmers were also identified on the basis of possessing sufficient scientific understanding of knowledge about the concept of bioremediation. Importance was also given for easy accessing of trial ponds. The identified farmers could able to communicate the results confidently to other farmers who had ammonia problem in their ponds. The neighbouring farmers were totally involved in monitoring of the farms during the trial period.

Total ten field trials were carried out in aerated zero water exchange systems cultured with tiger shrimp *Penaeus monodon* in Tamil Nadu for the period of 3 to 11 weeks further indicated as T1, T2, T3, T4, T5, T6, T7, T8, T9 and T10. In Gujarat, two trials were conducted for the period of 8-9 weeks, further indicated as T11 and T12.

In each shrimp farm, control ponds were also selected, where no bagasse was applied. Ammonia levels were monitored in treatment and control ponds at weekly and monthly intervals. Commercially available feed was used in all the ponds with frequency of feeding gradually increasing with the advance of culture period.

Analysis of soil and water quality parameters

The pH, salinity, alkalinity, dissolved oxygen (DO) and total ammonia nitrogen (TAN) of water samples were determined using standard methods^[26,27]. The pH and electrical conductivity in soil samples were measured with a pH meter and EC meter respectively. Soil water ratio for pH and EC measurement was 1: 2.5. Organic carbon was determined using the chromic acid digestion method. Each parameter was measured in triplicate from every treatment and control ponds.

Determination of total heterotrophic bacteria and vibrio counts

Total plate count (TPC) of bacteria in soil, water and bagasse biofilm was estimated in triplicate on nutrient agar by the spread plate method. For estimation of total *Vibrio* counts, TCBS agar was used. A known quantity (0.5 g) of bagasse was collected from T1 to T6 on 7th day of treatments and from T7 to T10 on 30th day of treatments. Then, samples were collected from all the ponds on the harvest day. Bagasse samples were rinsed twice to remove loosely adherent cells. The substrate was later re-suspended in phosphate buffered saline (PBS) and vortexed for 3 minutes to dislodge biofilm cells and the bacterial counts of the suspension were estimated as number of colony forming units per grams of substrate (CFU/g).

DNA extraction from soil samples and bagasse biofilm

The genomic DNA of the bacterial population was extracted from approximately 0.4-0.6 g of wet soil samples with a FastDNA spin kit for soil (UltraClean Soil DNA Kit, MO Bio laboratories, Carlsbad, California) using bead beating according to the manufacturer's instructions. DNA extraction procedures can miss entire groups of gram positive organisms that are difficult to lyse. Therefore, the genomic DNA was also extracted from composite soil samples using modified CTAB procedure^[25].

For extraction of DNA from bagasse biofilm, exact amount of bagasse (0.5g) was taken in triplicate in sterile centrifuge tubes containing phosphate buffer saline PBS and vortexed thoroughly for 2-3 min to dislodge the biofilm cells. The genomic DNA of the dislodged biofilm

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cells was extracted using modified CTAB procedure^[25]. The DNA concentrations were measured spectrophotometrically using UV-spectrophotometer (Shimadzu, Japan). The DNA thus obtained served as a template for polymerase chain reaction (PCR) and real time PCR. The PCR was also tested on autoclaved bagasse to make sure there was no cross reaction for the PCR.

PCR amplification of amoA

In the present study, the functional gene encoding *amoA* was amplified by PCR using Eppendorf thermal cycler (Master cycler gradient) according to protocol described previously^[22]. Following set of primers were used to PCR amplify 669 bp fragment of *amoA*.

- A-189F : 5'- GGN GAC TGG GAY TTC TGG-3'^[28]
- AMO2R : 5'- CCCCTCKGSAAAGCCTTCTTC-3'^[29]

Real-time PCR assay

The amplified amoA genes (669 bp) was purified and ligated using the pGEM- T Easy vector system (Promega, USA), which were then transformed into high efficiency competent cells (E. coli DH5-á) using the protocol described previously^[25]. Plasmid DNA concentration was measured spectrophotometrically using UV-spectrophotometer (Shimadzu), Concentration of plasmid containing cloned amoA (669 bp) was measured spectrophotometrically using UV-spectrophotometer (Shimadzu), which was used for standardization of real-time PCR. Amplification reactions were carried out in triplicate with real-time PCR master mix for the selected primer set on ABI real-time PCR. The thermocycling programme for the real-time PCR was as follows: 13 min 95°C initial denaturation, followed by 40 cycles of 20 s 95°C, 1 min 60°C and 1 min 68C. In all experiments, appropriate negative controls containing no template DNA (NTC) were subjected to the same procedure to exclude or detect any possible contamination.

Optimization of PCR using different primers.

Following primers sets were used for PCR amplification of 155 bp fragment of the *amoA* gene. AMOCIBA-F76+AMOCIBA-R83, AMOCIBA-F77+AMOCIBA-R83, AMOCIBA-F78+AMOCIBA-R83 AMOCIBA-F76 : 5'-CCATCGATCATGATTCC

	GGGTGC-3'.07 ^[25]
AMOCIBA-F77	: 5'-CCATCGAWCATGATT
	CCGGGTGC-3' ^[25]
AMOCIBA-F78	: 5'-CCATCGAHCATGAT
	WCCKGGTGC-3' ^[25]
AMOCIBA-R83	: 5'-ACGACAGGCAAGTGA
	GTCGGTC-3' (Present study)

RESULTS

Microphotography of biostimulator

The SEM image of biostimulator (Figure 1a,b) revealed high surface area for biofilm formation and there was no surface deformation during the process of preparation of biostimulator from raw bagasse. This also shows physical integrity of the biostimulator. Steam treatment has smoothened the surface by removing dust particles. These images also revealed high porosity and high surface area of the biostimulator.



Figure 1: Scanning electron microscopic images of bagassebiostimulator

Field demonstration of bagasse as biostimulator

Field trials were carried out by selecting shrimp farms, where ammonia was perceived as one of the

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major problems affecting the productivity of shrimps. The innovative way of using bagasse as biostimulator in shrimp pond has been demonstrated in Figure 2. The details of shrimp farms and the ranges of various soil and water quality parameters recorded on different sampling days are presented in TABLE 1.

Place	Experiment	Duration of bagasse treatment	Culture period	% ammonia increase(I) /decrease(D)	<i>amoA</i> in original soil samples	% gain in shrimp production
T1	Control		140	33I	+ve	
	Treatment	3	171	52D	+ve	5
T2	Control		132	17I	+ve	
	Treatment	3	132	38D	+ve	8
T3	Control		152	3%I	+ve	
	Treatment	2	152	29D	+ve	No significant gain
T4	Control		150	8I	+ve	
	Treatment	5	150	47D	+ve	20
Т5	Control		115	78I	+ve	
	Treatment	7	115	33I	+ve	8
T6	Control		147	39I	-ve	
	Treatment	11	138	23I	-ve	23
T7	Control		132	201	+ve	
	Treatment	11	132	23D	+ve	5
T8	Control		126	18I	+ve	
	Treatment	11	126	15D	+ve	4
T9	Control		94	56I	+ve	
	Treatment	6	94	2I	+ve	10
T10	Control		140	291	-ve	
	Treatment	11	140	8I	-ve	11
T11	Control		160	81I	+ve	
	Treatment	8	160	63D	+ve	28
T12	Control		147	1001	+ve	
	Treatment	9	145	75D	+ve	23

TABLE 1: D	etails of expe	rimental and	control shr	imp ponds
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T1 to T10: Shrimp ponds at tamil nadu, t11 and t12: shrimp ponds at gujarat

Effect of bagasse as biostimulator on ammonia levels in culture ponds

In the first field trial on bagasse biostimulator conducted in aerated shrimp pond T1, decrease in ammonia level from initial concentration of 0.25 mg/l to 0.21(16%), 0.15(40%) 0.12(52%) mg/l was observed after 1, 2 and 3 weeks of the treatment respectively as compared to steady increase in ammonia level (33%) in control pond from 0.24 to 0.32 mg/l in 3 weeks (Figure 3a). There was 5% gain in shrimp production in the treatment pond as compared to control pond.

In T2, ammonia level was (Figure 3b) decreased from the initial concentration of 0.78 mg/l to 0.64 mg/l (18%) over a period of 1 week of the treatment. Thereafter, there was a further decline and ammonia levels were 0.54 (31%) and 0.48 (38%) after 2 and 3 weeks (i.e up to harvest) of the treatment. In control pond, ammonia level increased from 0.75 to 0.88 mg/l in 3 weeks. In spite of short duration of bagasse application, there was an increase in shrimp production by 8% in the treatment pond as compared to control pond due to adequate aeration enhancing biofilm/periphyton formation onto bagasse.

In T3, ammonia decreased from initial concentration of 3.8 mg/l to 3.2 (16%) and 2.7 mg/l (29%) in 1 and 2 weeks (i.e up to harvest) of the treatment respectively (Figure 3c), whereas in control pond, ammonia level ranged from 3.6 to 3.7. There was no significant gain in shrimp production in the treatment pond as compared to control pond, due to the fact that there

abiotic stress on the animals.

In T4, ammonia decreased from initial concentra-

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was a short duration of bagasse application and very high initial ammonia concentration would have acted as

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Figure 2 : Demonstration of bagasse as biostimulator in shrimp pond

tion of 1.36 mg/l to 0.95(30%), 0.79(42%) and 0.72(47%) mg/l after 2, 4 and 5 weeks of bagasse application respectively (Figure 3d) as compared to control, where ammonia ranged from 1.22 to 1.32 mg/l.

There was an increase in shrimp production by 20% in the treatment pond as compared to control pond, which can be attributed to the longer duration of bagasse application, adequate aeration and the effective control of

ammonia level in the treatment pond, thereby reducing abiotic stress on the animals in the treatment pond

In control pond of T5, ammonia increased from an initial concentration of 0.492 mg/l to 0.606 mg/l and 0.876 mg/l after 8 and 16 weeks of the culture respectively, whereas in the other pond (initially without bagasse), ammonia slightly increased after 8 weeks of the culture from an initial concentration of 0.613 mg/l to 0.814 mg/l (Figure 3e). Bagasse was applied in this pond after 9 weeks of the culture. Ammonia level was decreased and maintained up to 0.782 mg/l till end of the culture (i.e. 16th week) due to bagasse application of 7 weeks, whereas in control pond, there was a steady increase in ammonia level from 0.492 to 0.876 mg/l (78%) during last 7 weeks of the culture. There was an increase in shrimp production by 8% in the treatment pond as compared to control pond.

In control pond of T6, ammonia increased from initial concentration of 0.271 mg/l to 0.549 mg/l as compared to ammonia increase in the pond (initially without bagasse) from 0.254 to 0.836 mg/l (Figure 3f) during first 8 weeks of the culture. Bagasse was applied after 9 weeks of the culture. Ammonia was maintained up to 0.836 mg/l till end of the culture (i.e. 20th week) due to bagasse application for 11 weeks. Ammonia increase was only 23% (0.678 to 0.836 mg/l) in the treatment pond as compared to 39% increase (0.394 to 0.549 mg/l) in the control pond. This can be due to the fact that AOB were undetectable in the original soil samples by PCR. Furthermore, less number of AOB were estimated in bagasse biofilm by real-time PCR. There was an increase in shrimp production by 23% in the treatment pond as compared to control pond, which can mainly be attributed to longer duration of bagasse application and adequate aeration enhancing the periphytic growth, which serves as a natural feed for the shrimp.

In the control pond of T7, ammonia has increased from initial concentration of 0.136 mg/L to 0.598 and 0.718 mg/L during second and fourth month of the culture, whereas in the treatment pond, ammonia has increased from initial concentration of 0.478 to 0.916 mg/L up to second month of the culture, thereafter ammonia has decreased up to 0.704 mg/L. (Figure 4a). There was 23% ammonia removal due to long duration of treatment with bagasse. There was increase in shrimp production by 5% in the treatment pond as compared to control pond. In control pond of T8, ammonia has increased from initial concentration of 0.416 mg/L to 0.624 mg/L and 0.739 mg/L during second and fifth month of the culture, whereas in the treatment pond, ammonia has increased from initial concentration of 0.623 mg/L to 0.943 mg/L up to second month of the culture, thereafter ammonia has decreased and level was maintained up to 0.804 mg/L (Figure 4b). There was 15% ammonia decrease due to long duration of treatment with bagasse. There was an increase in shrimp production by 4% in the treatment pond as compared to control pond.

In spite of longer duration of bagasse application in T7 and T8 ponds, higher shrimp production could not be achieved in these ponds due to inadequate supply of aeration. Furthermore, higher initial concentrations of ammonia could have acted as abiotic stress among the animals.

In control pond of T9, ammonia has increased from initial concentration of 0.371 mg/L to 0.58 mg/L up to fourth month of the culture, whereas in the treatment pond, ammonia has increased from initial concentration of 0.354 mg/L to 0.683 mg/L up to third month of the culture, thereafter ammonia level was maintained up to 0.698 mg/L due to application of bagasse during last 6 weeks of the culture before harvest (Figure 4c). There was a increase in shrimp production by 10% in the treatment pond as compared to control pond.

In control pond of T10, ammonia has increased from initial concentration of 0.269 mg/L to 0.702 mg/L as compared to ammonia increase in treatment pond from 0.419 to 0.899 mg/L (Figure 4d). In the treatment pond, after applying bagasse, ammonia increase was only 8% (from 0.82 to 0.899 mg/L) due to application of bagasse for 11 weeks as compared to 29% increase (0.415 to 0.702 mg/L) in the control pond without bagasse treatment. Data are shown with mean values. Ineffectiveness of ammonia removal was due to the undetectable nitrifying bacteria (PCR negative) in soil samples originally collected from this pond and also inadequate aeration in this pond. Furthermore, the low number of autotrophic nitrifying bacteria was estimated in bagasse biofilm by real-time PCR. There was an increase in shrimp production by 11% in the treatment pond as compared to control pond due to longer duration of bagasse application.

At Gujarat, in control pond of T11, ammonia has increased from initial concentration of 0.544 mg/L to



Figure 3: Ammonia detoxification using bagasse as biostimulator. Each point represents an average of triplicate measurements. (SPK, SPM-1, SPM-2, SPN, SPN-1, SPN-2 Shrimp ponds at Tamil Nadu)



Figure 4 : Ammonia detoxification using bagasse as biostimulator. Each point represents an average of triplicate measurements. (SPTN-1 to SPTN-4: Shrimp ponds at Tamil Nadu, SPGUJ-5 and SPGUJ-6: Shrimp ponds at Gujarat).

0.984 mg/L in 8 weeks, whereas in the treatment pond, ammonia has decreased from 0.521 mg/L to 0.194 mg/ L in two weeks after application of bagasse, thereafter ammonia level was maintained up to 0.414 mg/L during 57 days of bagasse treatment period till harvest (Figure 4e). There was increase in shrimp production by 28% in the treatment pond as compared to control pond.

In control pond of T12, ammonia has increased from initial concentration of 0.1 to 0.2 mg/L, whereas in treatment pond, there was a decrease in ammonia concentration from 0.2 to 0.05 mg/L during bagasse treatment period of 63 days (Figure 4f). There was increase in shrimp production by 23% in the treatment pond as compared to control pond.

Occurrence of amoA

AOB were detected qualitatively in soil samples by PCR amplification of 669 bp fragment of the *amoA* gene using *amoA* gene-specific primers (Figure 5a,b). The amplification confirms the presence of AOB in most of the soil samples originally collected from the ponds excepting T6 and T10, which revealed that nitrifying organisms were normal inhabitants of shrimp aquaculture environment.

PCR amplification of amoA for optimization of primers

Initially, PCR was used to screen the primer sets using plasmid DNA having cloned *amoA*. Amplification of expected bands on agarose gels provided as supplementary data (Figure.5c). PCR amplification of *amoA* using AOB specific primers sets (AMOCIBA-F76+AMOACIBA-R83, AMOCIBA-F77+AMOC IBA-R83, AMOCIBA-F78+AMOCIBA-R83) revealed that all three set of primers amplify expected fragment (155bp) of *amoA* gene.

Purified cloned *amoA* was used as a standard for real-time assay for the detection of nitrifying bacteria (Fig.6a,b,c). Based on the annealing temperature and G/C contents, AMOCIBA-F77+AMOCIBA-R83 was found to be the best primer set for development of realtime assay for nitrifying bacteria.

Estimation of nitrifying bacterial population in bagasse biofilm

In the present study, a quantitative real-time PCR assay targeting *amoA* was used to estimate ammonia oxidizing bacterial population size in bagasse biofilm.

The abundance of nitrifying bacteria onto bagasse biofilm increased in the samples during the course of experiment. Bagasse partially supply bacterial nutritional requirement thus facilitating better biofilm formation.

In the bagasse biofilm samples from T1 to T6, ammonia oxidizing bacterial count (no./g) was observed after first week of the treatment, reaching the highest value on the harvest day. In these samples, AOB counts were found to be in the range of 1372700-1609288, 1043040-1149570, 585915-723450, 1562822-1997158, 1845236-2489174 and 56728-221672 gene copies/g substrate respectively (Figure 7a) from 1 week to 3, 3, 2, 5, 7, and 11 weeks of the bagasse treatment. The autotrophic nitrifying bacterial biofilm population was found to be in the range of 10⁵-10⁶ amoA gene copies/g of the biostimulator. The highest number of nitrifying bacteria (106) were found in shrimp ponds-T1, T2, T4 and T5 due to adequate aeration (moderate aeration in T5) and longer duration of the treatment enhancing nitrifying growth onto bagasse biofilm, whereas in shrimp pond-T3, in spite of providing adequate aeration, AOB counts were found to be less (10^5) in biofilm by one order of magnitude, due to short duration (2 weeks) of the treatment with bagasse. In shrimp pond T6, in spite of longer duration of bagasse and providing adequate aeration, AOB counts were found to be less (10^5) by one order of magnitude due to undetectable nitrifying bacteria (PCR -ve) in soil samples originally collected from this pond.

In the bagasse biofilm samples from T7 to T10, ammonia oxidizing bacterial count (no./g) was observed after 4 weeks of the treatment, reaching the highest value on the harvest day. The highest number of amoA gene copies (10^5) were found in shrimp ponds-T7 and T8. AOB counts in T7 and T8 have increased from 294527 and 342561 to 689274 and 762179 gene copies/g substrate in 4 weeks to 11 weeks old bagasse biofilm, respectively (Figure 7b). In T9 sample, AOB counts have increased from 184218 to 368129 gene copies/g substrate in 4 weeks to 6 weeks old bagasse biofilm. In sample T10, AOB counts were found to be very limited in numbers (12882-49819 gene copies/g substrate) in 4 weeks to 11 weeks treatment with bagasse, which can be attributed to originally undetectable autotrophic nitrifying bacteria (PCR negative) in soil samples and also inadequate aeration.



(a) Detection of AOB through amplification of *amoA* gene (Lane 1 — 100 bp Marker, lane 2 - SPTN-1 sample, lane 3- SPTN2 sample, lane 4- SPTN3 sample, lane 5- SPGUJ-5 sample, lane 6- SPGUJ-6 sample).



(b). Detection of AOB though amplification of *amoA* gene (lane 1: 100-1000 bp marker, lane-2: SPK, lane-3: SPM1, Lane-4: SPM2, Lane-5: SPN, Lane-6: SPN1: Lane-7: SPN2).



(c). PCR amplification of 155 bp fragment of *amoA* using specific primers, Lane 1- 100 bp marker, lane-2 AMOCIBA-F76+AMOCIBA-R83, lane-3 AMOCIBA-F77 +AMOCIBA-R83, lane-4 AMOCIBA-F78+AMOCIBA-R83.

Figure 5: PCR amplification of amoA

Heterotrophic bacteria and Vibrio counts in bagasse treated and untreated ponds

In soil and water samples of control and treatment ponds, TPC of heterotrophic bacteria and *Vibrio* sp. were 10^5 - 10^7 CFU/g of soil (ml of water) and 10^2 - 10^3 CFU/g of soil (ml of water), whereas in bagasse biofilm, it was 10^5 - 10^6 and 10^3 CFU/g of bagasse respectively.

There was no significant difference in total *Vibrio* as well as in heterotrophic bacteria (TPC) counts between control and treatment ponds. The bacterial count on bagasse (no./g) was observed after the 30th day of the treatment, but the number increased gradually, reaching the highest value on the harvest day. The bacterial density per unit of bagasse was much higher than that in water.



(a). Quanutative detection of AOB using primer set ANIOCIDA-F /8 and ANIOCIDA-K85.



Slope: 3.308, Y-intercept: 31.912, Correlation coefficient: 0.998 (b). Quantitative detection of AOB using primer set AMOCIBA-F77+AMOCIBA-R83. Figure 6: SYBR Green real-time PCR assay for quantitative detection of AOB.

Effect of bagasse as biostimulator on other water quality parameters

Analyses of soil and water samples collected from treatment ponds indicated that soil parameters such as pH, electrical conductivity and organic carbon were found to be in the range of 7.46 to 8.88, 7.2-10.3 dS/m, and 0.43 to 1.92% respectively. Water quality parameters such as pH, alkalinity, dissolved oxygen (DO), were in the range of 7.21-8.8, 92—232 mg/l and 4.4 to 6.2 mg/l respectively. Data are shown with mean values. Salinity in T1 to T12 ponds were found to be in the range of 38-40, 32-53, 25-27, 35-38, 17-36, 29-45, 16-32, 13-30, 17-35, 27-48, 16-22, 15-20 ppt respectively. Soil and water quality parameters were

well within the safe limits, which indicate that there was no adverse effect of bagasse-biostimulator on the other water quality parameters. DO level was slightly lower in the ponds where bagasse was applied up to 11 weeks, which can be attributed to predominant heterotrophic biofilm formation, which accounts for the oxygen consumption^[30]. However, there was no significant difference in DO in control and treatment ponds.

Effect of periphyton on shrimp production

In Tamil Nadu, 4-23% higher production was achieved in the ponds treated with bagasse for 2-11 weeks. In the present study at Gujarat, shrimp production has increased from 23-28% in the ponds treated with bagasse for 8-9 weeks. Periphyton grown on the

bagasse could be a natural food for shrimp and could lead to enhanced shrimp production. Effective control of ammonia and higher shrimp productions in the bagasse treated ponds can solely be attributed to longer duration of bagasse application, adequate aeration and the presence of detectable AOB in original soil samples and sufficient numbers of AOB in bagasse biofilm. Furthermore, bagasse has higher water holding capacity which may be one of the factors responsible for efficient removal of ammonia. Previously, higher fish production has been reported in periphyton based aquaculture^[11-15]. Miller and Falace^[31] suggested two mechanisms for increasing fish production in artificial reefsbased systems: (1) the additional shelter provided by the substrate allows more of the resources to flow into fish biomass, and (2) the new primary production and attached benthic secondary production fostered by the artificial substrate support a new food web, part of which ends up in fish biomass.



Figure 7: Quantitative estimation of ammonia oxidizing bacteria in bagasse biofilm in different shrimp ponds. Each point represents an average of triplicate measurements. (a). Quantitative detection of AOB using primer set AMOCIBA-F78 and AMOCIBA-R83. (b). Quantitative detection of AOB using primer set

Estimation of difference in economic gain in treated crop over the untreated crop

Estimation of economic gain in treated pond as compared to untreated pond (1 ha size) for average 10% gain in production due to bagasse-biostimulation as compared to average production of 1500 kg in control pond has been given in TABLE -2. From the Table, it is evident that average economic gain in treated pond due to bagasse biostimulation is @ \$980/ha/crop.

TABLE 2 : Estimation of economic gain in treated pond as compared to untreated pond (1 ha size) for average gain of 10% in shrimp production due to bagasse-biostimulation with reference to average production of 1500 kg in control pond.

Expenditures (Per hectare/crop)				
Cost and transportation of bagasse :	\$20			
Labour charges :	\$20			
Cost of limestones :	\$30			
(Per hectare Pond/Crop)				
TOTAL (A):	\$70			
For 10% additional biomass/hectare/crop				
Average production in control ponds :	1500 kg			
Additional biomass due to bagasse-biostimulation :	150 kg			
Market shrimp price/kg :	\$7			
Price of additional biomass(B) 10% :	\$1050			
Economic gain due to bagasse-biostimulation(B-A):	\$980			

DISCUSSION

Lignocellulosic material consists of mainly three different types of polymers, namely cellulose, hemicellulose and lignin, which are associated with each other^[32]. Many factors, like lignin content, crystallinity of cellulose, and particle size, limit the digestibility of the hemicelluloses and cellulose present in the lignocellulosic biomass. Lignin is an amorphous heteropolymer, which is non-water soluble and optically inactive. The main purpose of lignin is to give the plant structural support, impermeability, and resistance against microbial attack and oxidative stress. Hemicellulose serves as a connection between the lignin and the cellulose fibers and gives the whole cellulose-hemicellulose-lignin network more rigidity^[32,33]. Earlier studies^[5-7] revealed that functional groups such as alcohol, ketones, and carboxylic groups of lignin present in bagasse can function as the reaction site and partly be involved in the adsorption of ammonium and nitrite ions by ion exchange mechanism with Ca^{++} and PO_4^{--} ions respectively. The authors also reported that the removal of ammonia in an aseptic condition is much slower than a non-sterile condition, which indicates that autotrophic periphytic growth is the key

player in ammonia removal as bagasse stimulates periphytic growth, which, in turn, removes ammonia from shrimp farm wastewater. Results of previous laboratory studies on the use of bagasse for ammonia removal have demonstrated that percentage ammonia removal was found to be decreasing with an increase in initial ammonia concentration.

There are several reports^[5-7,16] on the use of lignocellulosic materials for enhancing the growth of microorganisms. Azim et al.[13,14] reported lower ammonia concentration in the pond treated with bamboos and observed that periphyton improved water quality in aquaculture systems by increasing nitrification. The reduction in total ammonia content in bagasse based treatment has also been observed by Mridula et al.[15] and it was estimated that autotrophic productivity could be doubled by providing a substrate area similar to the pond water surface area. They also reported that ponds with substrates had lower total ammonia levels than control ponds and concluded that enhanced bacterial biofilms on the substrates might reduce ammonia levels through the promotion of nitrification. However, none of these studies have reported on the quantification of autotrophic nitrifying bacteria in the biofilm onto the bagasse. This study demonstrates for the first time the quantification of nitrifying bacteria onto bagasse-biofilm under field culture conditions. Bagasse-biostimulator enhanced autotrophic nitrifying biofilm growth in the range of 10^4 - 10^6 amoA gene copies / g of the biostimulator in the aerated ponds.

Pre-treatments of the lignocellulosic biomass have as a goal to enhance its digestibility^[34]. Each pre-treatment has its own effect(s) on the cellulose, hemicelluloses and lignin. Steam pre-treatment, lime pre-treatment, liquid hot water pre-treatments and ammonia based pretreatments are considered to be pre-treatments with high potentials. The main effects are dissolution of hemicellulose and alteration of lignin structure, providing an improved accessibility of the cellulose for hydrolytic enzymes. The solubilization of lignocelluloses components depends on temperature, moisture content and pH^[32]. In the present study, steam treatment has increased the digestibility of bagasse, which in turns, enhance biofilm formation onto bagasse with the result of increased shrimp production.

In the present study, pH was slightly alkaline in all the ponds, indicating favourable conditions for biological production. Because of proper aeration, DO level was maintained in T1, T2, T3, T4, T5, T6, T9, T11 and T12 ponds. The enhanced bacterial biofilm developed on the substrate would have brought down the ammonia level by nitrification. Such an observation was also made by Langis *et al.*^[35] who recorded lower ammonia levels in aquaria harbouring bacterial biofilm on glass panels.

Studies conducted by Burford et al.^[3] suggested that L.vannamei are capable of ingesting and retaining nitrogen derived from natural biota. This study suggests that natural biota, which in this system was largely flocculated particles, can contribute substantially to L.vannamei nutrition in a high-intensity zero-exchange system. Bratvold and Browdy^[36] studied changes in water quality and microbial community activity due to AquaMats substrate added to tanks stocked with L. vannamei post-larvae and found that tanks with substrates and sand sediment had higher pH and total photosynthesis, lower turbidity, ammonia and orthophosphate and higher nitrification. They also reported that among the two substrate-based treatments, survival as well as yield of L. vannamei was higher with bagasse than with paddy straw. Talwar and Jhingran^[37] demonstrated that L.fimbriatus production can significantly be increased with the introduction of biodegradable plant substrates into the culture tanks.

Increased growth and production of different species have been obtained in substrate-based pond culture by previous researchers. Production increases of 47% (Cyprinus carpio)^[9], 59% (Oreochromis mossambicus)^[10], 77% (Labeo rohita)^[11] and 42% (Tor khudree)^[12] have been recorded with various substrates. The growth of *P.monodon* in the ponds treated with bagasse was significantly higher than the control, the percentage increases being 4-23% in Tamil Nadu and 23-28% in Gujarat. The overall survival and production of shrimp was also higher in the treatment ponds. The enhanced shrimp growth observed in substratebased ponds indicated effective utilization of the microbial biofilm that developed on the substrate, which was the only variable different from the control. Growth of shrimp in control ponds could be attributed to the natural and supplemental feed.

Shrimp industry has suffered drastic collapses from decreased growth and survival coming from an increase in stocking density. Reduced growth and survival of

shrimp cultured at high densities is thought to result from a combination of factors, which include a decrease of favourable space and natural food sources, an increase in adverse shrimp behaviour such as cannibalism, a degradation of water quality and an accumulation of undesirable sediment^[38,39]. During the last decade, adding artificial substrates to penaeid shrimp culture systems has successfully been demonstrated in USA, Turkey, Australia, Brazil and China to overcome the negative effect of increased stocking density on growth and survival^[36,39-44].

For the advantage of the artificial substrates on shrimp growth, various researchers have carried out experiments on various aspects^[3,36,39-40,42-43,45-50]. They obtained different results and gave different explanation including improvement of the water quality, addition of the natural food supplement, limited reproduction of pathogenic bacteria provide refuge for shrimp to escape any negative behavioural interactions and adding living space. However, there is not an agreement about the predominant factor among those factors. Ballester et al.[43,49] determined that growth and survival of shrimp were not enhanced in the presence of floated cages that had their biofilm periodically removed, suggested that the importance of using substrates for shrimp is not related to the space but to the availability of food provided by biofilm formed on the substrate, while recent study by Zhang^[51], which studied the effects of artificial substrates on the spatial distribution of shrimp in the intensive culture condition, suggested that the difference of the shrimp growth and survival were affected mainly by living space added with the addition of artificial substrates. Therefore, a better understanding of the effect of artificial substrate on shrimp performance is necessary. The results of present study have successfully demonstrated the advantage of bagasse as a biostimulator for enhancement of bacterial biofilm in maintaining ammonia and achieving higher shrimp production under real conditions of coastal aquaculture.

Nakano *et al.*^[52] developed microbial consortium for nitrogen removal from aquaculture through the coupling of ammonia-oxidation using *Nitrosomonas* sp., and denitrification using *Pseudomonas* sp. and *Alcanivorax* spp. Diep *et al.*^[53] isolated *Pseudomonas stutzeri* strains from catfish pond, which were effective in lowering soluble N (NH₄, NO₂ and NO₃) levels in fishpond water from 10 mg/l to negligible amounts after 4 days. The present study indicates that formation of autotrophic biofilm onto bagasse mainly depends on the adequate aeration, presence of nitrifying bacteria as well as duration of the treatment. The nitrifying organisms are aerobic and have a high requirement for oxygen. Fernandes *et al.*^[54] has demonstrated that in high-density ponds, the aerators served to stimulate bacterial growth and activity which consequently maintained the quality of the water to match that of lowdensity ponds. They observed a marked increase in ammonium content in the non-aerated pond at the end of the culture period. The result from the present study in shrimp pond T10, is in agreement with those of Fernandes *et al.*^[54] who reported that the removal of ammonia was not significant due to lack of aeration.

Fu *et al.*^[55] set up biological aerated filter bioaugmented with heterotrophic nitrifying bacterium *Lutimonas* sp. H10 for ammonia removal treatment of the circulation water in a marine aquaculture, where the ammonia removal was not improved. This bioaugmentation failure was attributed to the poor biofilm forming ability of the inoculated strain. The result from the present study in shrimp pond-T6 and T10 is in agreement with those of Fu *et al.*^[55] who reported that the removal of ammonia was not significant due to undetectable nitrifying bacteria (PCR -ve) in soil samples originally or less numbers of nitrifying bacteria in bagasse biofilm.

CONCLUSION

For biostimulation purposes, an important choice criterion is the substrate cost, which, combined with the interest in byproducts recycling, has been leading to an increasing search for cheap and available potential biofilm carriers. For this reason, in the present study, the innovative way of using inexpensive abundantly available bagasse as biostimulator has successfully been demonstrated for supporting biofilm formation, ammonia detoxification and higher shrimp production in coastal aquaculture. Ammonia oxidizing bacteria flourished as biofilm on the bagasse-biostimulator in the aerated shrimp pond and could maintain ammonia-N concentration within permissible levels. Bagasse biostimulation technology is a simple, cost effective bioremediation technology without much technical sophistication. The present study revealed that bagasse can very well be

placed in the ponds for three months, thereafter it starts biodegrading. Integration of this technology in zero exchange and water reuse systems could be advantageous for water and area savings, reduced risk of contamination and better environmental control. Based on the present research findings, following recommendations have been made: Application of 10 kg bagasse biostimulator/hectare shrimp pond; Longer duration of bagasse application, which should be 2-3 months before harvest; Supply of adequate aeration using long arm aerators; Presence of nitrifying bacterial population; Regular monitoring of ammonia and DO. However, further research is needed to determine the optimal ways to produce natural biota, principally microalgae and phytoplankton, and optimize the nutritional composition. It would also be beneficial to determine the role of natural biota in supplying the other nutritional requirements of the shrimp, and ultimately to determine the effect of the natural biota on shrimp growth.

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