



# Research Journal of **Microbiology**

ISSN 1816-4935



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## ***Bacillus subtilis* Isolated from Sugarcane Rhizosphere Produces PHA to Defend NaCl Induced Stress**

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### **ABSTRACT**

*Bacillus subtilis* isolated from sugarcane rhizospheric soil, was found to survive upto 9% NaCl, pH range of 5.5-9.0. It harboured a plasmid of 20 kb which conferred salinity tolerance in transformed *E. coli* colonies allowing the later to survive 5% NaCl stress. Both *Bacillus* and transformed *E. coli* colonies produced PHA, stained positive with Nile blue. At low pH and in presence of NaCl, both *B. subtilis* and plasmid transformed *E. coli* survived better and produced PHA, but cells did not sustain alkaline pH. Restriction enzyme digested fragments showed homology with genes encoding DHQase1, histidine kinase, dehydratase from Hot\_dog family, in Blast search analysis and are found to be associated with PHA synthesis.

**Key words:** *Bacillus*, PHA, salinity, plasmid

### **INTRODUCTION**

Plastics have become an important part of our modern life, used in different sectors of operations like packaging, building materials and consumer products. Each year, about 100 million tonnes of plastics are produced worldwide. Demand for plastics in India reached about 4.3 million tonnes and is expected to increase and the exact time for their degradation is unknown (Kalia *et al.*, 2000). Presently plastic and synthetic polymers are mainly produced using petro-based raw material which do not decompose easily, contributing to the environmental pollution (Ojumu *et al.*, 2004). Biodegradable plastics have emerged as alternative to overcome the environmental pollution. Polyhydroxyalkanoates (PHAs) are polyesters of hydroxyacids and are naturally synthesized numerous microorganisms having dual function as a reserve compound and as a stress metabolite accumulating in response to stress condition. PHAs have properties of biodegradable thermoplastics and their synthesis with sustained industrial scale production of polymers at low cost will boost the biodegradable plastic industry (Soam *et al.*, 2012).

*B. subtilis* is Generally Regarded As Safe (GRAS) organism by Food and Drug Administration and is one among the most widely used microbes for large scale production of recombinant proteins, amino acids and fine chemicals. *Bacillus* spp. among gram positive bacteria, are widely known for production of valuable metabolites, bioremediation and generation of bioenergy and some of them produce PHA upto 11-65% of their biomass. Various sources industrial waste, soya, malt waste and unrefined natural substrates have been used to produce PHA to reduce the cost of production (Anderson and Dawes, 1990; Akiyama *et al.*, 2003; Liu *et al.*, 2008; Sangkharak and Prasertsan, 2008).

Bacterial cells in soil face different stress conditions, such as low nutrient availability and detrimental physical, chemical, or biological factors, all fluctuating in time and space. To cope with this changing oligotrophic environment, soil bacteria have developed various survival strategies (Van Elsas and van Overbeek, 1992). Accumulation and degradation of PHA is one such strategy by which bacteria survive and sustain its growth (Okon and Itzigsohn, 1992). PHA is usually formed as intracellular inclusions during unbalanced growth and is accumulated during excess carbon in response to the limitation of essential nutrients and also serves as an internal reserve of carbon. Capability of bacterial cells to survive extended periods of starvation in the soil has been attributed to its ability to synthesize and degrade PHB (Flora *et al.*, 2010; Mercan *et al.*, 2002; Yuksekdag *et al.*, 2004). *Bacillus subtilis* isolated from the root rhizosphere of sugarcane was found to protect the plants from salinity stress (data not shown) and possess the capability to produce PHA in a salinity stress.

## MATERIALS AND METHODS

**Isolation of *Bacillus* spp.:** Soil sample from the root rhizosphere of sugarcane variety CoM 0265 (saline tolerant) was collected from Sugarcane Research Station, Padegoan, Maharashtra, India. Soil suspension (1:100 w/v) in sterile water was serially diluted and plated on LB agar containing 1-5% NaCl. *Bacillus* colonies that grew on 5% NaCl media were isolated, verified for their salinity tolerance and pure colonies were maintained on LB containing 5% NaCl (Fig. 1).

**Isolation and transformation of *Bacillus* plasmid:** Overnight grown *Bacillus* cultures were transferred to 50 mL LB medium containing 5% NaCl and grown on rotary shaker for 3 h at 30°C. Bacterial cells were recovered by centrifugation at 4000 rpm for 10 min at 4°C. Plasmid DNA was isolated by alkali lysis method and examined on agarose gel using Hind III digested lambda DNA as marker. Agarose gel purified *Bacillus* plasmid was transformed in to *E. coli* strain DH5 $\alpha$  using standard protocol and selected on LB agar plates containing 5% NaCl and confirmed the presence of plasmid (Sambrook *et al.*, 1989).

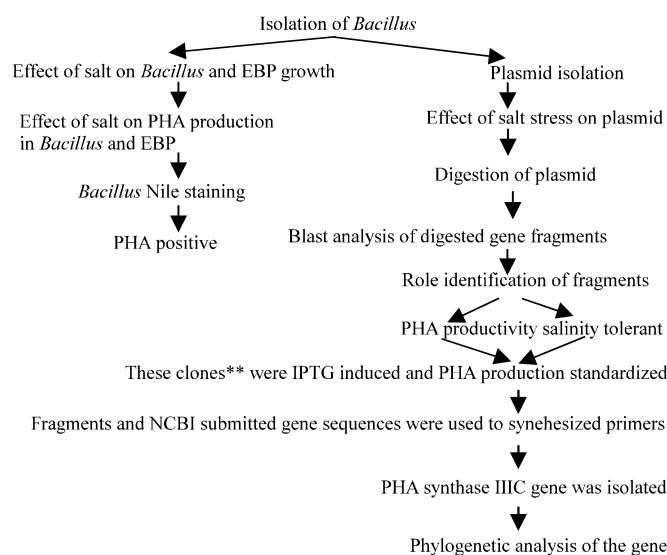


Fig. 1: Representation of the methodology used during experiment. \*EBP: Transformed *E. coli* with *Bacillus* plasmid. \*\*Clones: *E. coli* DH5 $\alpha$  with inserts of *Bacillus* plasmid gene fragments

**Growth curve analysis of *Bacillus* and *E. coli* transformed with *Bacillus* plasmid (EBP):**

Overnight grown culture (~12 h, 120 rpm at 30°C) was inoculated to LB media (1:100 v/v) with suitable concentrations of sodium chloride and incubated at 30°C upto 48 h on a rotary shaker set to 130 rpm. Concentration of NaCl used was 5, 7 and 9 and media pH at 5.5 and 9.0. Overnight grown cultures with and without 5% NaCl (OD at 600 nm ~1.0, 1:50 v/v) were used as inoculum. Growth of the organisms was monitored by recording the absorbance at 600 nm.

**Detection and assay for PHA:** Bacteria (*Bacillus* and EBP) were grown on LB (with and without NaCl amendment) at 30°C. Culture suspension was centrifuged at 10000 rpm for 10 min. Pellet was washed with sterile water, dried and weighed. Pellet was suspended in 1/10th volume sterile water and sonicated (15 sec, 8 cycles). Suspension was centrifuged at 10000 rpm for 10 min. Pellet was washed with ethanol: Acetone (1:1v/v) mixture and centrifuged at 10000 rpm for 5 min. PHA was extracted with two volume of chloroform overnight at room temperature on a rotary shaker at 150 rpm. Centrifuged at 10000 rpm for 20 min and chloroform extract was dried at 40°C, dry weight was recorded (Bonartseva and Myschkina, 1985; Kuniko *et al.*, 1988). Nile blue A was used to stain PHA using the protocol described by Ostle and Holt (1982).

**Digestion of plasmid DNA:** *Bacillus* plasmid was co-digested with EcoR1 and HindIII (1:1) and cloned into MCS site in pUC18. Recombinants were transformed into *E. coli* DH5 $\alpha$  and selected on LB medium with ampicillin, X-gal and IPTG (Sambrook *et al.*, 1989). Before sequencing, plasmids were isolated from the selected colonies, re-digested with same restriction enzymes and checked for the presence and size of the insert.

## RESULTS AND DISCUSSION

**Isolation of *Bacillus*:** *Bacillus* isolated from sugarcane rhizosphere exhibited salinity tolerance upto 5% on LB media. They showed characteristic translucent white slimy growth on the saline media. However, on saline free media they showed morphological characteristics similar to *Bacillus*. This lead us to speculate that slimy polymer production is salinity inducible, possibly protect the cells from salt stress and trait may be harboured on the extra chromosomal element (Fig. 2a, b). Growth analysis revealed that slimy polymer production is initiated at an early phase of growth in NaCl amended media and accumulates with time. To minimize the interference of the slimy polymer produced in DNA isolation, the cells were incubated for 3 h and harvested. Analysis revealed that it harboured a plasmid of ~20 kb. To examine the possibility that the plasmid might harbour the genes encoding for the salinity tolerance and slimy polymer production, it was transformed into *E. coli*, strain DH5 $\alpha$  (EBP) (Fig. 2c, d). EBP cells showed tolerance upto 5% NaCl and produced characteristic white translucent slimy polymer (Fig. 2d). This result suggests that *Bacillus* plasmid harbor genes that confer salinity tolerance as well as for polymer production.

Since, there was a strong correlation with salinity tolerance and polymer production, tests were carried out to examine its chemical nature. Tests for polysaccharides were negative and positive to nile blue staining. Nile blue staining showed bright orange fluorescence under UV fluorescence, a typical detection for PHA (Ostle and Holt, 1982). This suggested us that the slimy polymer produced by *Bacillus* and EBP under salinity stress is PHA (Fig. 3). Since, biodegradable plastics have emerged as alternative to overcome the environmental pollution using raw materials like Poly  $\beta$ -hydroxybutyrate and hydroxyl acetates, further studies were targeted to establish the conditions for PHA production.

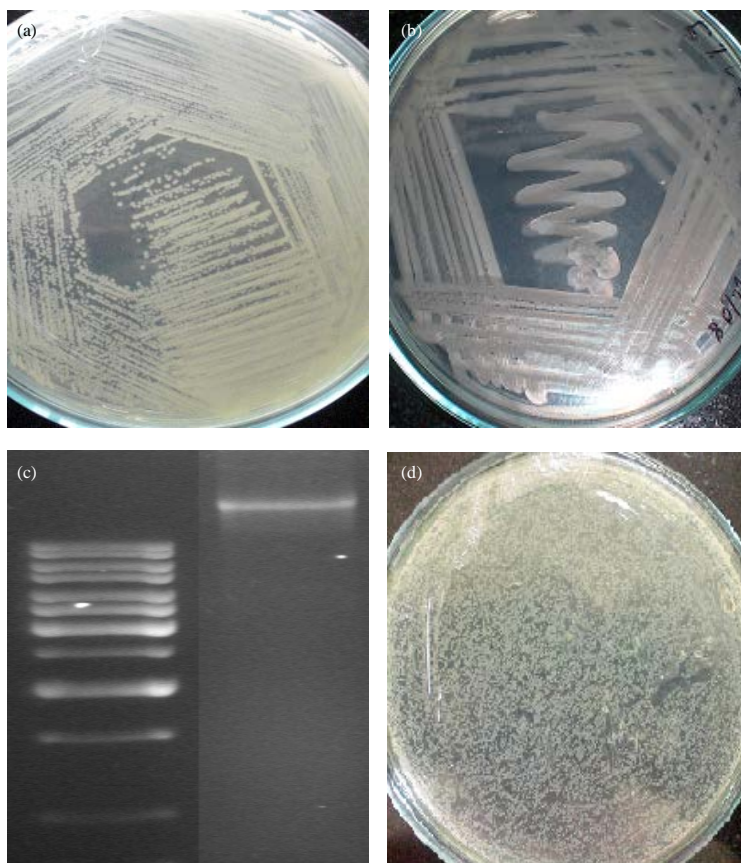


Fig. 2(a-d): *Bacillus* with salinity tolerance (a) LB with 10% soil extract, (b) *Bacillus* on LB with 5% NaCl, (c) Isolated plasmid ~20 kb on 0.8% agarose gel, Lane 1: Marker 1 kb, Lane 2: *Bacillus* plasmid and (d) *Bacillus* plasmid transformed *E. coli* (EBP) plated on LB with 5% NaCl, shows mat-like growth due to the excessive production of polysaccharide

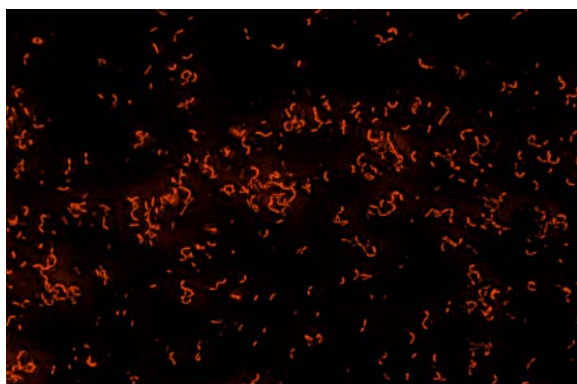


Fig. 3: Fluorescence microscopy of *Bacillus subtilis* isolated from root rhizosphere of sugarcane variety CoM 0265 showing PHA positive reaction with Nile blue staining (in collaboration with Agharkar Research Institute, Pune)

**Effect of sodium chloride on growth of *Bacillus* and EBP:** Factors like temperature, salt, pH affect the growth of organisms. Most of the bacteria grow better at pH range 6.0-8.0 and they activate transport mechanism to maintain normal physiological  $H^+$  ion concentration within the cell during stress. Growth kinetics in presence of NaCl was carried out at pH 7.0 at 30°C in LB media. *Bacillus* as well as EBP showed similar growth pattern upto 12 h and reached stationary phase after 24 h (Fig. 4a, b). *Bacillus* reached exponential phase within 20 h of inoculation at 5%, also showed vigorous growth at 7% NaCl (Fig. 4a), whereas significant reduction in growth was noticed at 9% NaCl. Growth of *Bacillus* cells slowed after 12 h and started undergoing sporulation. Biomass recorded after 24 h of incubation was 2.12, 1.75 and 1.52 g/100 mL for control, 5 and 7% NaCl concentration, respectively (Fig. 5a). Further incubation upto 32 h also showed relative increase in biomass at all concentrations of NaCl. Further, cells growing on 9% NaCl media showed significant recovery in growth after 28 h of incubation. Cells hardly survived in pH 9.0 with 5% NaCl. However, acidic media (pH 5.5) favoured the growth of *Bacillus* (2.10-2.42 g/100 mL) whereas reduction in biomass was significant at pH 9.0 (1.74 g/100 mL). In general, an exponential increase in cell growth was observed in media with amendments, except with pH 9.0 containing 5% NaCl and achieved stationary phase around 24-28 h, respectively.

On the contrary, EBP cells showed significant reduction in growth in presence of 5% NaCl and with increase in NaCl concentration (7 and 9%) growth was drastically reduced (Fig. 5c). Biomass was recorded 1.34 g/100 mL at 24 h in control, whereas it was 0.79 g/100 mL at 5% NaCl. EBP cells sustained NaCl concentration upto 5% (Fig. 4b) which is significantly higher compared to NaCl requirement for the normal growth of *E. coli* (Abdulkarim *et al.*, 2009). These results suggest that *Bacillus* cells have the ability to adapt for higher concentration of NaCl, to certain extent higher pH but not together. Earlier studies report that cells undergo dehydration under hypertonic conditions, cell wall rupture under hypotonic conditions and affects phospholipid composition as well as properties of cytoplasmic membrane. Organisms belonging to *Bacillus* spp. when exposed to severe stress undergo sporulation and survive (Bergmann *et al.*, 2013; Ramadas *et al.*, 2009).

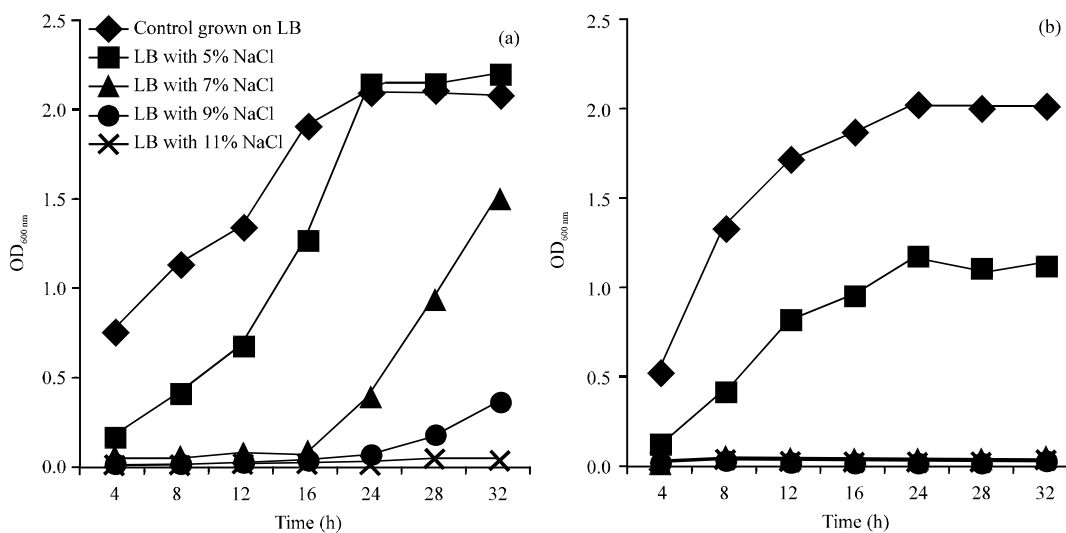


Fig. 4(a-b): Effect of NaCl on growth of (a) *Bacillus* and (b) EBP. The overnight grown culture was grown for various intervals of time in LB with modifications and the cell density was recorded as measure of OD<sub>600 nm</sub>

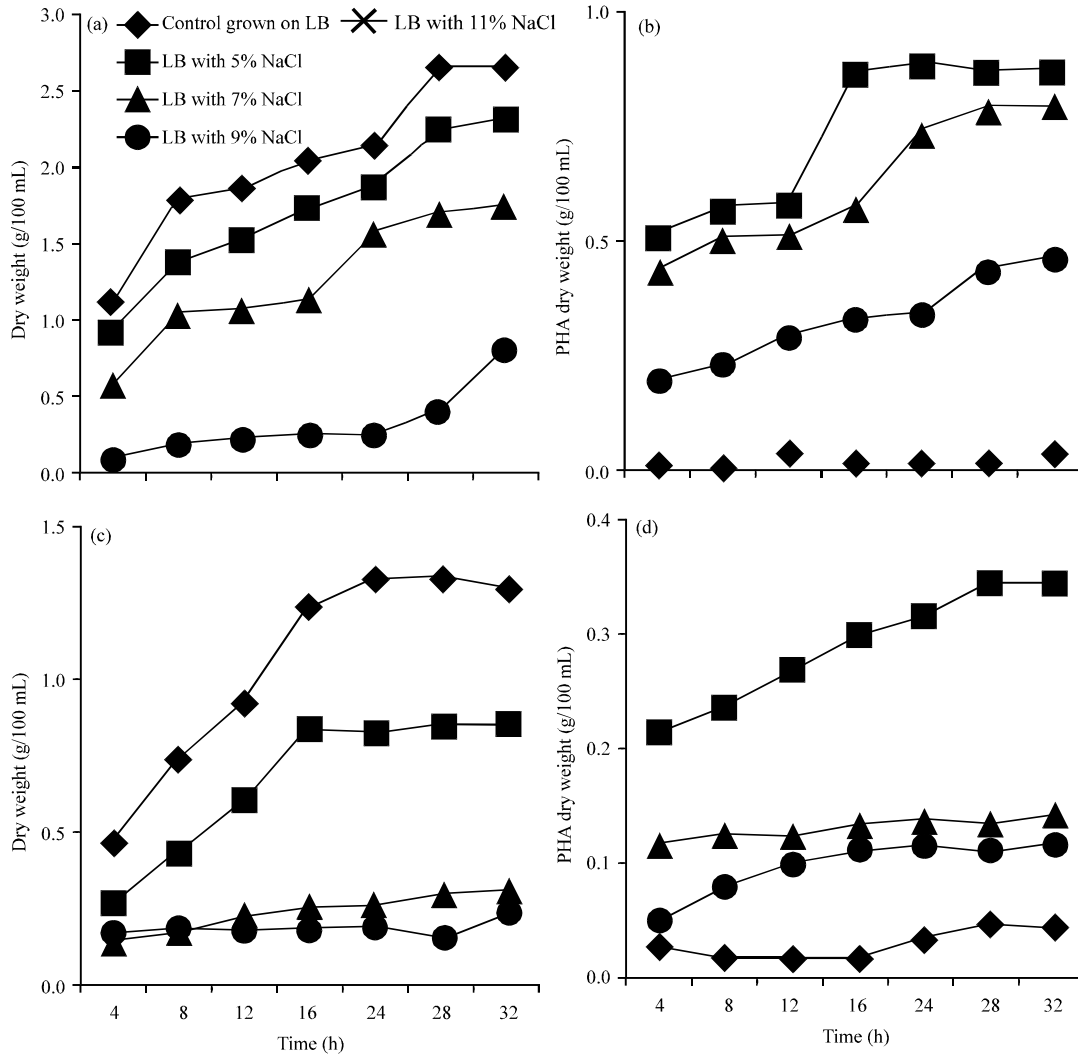


Fig. 5(a-d): Effect of NaCl on biomass and PHA content in (a, b) *Bacillus* sp. and (c, d) EBP

Further results of the study suggest that plasmid encoded genes may have played a significant role in facilitating cellular processes that provide protection and turgidity to cell wall and/or synthesising osmoprotectants in EBP cells.

**Effect of NaCl on PHA production:** PHA content of the *Bacillus* grown on 5% NaCl containing media was highest followed by 7% NaCl. PHA accumulation was slow upto 12 h of incubation followed by a steep rise till 24 h. After 24 h of incubation, PHA content was 0.89 and 0.74 g/100 mL at 5 and 7% NaCl which was significantly higher than the cells grown in control and media containing 9% NaCl (0.38 g/100 mL; Fig. 5a, b). PHA production was observed in EBP treated with 5% NaCl (0.32 g/100 mL) which was higher than EPB grown on 7.0 and 9.0% NaCl (Fig. 5d; 0.10 and 0.09 g/100 mL, respectively). These findings suggest that the ability of *E. coli* to produce PHA in NaCl inducible manner is characteristic of the genes encoded by the *Bacillus* plasmid which otherwise barely survive NaCl stress. Polyhydroxyalkanoate(s) (PHAs) are natural biopolymers, synthesized by numerous microorganisms, have dual function as reserve carbon and

metabolite accumulating in response to stress condition (Rehm, 2007; Shrivastav *et al.*, 2010). Organisms are known to accumulate PHAs generally upto 90% of cell dry weight (Verlinden *et al.*, 2007). Effect of NaCl concentration on PHA accumulation was found to correlate with activity of enzymes directly associated with polymer biosynthesis (Hiroe *et al.*, 2012; Singh *et al.*, 2009). PHA accumulation in response to high salt concentration, nutrient limitation (such as N, P, S or Mg) and excess carbon has been documented (Poblete-Castro *et al.*, 2012).

**BLAST analysis of plasmid encoded genes:** Our studies suggest that plasmid encoded genes play significant role in imparting the salinity tolerance and salinity induced PHA synthesis. Hind III and EcoR1 digested fragments of *Bacillus* plasmid were cloned and selected for blue/white colonies. Sequences of the positive clones were subjected to BLAST analysis (Table 1). Individual colonies were subjected to screening on 0.5 M IPTG and 3% NaCl saline media. Among colonies having clones RSC1023 (Plasmid stability factor), RSC1028 (3-dehydroquinate dehydratase), RSC1042 (Signal transduction histidine kinase), RSC1047 (Sensor histidine kinase) as well as RSC1018 and RSC1032 showed PHA production (Fig. 6). Although clones RSC1018 and RSC1032 did not show any similarity with known gene sequence in BlastN analysis, did produce significant amount of PHA in about 32 h (Fig. 6a, 0.63 g/100 mL) and 24 h (Fig. 6d, 0.68 g/100 mL) of growth with IPTG induction in presence of NaCl. However, IPTG induction alone did not facilitate PHA synthesis. This result suggests that these two ORFs codes for proteins which have significant functional role in production of PHA under salinity stress. RSC1023 (plasmid stability factor, TubZ) and RSC1028 (dehydroquinate dehydratase) induced PHA synthesis within 4 h of growth (0.80 and 0.50 g/100 mL). Dehydroquinate dehydratase triggered the PHA synthesis (0.60 g/100 mL) in the normal media in about 8 h of its growth (Fig. 6b, c). Members of plasmid stabilization family of proteins, ParA proteins, are responsible for plasmid-DNA segregation and maintaining stability (Bouet *et al.*, 2007). TubZ is closely related to GTPase superfamily and tubZ gene is co-transcribed with tubR which encodes a protein with DNA-binding capability (Larsen *et al.*, 2007). Although, it is difficult to explain, how the plasmid stability factor induces the synthesis of PHA, but it is possible that dehydroquinate dehydratase an enzyme associated with carbon catabolism, is possibly involved in generating the required carbon skeletons for PHA biosynthesis (Battah, 2009; Kempa *et al.*, 2008).

Histidine kinases (Hiks) are known as cellular sensory proteins and Hik domain is specifically phosphorylated in response to changes in environmental conditions (Kanesaki *et al.*, 2002). RSC1042, encodes for signal transduction histidine kinase, hardly induces PHA production (0.18 g/100 mL at 12 h) in presence of IPTG and NaCl (Fig. 6e). However, sensor histidine kinase (RSC1047) expression resulted in significant amount of PHA production till 12th h of growth (~0.34 g/100 mL) with IPTG induction and then declined. In general, stress mediated signalling mechanism involves kinase mediated activation of downstream cellular responses to mitigate the stress imposed and histidine kinases play a major role in triggering the signalling cascade (Doebber *et al.*, 2008; Marin *et al.*, 2003). This explains the significant enhancement of PHA synthesis in 4 h (0.62 g/100 mL) which decreased after 12th h (Fig. 6f) in presence of NaCl. Such specific His kinases (Hiks) and response regulators (Rres) have been reported to be involved in the perception and transduction of signals due to low temperature, salt stress, osmotic stress and metal ion deficiency (Paithoonrangsarid *et al.*, 2004; Suzuki *et al.*, 2004).

The cells harbouring PHA synthase IIIC clone (Fl31, HE591385) failed to produce PHA in the absence of IPTG induction. However, with IPTG, PHA production increased sharply after 12 h and



Table 1. Details of BLAST analysis of gene sequences related to salinity and PHA production

BLAST N analysis		BLAST X analysis		BLAST P analysis		NCBI pub med Search				
<i>Bacillus</i> gene Fragment/ Accession No.	Match	Query coverage/ identities/ AT-GC%	Gene product	ORF No.	E-value/ identity (%)	bStart/ Stop (largest ORF)	cFrame length (amino acids)	Conserved amino acids/sites/domain	Probable function	References
RSC1014/ GS887724	<i>B. megaterium</i>	76%/72% AT(64.1%) GC(35.9%)	Hypothetical protein bthur0013_47010	3	5E-119/99%	++	220	Lactamase_B superfamily (Metal dependent hydrolases)	Role in Lactic acid and glutathione, synthesis, Act as transporter for DNA uptake.	Yamamura <i>et al.</i> (2009)
RSC1016/ GS887727	<i>Geobacillus</i> sp.	12%/74% AT(59.7%) GC(40.3%)	Histidinol-phosphatase	4	1.00E-90/98%	+/+	89	histidinol-phosphatase (His J family)	Role in histidine biosynthesis	Coq <i>et al.</i> (1999)
RSC1018/ GS887728	<i>A. flavithermus</i>	AT(73.7%) GC(26.3%)	NSM	-	-	-	-	-	-	-
RSC1023/ GS887731	<i>B. megaterium</i>	AT(63.0%) GC(37.0%)	Plasmid stability	2	3E-29/98%	+/+	86	-	Maintenance of plasmid structure.	Larsen <i>et al.</i> (2007)
RSC1028/ GS887737	<i>B. megaterium</i>	98%/97% AT(56.4%) GC(43.6%)	3-dehydro quinate dehydratase	3	3E-36/92%	+/+	64	DHQaseI, catalytic active site (Arg, Ala, Gln), site	Aromatic acid (phenylalanine, tyrosine, tryptophan) and chorismate biosynthesis.	Battiah (2009), and Kempa <i>et al.</i> (2008)
RSC1032/ GS887740	-	-/- AT(76.8%) GC(23.2%)	NSM	-	-	-	-	-	-	-
RSC1040/ GS887719	No significant match	AT(69.2%) GC(30.8%)	Putative lipoprotein (ydhF)	6	3E-10/67%	-/+	49	-	pho-ydhf work together, phob-part of PHA synthetic pathway.	Eichenberger <i>et al.</i> (2003) and Stubbe <i>et al.</i> (2005a, b)
RSC142/ FN645942	<i>B. cereus</i>	74%/74% AT(59.7%) GC(40.3%)	Signal transduction histidine kinase	1	1.00E-27/68%	+/-	130	HAMP superfamily	Bacterial signalling cascade.	Marin <i>et al.</i> (2003) and Doebber <i>et al.</i> (2008)
RSC1047/ GS887707	<i>B. cereus</i>	86%/85% AT(59.8%) GC(40.2%)	Sensor histidine kinase	3	4.00E-101/57%	+/+	353	HATPase domain, Mg <sup>2+</sup> binding site, G-X-G motif	Act as Heat Shock Protein, DNA mismatch repair, Salt tolerance, cellular pH maintenance	Shima <i>et al.</i> (2008)

Table 1: Continue

BLAST N analysis		BLAST X analysis				BLAST P analysis		NCBI pub med Search		
Accession No.	Match	Query coverage/ identities/ AT-GC%	Gene product	ORF No.	E-value/ identity (%)	bStart/ Stop (largest ORF)	cFrame length (amino acids)	Conserved amino acids/sites/domain	Probable function	References
RSC1049/ GS887708	<i>B. cereus</i>	66/75% AT(59.9%) GC(40.1%)	Signal transduction	1	4.00E-19/68%	+/-	176	HAMP superfamily	Signalling domain present in every signal molecule	Marin <i>et al.</i> (2003) and Doebber <i>et al.</i> (2008)
RSC1050/ GS887709	<i>B. megaterium</i> plasmid pBM100	22/86% AT(63.2%) GC(36.8%)	histidine kinase Hypothetical protein pBM100-7	3	8.00E-27/55%	+/+	148	HTH_LUXR, helix_turn_helix, lux regulon	Controls expression of bioluminescence and increases during osmotic shock	Zavilgelskii and Kotova (2003)
RSC1051/ GS887721	<i>B. licheniformis</i>	8/76% AT(57.7%) GC(42.3%)	Putative integral inner membrane protein	6	1E-118/98%	+/+	245	Cneg_AbrB_dup superfamily	AbrB protein in nutrient depletion, express gene to integrate metabolic and environmental information.	Strauch <i>et al.</i> (2005) and Nagorska <i>et al.</i> (2008)
RSC1057/ GS887749	<i>B. licheniformis</i>	47/98% AT(62.6%) GC(37.4%)	Hypothetical protein BH01933	2	8E-72/75%	+/+	198	Ras_like_GTPase family. proteins like the GTP translation factors and G-alpha chain of the heterotrimeric G proteins	Regulates gene expression, cytoskeletal reorganization, vesicle trafficking, nucleo cytoplasmic transport, Microtubule organization.	Wicker-Planquart <i>et al.</i> (2009)

ORF No. was found using NCBI ORF finder tool. Symbolizes the presence of start and stop codon in the gene fragment using NCBI ORF finder tool. Frame length was calculated using NCBI ORF finder tool. \*These inserts are present in pairs in the clone. NSM: No significant match

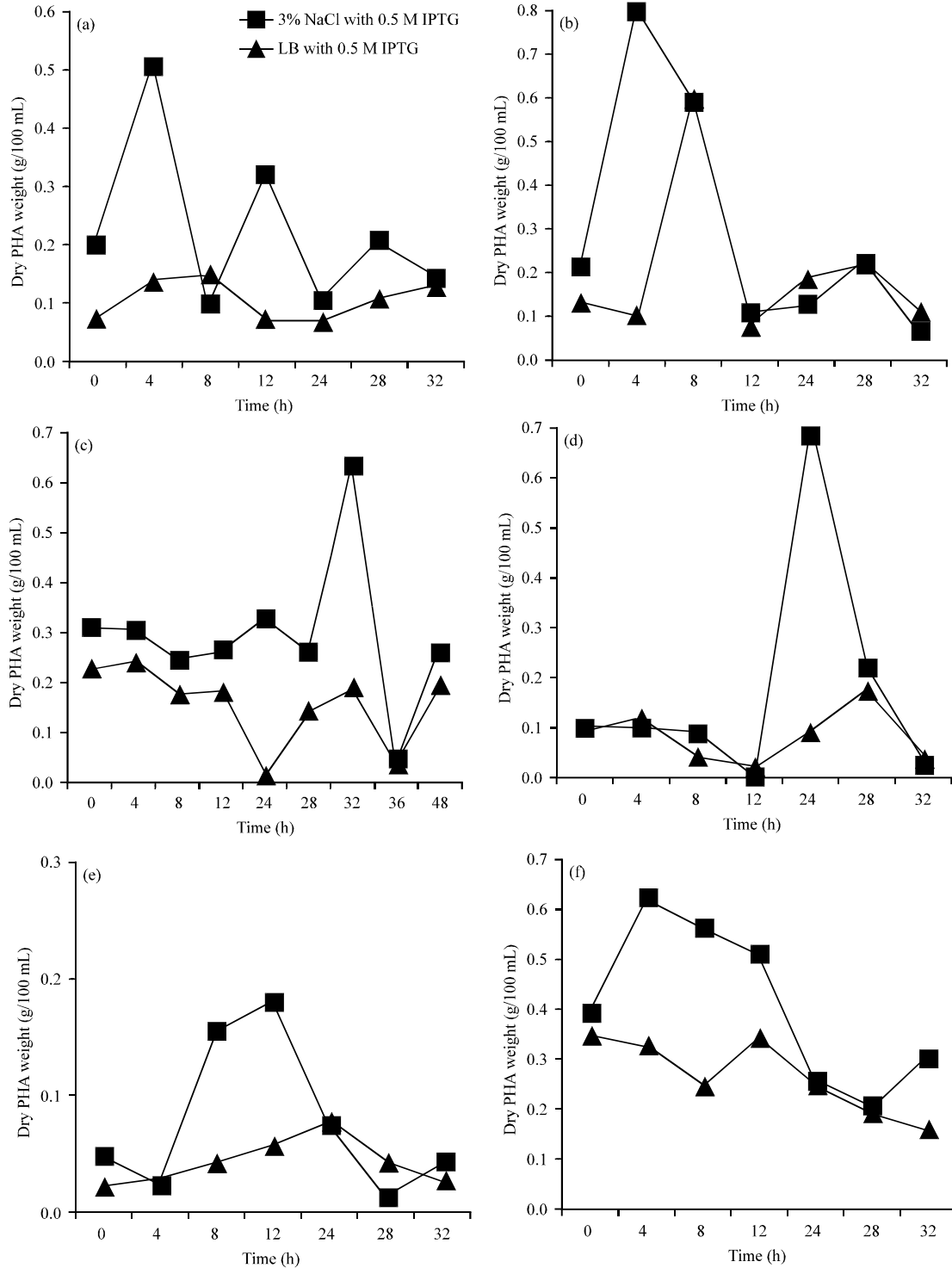


Fig. 6(a-f): PHA production in *E. coli* harboring recombinant clones of *Bacillus* plasmid-clones (a) RSC1018 (GS887728), (b) RSC1023 (GS887731), (c) RSC1028 (GS887737), (d) RSC1032 (GS887740), (e) RSC1042 (FN645942) and (f) RSC1047 (GS887707). The clones were grown on LB containing 3% NaCl with 0.5 M IPTG and LB with 0.5 M IPTG

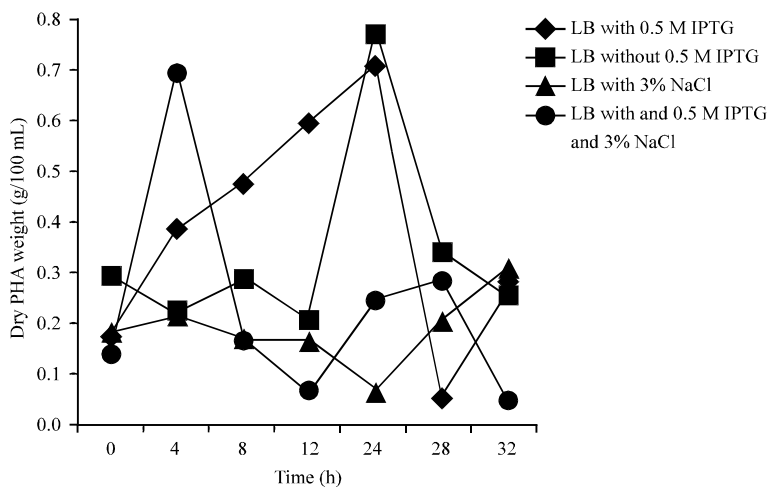


Fig. 7: PHA production in *E. coli* harboring recombinant clones of *Bacillus* plasmid, clones. Clone F131 (HE591385) encoding for *Bacillus* PHA synthase IIIC (HE591385) was expressed in *E. coli* with IPTG and NaCl for PHA production

reached maximum in about 24 h of growth (0.78 g/100 mL, Fig. 7) and decreased sharply within 28 h. With NaCl alone, PHA synthesis gradually increased over a period of time and reached maximum at 24 h (0.72 g/100 mL). In contrast to this in saline media with IPTG induction, onset of PHA syntheses began early and reached maximum within 4 h (0.71 g/100 mL) and declined within 8 h. These results put together suggest that salinity triggers/activate cellular metabolic pathways that divert the carbon precursors for PHA biosynthesis to alleviate salinity stress in their early phase of growth.

## CONCLUSION

*Bacillus* spp., Generally Regarded As Safe (GRAS) organism by Food and Drug Administration (FDA), have been shown to accumulate PHA/PHB during the sporulation (Katircioglu *et al.*, 2007). PHAs are good replacement for plastics and are biodegradable to carbon dioxide and water by natural process. PHA/PHBs are being extensively used in pharma industry and in encapsulation and controlled release of drugs (Davis *et al.*, 2008). *Bacillus* isolated from the root rhizosphere of sugarcane variety CoM 0265 exhibited tolerance to NaCl and produce PHA in salt inducible manner. Analysis of the *Bacillus* genome revealed the presence of ~20 kb plasmid and *E. coli* transformed with this plasmid were able sustain 5% NaCl stress and produced PHA. *Bacillus* was able to produce high levels of PHA at 5% NaCl concentration pH was favourable of the PHA synthesis. Dissecting the plasmid and their expression in *E. coli* revealed that plasmid stability factor, 3-dehydroquinate dehydratase, signal transduction histidine kinase and sensor histidine kinase as well as RSC1018 and RSC1032 (with no homologous sequences in NCBI BLAST analysis) are associated with PHA synthesis. Further, we also identified PHA synthase IIIC homologue harboured on the plasmid and its expression can trigger the synthesis of PHA. Studies are in progress to characterize PHA synthase IIIC and its role in PHA biosynthesis.

## ACKNOWLEDGMENT

Financial support of VSI, Pune to GE and Agharkar Research Institute, Pune for technical support to analysis PHA is gratefully acknowledged.

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