Chapter 2

Materials and Methods

2.1 Bacterial strains and Plasmids

All the wild type (WT) and genetically modified *Escherichia coli*, *Bacillus subtilis* strains are listed in **Table 2.1**. The plasmids used and constructed in the present study are given in **Table 2.2**. *E. coli* DH10B was used for all the standard molecular biology experiments wherever required. *B. subtilis* DK1042 was obtained from *Bacillus subtilis* Genetic Stock Center (BGSC), Ohio, U.S.A. pUC816 containing *Vitreoscilla* hemoglobin (*vgb*) gene was generously gifted by Dr. B. C. Stark, Illinois Institute of Technology, USA. pSW4-GFPopt shuttle vector containing *B. subtilis* codon optimized *gfp* was generously gifted by Dr. Stephen H. Leppla, National Institute of Health (NIH), Bethesda, Maryland, USA.

Bacillus subtilis NCIB 3610 strain (abbreviated as 3610) is an undomesticated derivative of Marburg which has retained the ability to form multicellular structures with complex architecture and cell organization (Branda et al., 2001; Branda et al., 2006). *B. subtilis* NCIB 3610 shares genomic similarity with *B. subtilis* 168 (Earl et al., 2007). However, *B. subtilis* 168 and other laboratory strains have lost the ability to form pellicle biofilms, swarming motility, exopolysaccharide capsule, surfactant, and the production of antimicrobials are just a few examples of the many phenotypes that have been lost from (Kearn et al., 2004; Earl et al., 2007). NCIB 3610 also harbors an 84 kb plasmid (pBS32) that encodes 102 genes including *comI*, the recent identification of ComI as a genetic competence inhibitor allowed for the generation of the naturally competent 3610 comIQ12L (DK1042) strain (Konkol et al., 2013), facilitating genetic study of the complex phenotypes associated with undomesticated strains which is why *B. subtilis* DK1042 has been chosen to study the effect of metabolic engineering without compromising much on the genetic make-up of natural isolates.

Table 2.1: List of Bacterial strains used in this study. Amp= Ampicillin; Km= Kanamycin; Gm = Gentamycin; Str = Streptomycin; Neo = Neomycin; r = resistant.

Bacterial Strains	Genotype	Reference
E. coli DH10B	F^-*endA1 recA1 galE15 galK16 nupGrpsL ΔlacX74 Φ80lacZΔM15 araD139 Δ (ara, leu) 7697 mcrA Δ(mrr-hsdRMS-mcrBC)* $^{\lambda-}$	Invitrogen

Bacterial Strains	**Genotype**	**Reference**
DH10B (pNRM1)	*E. coli* DH10B with plasmid pNRM1; Amp^r	This study
DH10B (pNRM2)	*E. coli* DH10B with plasmid pNRM2; Amp^r	This study
DH10B (pNRM4)	*E. coli* DH10B with plasmid pNRM4; Amp^r	This study
DH10B (pNRM5)	*E. coli* DH10B with plasmid pNRM5; Amp^r	This study
DH10B (pNRM6)	*E. coli* DH10B with plasmid pNRM6; Amp^r	This study
DH10B (pNRM11)	*E. coli* DH10B with plasmid pNRM11; Amp^r; Km^r; Neo^r	This study
DH10B (pNRM157)	*E. coli* DH10B with plasmid pNRM157; Amp^r; Km^r; Neo^r	This study
DH10B (pNRM1113)	*E. coli* DH10B with plasmid pNRM1113; Amp^r; Km^r; Neo^r	This study
DH10B (pNRM1114)	*E. coli* DH10B with plasmid pNRM1114; Amp^r; Km^r; Neo^r	This study
DH10B (pNRM11O)	*E. coli* DH10B with plasmid pNRM11O; Amp^r; Km^r; Neo^r	This study
DH10B (pNRM13)	*E. coli* DH10B with plasmid pNRM13; Amp^r; Km^r; Neo^r	This study
DH10B (pNRM14)	*E. coli* DH10B with plasmid pNRM14; Amp^r; Km^r; Neo^r	This study
B. subtilis DK1042	NCIB 3610 with plasmid pBS32 carrying *ComI*^{Q12L}	BGSC, Konkol et al., 2013
NRM1113	*B. subtilis* DK1042 *amyEP43-lox71-Kan-lox66-P43-vgb-gfp*	This study
NRM1114	*B. subtilis* DK1042 *amyEP43-lox71-Kan-lox66-(P43)*$_4$*-vgb-gfp*	This study
NRM11O	*B. subtilis* DK1042 *amyEP43-lox71-Kan-lox66-(P43)*$_4$*-vgb-gfp-P43-FpOAR-oah*	This study

Table 2.2: List of plasmids used in the present study.

Plasmid	Feature	Reference
pSW4-GFPopt	pSW4 (Gram-positive/Gram-negative) shuttle plasmid containing 729-bp AseI/BamHI-cloned *gfpopt*, Amp^r; Km^r; Neo^r	Sastalla et al., 2009
pUC18	High copy number cloning vector, Amp^r	Laboratory stock
pUC19	High copy number cloning vector, Amp^r	Laboratory stock
pUC816	Derived from the high-copy number vector pUC8 by insertion of a *vgb* gene; Amp^r	Anand et al., 2010
pDK	*B. subtilis* integration vector with *amyE* locus; Amp^r; Km^r; Neo^r	BGSC, Yuan and Wong, 1995
pUC57*oah*	pUC57 vector containing codon optimized *oah*	Synthesized commercially
pUC57*FpOAR*	pUC57 vector containing codon optimized *FpOAR*	Synthesized commercially
pUC57P43	pUC57 vector containing tandem repeat of three P43 promoters, EcoRI/BamHI	Synthesized commercially
pNRM1	pUC19 containing codon optimized green fluorescent protein (*gfp-opt*) gene, XbaI/BamHI	This study, Ch. 3
pNRM2	pNRM1 containing P43 promoter and *Vitreoscilla* hemoglobin (P43-*vgb*) gene, HindIII/XbaI	This study, Ch. 3
pNRM4	pUC18 containing oxaloacetate acetyl hydrolase (*oah*) gene, PstI/HindIII	This study, Ch. 4
pNRM5	pNRM4 containing P43 promoter and oxalate transporter gene (*P43-FpOAR*), SalI/PstI	This study, Ch. 4
pNRM6	pNRM5 containing P43-*vgb-gfp*, KpnI/BamHI	This study, Ch. 4
pNRM11	*B. subtilis* integration vector carrying *P43-lox71-Kan-lox66* cassette	This study, Ch. 3
pNRM157	pNRM11 containing tandem repeats of P43 promoters, EcoRI/BamHI	This study, Ch. 3 and 4

pNRM13	PNRM11 containing P43-*vgb-gfp-P43-FpOAR-oah* operon BamHI/KpnI	This study
pNRM14	pNRM157 containing *P43-vgb-gfp-P43-FpOAR-oah* operon BamHI/KpnI	This study
pNRM1113	pNRM11 containing P43-*vgb-gfp* operon	This study, Ch. 3
pNRM1114	pNRM11 containing P43 tandem repeats and *P43-vgb-gfp*	This study, Ch. 3
pNRM11O	pNRM157 containing *P43-vgb-gfp-P43-FpOAR-oah* operon	This study, Ch. 4

2.2 Media and Culture conditions

The *E. coli* and *B. subtilis* DK1042 were cultured and maintained on Luria broth and Luria Agar (LA) (Hi-Media Laboratories, India) at 37 ºC. For growth in liquid medium, shaking was provided at the speed of 200 rpm. The plasmid transformants of *E. coli* and *B. subtilis* were maintained using respective antibiotics at the final concentrations as mentioned in **Table 2.3** as and when applicable. *E. coli* and *B. subtilis* wild type strains, plasmid transformants and integrants were grown in Luria broth containing appropriate antibiotics and cell pellet were used to prepare glycerol stocks which were stored at -20 ºC.

Table 2.3: Recommended amounts of antibiotics used in this study (Sambrook and Russell, 2001, Integration vector manual, BGSC).

Organism	**Antibiotic**	**Rich Medium**	**Minimal Medium**
E. coli	Ampicillin	100 µg/ ml	100 µg/ ml
	Kanamycin	50 µg/ ml	50 µg/ ml
	Streptomycin	100 µg/ ml	100 µg/ ml
B. subtilis	Kanamycin/Neomycin	5 µg/ ml	5 µg/ ml

2.2.1 M9 minimal medium

Composition of M9 minimal broth was according to Kleijn et al., (2010) consisting 8.5 g of $Na_2HPO_4.2H_2O$, 3 g of KH_2PO_4, 1 g of NH_4Cl, 0.5 g of NaCl. The following components were sterilized separately and then added (per liter of final medium) 1ml of 0.1 M $CaCl_2.2H_2O$, 1 ml of 1 M $MgSO_4.7H_2O$, 1 ml of 50 mM $FeCl_3.6H_2O$, and 10 ml of trace salt solution. The trace salts solution contained (per liter) 170 mg of $ZnCl_2$, 100 mg of $MnCl_2.4H_2O$, 60.0 mg of $CoCl_2.6H_2O$, 60.0 mg of $Na_2MoO_4.2H_2O$, and 43.0 mg $CuCl_2.2H_2O$. When preparing the medium, the base salts were added first followed by $CaCl_2$, $MgSO_4$, $FeCl_3$, and finally the trace elements. Filter-sterilized glucose and malate, alone or in combination with each other were used as carbon sources.

2.2.2 Phosphate and Potassium solubilization Medium

Pikovaskaya agar medium (HiMedia) was used to detect the phosphate solubilization activity of WT *B. subtilis* DK1042 and the integrants. The components (g/l) were as follow Yeast extract 0.5, Dextrose 10.0, Calcium phosphate 5.0, Ammonium sulphate 0.5, Potassium chloride 0.2, Magnesium sulphate 0.1, Manganese sulphate 0.0001, Ferrous sulphate 0.0001 and agar 15.0.

Aleksandrov agar medium (HiMedia) was used to detect the potassium solubilization activity by WT *B. subtilis* DK1042 and the integrants. The components (g/l) were as follow Magnesium sulphate 0.5, Calcium carbonate 0.1, Potassium alumino silicate 2.0, Glucose 5.0, Ferric chloride 0.005, Calcium phosphate 2.0, and agar 20.0, Final pH (at 25°C) 7.2±0.2.

2.2.3 Tris buffered medium

The media composition included 50 mM Tris-HCl (pH-8.0), 100mM; NH_4Cl, 10mM; KCl, 10mM; $MgSO_4$, 2mM; $CaCl_2$, 0.1mM; micronutrient cocktail; Glucose, 100mM. The micronutrient cocktail was constituted of $FeSO_4.7H_2O$, 3.5 mg/L; $ZnSO_4.7H_2O$, 0.16 mg/L; $CuSO_4.5H_2O$, 0.08 mg/L; H_3BO_3, 0.5 mg/L; $CaCl_2.2H_2O$, 0.03 mg/L and $MnSO_4.4H_2O$, 0.4 mg/L. (Gyaneshwar et al., 1998). 1mg/ml Senegal Rock phosphate (RP) was used as P source. Each ingredient was separately autoclaved at a particular stock concentration and a fixed volume of each was added to pre-autoclaved

flasks containing sterile distilled water (prepared as in Section 2.2.2) to constitute complete media.

2.2.4 Lysogeny broth (LB), Lysogeny broth containing glycerol and manganese (LBGM) medium and Minimal Salt Glycerol Glutamate (Msgg) Medium to study biofilm formation

Lysogeny broth (LB) medium containing 10 g of tryptone, 5 g of yeast extract, and 5 g of NaCl per liter was used to study colony and pellicle biofilm (Shemesh and Chai, 2013). LBGM (Shemesh and Chai, 2013) is a biofilm promoting rich medium consisting of LB 1% [vol/vol] glycerol and 0.1 mM $MnSO_4$. Msgg is a minimal medium promoting biofilm formation (Branda et al., 2004). It consists of 5 mM potassium phosphate (pH 7), 100 mM morpholinepropane sulfonic acid (pH 7), 2 mM $MgCl_2$, 700 μM $CaCl_2$, 50 μM $MnCl_2$, 50 μM $FeCl_3$, 1 μM $ZnCl_2$, 2 μM thiamine, 0.5% glycerol, 0.5% glutamate, 50 μg of tryptophan/ml, 50 μg of phenylalanine/ml. Pellicle biofilm formation was studied in 6 well plates and colony biofilm formation was studied in 90 mm petri plates. Agar concentration was kept 1.5 % for all the colony biofilm experiments. Antibiotics were not added while studying biofilm formation.

2.2.5 Starch Agar Medium for detection of integrants

Starch agar medium (HiMedia) was used to screen the integrants having desired gene cassettes integrated at *amyE* locus. It consisted of following components (g/l) Meat Extract 3.0, Peptic digest of animal tissue 5.0, Starch, soluble 2.0 and agar 15.0, Final pH (at 25°C) 7.2±0.1.

2.3 Molecular biology tools and techniques

2.3.1 Isolation of plasmid and genomic DNA

2.3.1.1 Plasmid DNA isolation from *E. coli*

The plasmid DNA from *E. coli* was isolated using standard alkali lysis method (Sambrook and Russell, 2001).

2.3.1.2 Genomic DNA isolation from *B. subtilis*

Genomic DNA was isolated from *B. subtilis* using HiPurA™ Bacterial Genomic DNA Purification Kit (HiMedia). Cells were harvested from overnight grown culture broth by centrifugation at 13,000 rpm for two minutes. Supernatant was removed and the cells were resuspended in 200 µl of lysozyme solution (45 mg/ml) and incubated for 30 minutes at 37°C. 20 µl of the Proteinase K solution (20 mg/ml) was added to the sample for lysis. 20 µl of RNase A solution was added and mixed using pipette and incubated for five minutes at room temperature for removal of RNA. 200 µl of lysis solution (C1) was added and vortexed thoroughly for a few seconds and incubated at 55 °C for 10 minutes. 200 µl of ethanol (95-100%) was added to the lysate and mixed thoroughly by vortexing for few seconds. The lysate was transferred onto HiElute Miniprep Spin Column (Capped) provided in the kit. The column was centrifuged at 10,000 rpm for one minute at room temperature. The flow-through liquid was discarded and the spin column was placed in same two ml collection tube. 500 µl of prewash solution was added to the column and was centrifuged at 10,000 rpm for one minute at room temperature. The flow-through liquid was discarded and the column was again washed with 500 µl of diluted Wash Solution (WS) by centrifugation for three minutes at 13,000 rpm at room temperature. The flow through was discarded and the column was spinned again at same speed for the additional one minute to dry the column. After drying, the HiElute Miniprep Spin Column (Capped) was transferred to fresh uncapped collection tube. 200 µl of the Elution Buffer (ET) was added directly into the column without spilling to the sides and incubated for one minute at room temperature followed by centrifugation at 10,000 rpm for one minute at room temperature to elute the DNA. The eluted DNA was stored at -20°C until further use.

2.3.2 Polymerase Chain Reaction (PCR)

For cloning purposes Q5 hotstart High-Fidelity 2X Master Mix (NEB) and PrimeSTAR® HS DNA Polymerase (premix) (Takara) were used, and Emerald GT 2X master mix was used for post cloning confirmation. Thermocycling conditions and the PCR reaction were set up according to the instructions provided by the manufacturer **(Table 2.4 and 2.5)**.

Table 2.4: Thermocycling condition used in the study. *Exact primer annealing temperature and primer extension time varied with primers and the length of the desired gene. Primers were designed insilico using oligoanalyzer tool provided on the Integrated DNA Technology website (https//eu.idtdna.com/calc/analyzer). Primers were obtained from Integrated DNA Technologies, India **(Table 2.6)**.

Thermocycling Conditions		**EmeraldAmp GT (Takara)**		**PrimeSTAR® HS (Premix) (Takara)**		**Q5® Hot Start High-Fidelity 2X Master Mix (NEB)**	
		Temperature	**Time**	**Temperature**	**Time**	**Temperature**	**Time**
Initial denaturaion		98 °C	30 sec	98 °C	30 sec	98 °C	30 sec
Second denaturation	**25-35 cycles**	98 °C	10 sec	98 °C	10 sec	98 °C	5–10 sec
Annealing		60 °C	10 sec	60 °C	5 sec	*50–72 °C	10–30 sec
Extension		72 °C	1 min/kb	72 °C	1 min/ kb	72 °C	20–30 sec/kb
Final Extension		72 °C	5 min	72 °C	5 min	72 °C	2 min
Hold		4–10 °C	∞	4–10 °C	∞	4–10 °C	∞

Table 2.5: PCR reaction set up used in the present study. PCR amplifications were performed in Applied Biosystem, Veriti 96-Well thermal cycler. The PCR products were analyzed on 1.0% agarose gel along with appropriate molecular weight markers.

Reaction set up Protocol for PrimeSTAR® HS (Premix) (Takara)		
Components	**Volume**	**Final concentration**
PrimeSTAR HS (Premix)	25 µl	1X
Primer 1	10 - 15 pmol	0.2 - 0.3 µM
Primer 2	10 - 15 pmol	0.2 - 0.3 µM
Template		< 200 ng
Sterile purified water	up to 50 µl	
Total	50 µl	

Reaction set up Protocol for Q5® Hot Start High-Fidelity 2X Master Mix (NEB)		
Components	**Volume**	**Final Concentration**
Q5 High-Fidelity 2X Master Mix	25 µl	1X
10 µM Forward Primer	2.5 µl	0.5 µM
10 µM Reverse Primer	2.5 µl	0.5 µM
Template DNA	variable	< 1,000 ng
Nuclease-Free Water	to 50 µl	
Total	50 µl	
Reaction set up Protocol for EmeraldAmp® GT PCR Master Mix (Takara)		
Components	**Volume**	**Final concentration**
EmeraldAmp GT PCR Master Mix (2X Premix)	25 µl	1X
Forward Primer		0.2 µM
Reverse Primer		0.2 µM
Template		< 500 ng
dH2O	up to 50 µl	
Total	50 µl	

Table 2.6: Primers used for cloning different genes and operons

Sr No.	Primer	Sequence 5'- 3'	Total Length (bp)	template binding region		
				Length (bp)	% GC content	Tm (°C)
1	*vgb*F1	CC AAG CTT ATT TTA CAT TTT TAG AAA TGG GCG TGA AAA AAA GCG CGC GAT TAT GTA AAA TAT AAA TAA AGT GAT ATA TTA AGA GG AGG AG	90	45	26.7	57.4
2	*vgb*F2	GC GAT TAT GTA AAA TAT AAATA AAG TGA TAT ATT AAG AGG AGG AGGAC CCT CAT GTT AGA CCA GCA AAC	69	24	50.0	57.7

3	*vgb*R	TGC TCT AGA TTA TTC AAC CGC TTG AGC GTA CAA ATC TG	38	29	41.4	59.2
4	*gfp*F	GCTCTAGAGCATATTAAGAGGAGGAG A ATACAAATGTCAAAAGGAGAAGAATT ATTTACAGGGGTAGT	68	35	34.3	59.1
5	*gfp*R	CGGGATCCGCTGCACTGCAGGTCACGA GCTCGTTACTTATATAATTCATCCATTC CGTGTGTAATTCCTGC	71	40	33.3	59.5
6	*oah*F	TGCACTGCAGAT ATT AAG AGG AGG AGA ATA CAA ATG AAG GTG GAC ACC CCG GAT A	55	22	54.5	60
7	*oah*R	CCAAGCTTTCACACG CCGTTGGCGAAA	27	19	57.9	60.6
8	*FpoAR* F1	GCGTCGACATTTTACATTTTTAGAAAT GGGCGTGAAAAAAAGCGCGCGATTAT GTAAAATATAAATAATATTAAGAGGA GGAGAATA	87	46	30.4	60.3
9	*FpOA R*F2	GCGCGCGATTATGTAAAATATAAATAA TATTAAGAGGAGGAGAATACAAATGA CCGATCTGCACCGCAG	69	20	60	60.1
10	*FpOA R*R	TGCACTGCAGTCACAGCAGGTCCTCTT GACGC	32	22	59.1	61.4
11	KAN1 F	CATACATTATACGAAGTT ATATATTAAGAGGAGGAGAATACAAA TGAGAATAGT GAATGGACCAATAATAATGACTAGAG AAGAAA	86	43	30.2	59.7
12	KAN2 F	AAA GCG CGC GAT TAT GTA AAA TAT AATACCGTTCGTATAGCATACATTATA	89	49	28.6	59.4

		CGAAGTTAT ATA TTA AGA GG AGG AGA ATA CAAATG AGA				
13	KAN3 F	CGCGTCGAC ATT TTA CAT TTT TAG AAA TGG GCG TGA AAA AAA GCG CGC GAT TAT GTA AAA TAT AATACCGTTC	73	34	35.3	59.1
14	KANR	CCGGAATTC TACCGTTCGTATAATGTATGCTATACG AAGTTATTCAAAATGGTATGCGTTTTG ACACATCCACTATATATC	81	38	34.2	59.2
15	*vgb*F1 KPN1	CGGGGTACC ATT TTA CAT TTT TAG AAA TGG GCG TGA AAA AAA GCG CGC GAT TAT GTA AAA TAT AAA TAA AGT GAT ATA TTA AGA GG AGG AG	91	45	26.7	57.4
16	*gfp*R1 6	CGGGATCCCGATTTGCGGCCGCCCTTA ATTAAGGTT GGCGCGCCAATTACTTAT ATAATTCATCCATTCCGTGTGTAATTC CTGC	85	39	33.3	59.5
17	57F	GTA AAA CGA CGG CCA GTG	18	18	55.6	54
18	57R	GGA AAC AGC TAT GAC CAT G	19	19	47.4	50.8

2.3.3 Agarose gel electrophoresis

The DNA samples were mixed with appropriate volume of 6X purple loading dye (NEB) subjected to electrophoresis through 0.8% agarose (containing 1µg/ml ethidium bromide) gel in Tris-acetate-EDTA (TAE) buffer 0.5 - 2 h. The DNA bands were visualized by fluorescence under the UV-light using UV trans-illuminator or Bio-Rad gel documentation system.

2.3.4 Restriction enzyme digestion analysis

0.5-1.0 µg DNA sample was used for each restriction enzyme digestion. 1-3 U of the restriction endonuclease (RE) was used with the appropriate 10X or 20X buffers supplied by the manufacturer in a final reaction volume of 10µl. The reaction mixture was

incubated for 4-6 h at 37 °C. The DNA fragments were visualized by ethidium bromide staining after electrophoresis on 0.8% agarose gels and images were taken using Bio-Rad gel documentation system. In case of double digestion, a compatible buffer for the two REs was checked. If not available, digestion with one enzyme is performed followed by purification and subsequent digestion with the other enzyme, using respective buffers.

2.3.5 Gel elution and purification

The DNA fragments of desired sizes were recovered from the gel by cutting the agarose gel slab around the DNA band. The purification was done by using kit method (Nucleo-pore® Quick Gel Recovery Kit, Genetix Biotech Asia Pvt. Ltd.) and following the instructions described by manufacturer. The purification efficiency was checked by subjecting one µl DNA solution to gel electrophoresis and visualizing the sharp DNA band of desired size. The purified DNA was used for ligation experiments.

2.3.6 Ligation

The ligation reaction was usually done in 20 µl volume containing the following constituents Purified vector and insert DNA (volume varied depending on the respective concentrations); 10X T4 DNA Ligase buffer, 2 µl; T4 DNA ligase (MBI Fermentas), 0.5-1.0U and sterile double distilled water to make up the volume. The cohesive end and blunt ligation reaction was carried out at 22 °C for 4 h, with vector to insert molar ratio (molar concentrations calculated using insilico ligation calculator available at http//www.insilico.uni-duesseldorf.de/Lig_Input.html website) of 13 and 16respectively. Each ligation reaction consisted a total of 50-150 ng of DNA.

2.3.7 Transformation of plasmid DNA

2.3.7.1 Transformation of plasmid DNA in *E. coli*

The transformation of plasmids in *E. coli* using $MgCl_2$-$CaCl_2$ method was carried out according to Sambrook and Russell (2001).

2.3.7.2 Chromosomal integration of genes in *B. subtilis* DK1042

Desired gene cassettes were cloned in the integration vector based on homologous recombination at *amyE* locus. These integration vectors were transformed in *B. subtilis* DK1042 using natural competence (Juhas and Ajioka, 2015). Single colony of *B. subtilis* DK1042 was first inoculated into 10 ml minimal medium composed of 2 ml of 5X minimal salts solution (2 g ammonium sulphate, 14.8 g potassium hydrogen phosphate, 5.4 g potassium dihydrogen phosphate, 1.9 g sodium citrate, 0.2 g magnesium sulphate heptahydrate dissolved in 200 ml of deionised water), 0.1 ml glucose (50% w/v), 0.1 ml casamino acids (2% w/v), 0.02 ml tryptophan (10 mg/ml), 0.01 ml iron ammonium citrate (2.2 mg/ml) in a deionised water. Inoculated cells were grown at 200 rpm on a rotatory shaker at 37 °C for 18 h. Then, 1.4 ml of the *B. subtilis* culture was inoculated into 10 ml of the fresh minimal medium and grown for another 3 h. Subsequently, 11 ml of the starvation medium composed of 2.2 ml of 5X minimal salt solution and 0.11 ml glucose (50% w/v) in deionised water was added to the *B. subtilis* culture and cells were grown for additional 2 h and 45 minutes. 0.3 ml aliquots were transferred into 15 ml polypropylene tubes and transformed with 15 µl (7-8 µg) of integration vector. Transformed *B. subtilis* cells were incubated at 37 °C with shaking at 200 rpm on a rotatory shaker for 1 h prior to the addition of 700 µl Luria broth. Cells were then continued to grow for 1.5–2 h. 20–200 µl of this culture was plated onto antibiotic selection Luria agar plates. Antibiotic resistant colonies were further reexamined on starch agar plate to ensure successful integration of gene cassettes at *amyE* locus.

2.3.8 Gene Expression Analysis

2.3.8.1 RNA isolation from *B. subtilis* biofilm

RNA was isolated using Macherey-Nagel Nucleospin RNA isolation mini kit. 10^9 bacterial cells/columns or reactions were subjected for RNA isolation. Cells were scraped from the biofilm and resuspended in 100 µl TE buffer (10mM Tris-HCl, 1 mM EDTA; pH 8) containing 2-3 mg/ml lysozyme by vigorous vortexing. Incubate at 37 °C for 10 minutes for homogenization. Lysis was carried out by adding 350 µl Buffer RA1 and 3.5 µl β-mercaptoethanol to the suspension followed by vigorous vortexing. The lysate was filtered through Nucleospin Violet ring filters to reduce viscosity and turbidity of the solution and

was centrifuged for one minute at 11000 x g. 350 µl ethanol (70 %) was added to the lysate and was mixed by vortexing. In order to bind the RNA to the column, the lysate was passed through the Nucleospin RNA Column (light blue ring) and was centrifuged for 30 seconds at 11000 x g. The process was repeated till the entire volume of lysate was processed. Silica Membrane was desalted by adding 350 µl MDB – Membrane Desalting Buffer (MDB), and was centrifuged at 11000 x g for one minute to dry the membrane**.** DNase reaction mixture was prepared in a sterile 1.5 ml microcentrifuge tube by adding 10 µl reconstituted rDNase to 90 µl reaction buffer for rDNase. 95µl DNase reaction mixture was added directly onto the column center of the silica membrane of the column and was incubated at room temperature for 15 minutes for removal of genomic DNA traces. Silica Membrane was washed and dried three times with 200 µl RAW2, 600 µl of RA3 and 250 µl RA3 buffers respectively. After washing, the column was placed into a nuclease-free collection tube supplied with the kit**.** Column bound RNA was eluted in 60 µl RNase-free H_2O by centrifugation at 11000 x g for one minute.

2.3.8.2 cDNA synthesis

Three µg of total RNA was used for first strand cDNA synthesis by using QuantiNova™ Reverse Transcription Kit method. The purified RNA sample was incubated in QuantiNova gDNA Removal Mix at 45°C for two minutes to effectively reduce contaminating genomic DNA. After removal of genomic DNA, the RNA sample was then used directly in reverse transcription. The RNA was mixed with QuantiNova Transcription Enzyme with QuantiNova Reverse Transcription Mix and was incubated at 25 °C for three minutes and at 45 °C for 20 minutes. The reaction was stopped by incubation at 85 °C for five minutes.

2.3.8.3 Real-time quantitative PCR

In order to determine the genetic basis of the altered biofilm phenotype due to VHb expression, gene expression analyses of *epsE*, *tasA* and *bslA* genes coding the structural components of biofilm was carried out. Real-time PCR was performed to compare the level of *epsE*, *tasA*, *bslA, spo0Av and spo0As* gene expression in *B. subtilis* DK1042 and NRM1113 colony biofilms grown on LB and LBGM medium with the Evagreen PCR

master mix (Bio-Rad). Gene specific primers are listed in **Table 2.7**. Oneμl of cDNA samples were used as the template for real-time PCR. PCR was performed in the CFX connect (Bio-Rad) by using the following program one cycle of 95°C for 30 sec and 40 cycles of 95°C for 5 sec, 58°C for 10 sec, and 72°C for 15sec and the melting curves were determined from 65.0°C to 95.0°C for 6.5 minutes. *rpoB* and *gyrB*genes were used as internal controls and the geometric mean of cycle threshold (C_T) values of these two transcripts were used to normalize the C_T values of target gene transcripts (Ho et al., 2011; Vargas-Bautista et al., 2014). All quantitative PCR results were analyzed using the $\Delta\Delta C_T$ method (Pfaffl, 2004).

Table 2.7: Primers used for quantitative real-time PCR in this study

S.no.	Gene	Primer Sequence 5' to 3'	Tm	GC%	Length	Product size
1	*bslA* F	TGA ATC TAC ATC AAC TAA AGC TCA TAC TGA ATC CAC TAT	59.7	33.3	39	209 bp
2	*bslA* R	TCA ATG TGT CTT TCG TGT TTG CAG TAA ATC C	59.5	38.7	31	
5	*epsE* F	ATG CAT TCA AAC GGC GGT CAT TTA CG	60.3	46.2	26	206 bp
6	*epsE* R	CTA TTC ATG CTT GAC AAG CCC TTC CTT TTG	59.8	43.3	30	
9	*Spo0Av* - F	AAG ATT TTT CGA CAA ATT CAC GTT TCC TTG TTT GTC	60	33.3	36	200 bp
10	*Spo0Av*- R	ACG TTT CTT CCT CCC CAA ATG TAG TTA ACA	59.7	40	30	
11	*Spo0As* - F	AAC ATG TAG CAA GGG TGA ATC CTG TTA ACT	59.8	40	30	198 bp

12	*Spo0As* - R	GAT CTT TTT CTT TAA ACA GCG ACA GGC ATT CC	59.9	40.6	32	
13	*tasA*-F	CTA ATG GCG CTT AAT TAT GGA GAT TTT AAA GCA AAC G	59.8	35.1	37	205 bp
14	*tasA*-R	TTT CAG CAG CCG CTG CAT CAT TTT TA	59.9	42.3	26	
15	*rpoB* F	AAG TAA AAG GAA ACC TGG ATA AAT ACA GCC TGC	60.2	39.4	33	205 bp
16	*rpoB* R	TTG TAG CCA TCC CAC GTC ATG AAG	59	50	24	
17	*gyrB* F	AAC ATT TAC TCG TTT ACA AAC AAC ATT AAC ACG TAC G	59.3	32.4	37	206 bp
18	*gyrB* R	TCA AAC TGC GGA TCA GGG TGT TTG	59.8	50	24	

2.4 Phosphate and Potassium solubilization phenotype

P-solubilizing and K-solubilizing ability of the *B. subtilis* DK1042 integrants were tested on Pikovaskaya and Aleksandrow agar medium. Five µl of the culture (O. $D._{600}$ = 1.0) was aseptically spotted on the above mentioned agar plates and was allowed to dry completely followed by incubation at 37°C for 4-7 days. P and K solubilization ability was determined by monitoring the zone of clearance surrounding the bacterial colony mainly due to organic acid secretion on respective agar plates.

2.5 Physiological experiments

The physiological experiments were carried out using wild type *B. subtilis* DK1042 and its integrants in Lysogeny broth (LB) and M9 minimal broth. Growth curve was monitored in both LB and M9 broth while pH profile and extracellular oxalic acid detection using HPLC as carried out in M9 broth only. *E. coli* BL21DE3 and its plasmid

transformants were also examined for oxalic acid secretion in Tris rock phosphate broth using HPLC.

2.5.1 Growth characteristics and pH profile

Overnight luria broth grown cultures of *E. coli*, *B. subtilis* DK1042 and their transformants/integrants were inoculated in fresh luria broth and were allowed to grow till midlog phase ($O.D._{600}$ – 0.4 to 0.6) and were inoculated at 1% v/v in 100 ml M9 minimal broth and LB broth in 200 ml Erlenmeyer flasks. The batch culture studies were performed under aerobic conditions in Orbitek rotary shaker at 37 °C with shaking at 100 and 200 rpm as and when required. Two ml of samples were aseptically harvested at regular intervals and were subjected to various analytical techniques.

2.5.2 Analytical techniques

The cell density was monitored spectrophotometrically at 600 -nm as monitored. Change in absorbance was considered as the measure of growth and drop in pH of the media was taken as the measure of acid production. In all the cases, the observations were continued till the slow down phase of growth. Two ml aliquots withdrawn aseptically at regular time intervals were used to measure $O.D._{600}$ and pH. For extracellular oxalic acid determination, the samples were centrifuged at 10000 rpm for 10 minutes at 4 °C and the culture supernatant was filtered through a 0.22 micron membrane filter (Millipore, Bedford, MA, USA) before injection. Quantitative HPLC was performed on a Shimadzu HPLC, a 210nm wavelength UV–vis detector and Acclaim OA HPLC column was used. The mobile phase was composed of 100 mM Na_2SO_4 (adjusted to pH 2.65 with methane sulfonic acid). It was filtered through a 0.45 mm membrane filter (Millipore) and pumped to the column at a flow rate of 0.6 ml min^{-1}. The run time was set at 15 minutes and the column temperature was maintained at 25 ° C. The volume of injection was five µl. Prior to injection of the samples, the column was equilibrated for at least 30 minutes with the mobile phase flowing through the systems. Detection was carried out at 210 nm. The resolution peaks were recorded on the HPLC chart according to the retention time of each compound. Measurements of area under peak with the standard oxalic acid of known concentration were used for quantification.

2.6 Biofilm formation and sporulation in *B. subtilis* DK1042

Biofilm formation and sporulation by *B. subtilis* DK1042 and the integrants were monitored according to the method described by Shemesh and Chai (2013). *B. subtilis* cells were grown in LB broth till mid-log phase (OD_{600} = 0.4 -0.6) at 37 °C. Two µl of culture was spot inoculated on LB, LBGM and Msgg medium with 1.5% agar concentration and plates were incubated at 30 °C for 72 h before examination of colony morphology and sporulation efficiency. Diameters of colony biofilms were measured to quantify biofilm expansion. Pellicle formation was monitored in LB, LBGM and Msgg broth. Images were taken using Nikon D3400 digital camera. Biofilm-associated sporulation was monitored as described by Shemesh and Chai (2013). Briefly, biofilms were scraped from the agar surface and suspended in 0.85% saline. Biofilms were subjected to mild sonication (three rounds of 12 one-second pulses at 20% power) to free single cells. Cells were serially diluted and heat kill was performed at 80°C for 20 minutes in a water bath. Total cell numbers before and after heat kill were quantified by the plating method. Sporulation efficiency was calculated by dividing the total number of viable spores after heat kill by the total number of cells before heat kill.