Biochemical and phenotypic profiling of *Bacillus clausii*: a potent commercial probiotic

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Abstract

The popularity of the probiotics in India has expanded exponentially as they facilitate the host animals in improving the intestinal imbalance of microbial flora. They can be administered as pellets, capsules, paste, powder, curd, granules and also in form of suspensions. In some countries probiotics are also taken as prophylactic agents. *Bacillus clausii* spore suspension is a probiotic of choice in the treatment of diarrhoea and prevention of antibiotic associated diarrhoea. The spores have catapulted themselves as the prescription choice for the treatment of diarrhoea in India. In the present study, three commercially available *Bacillus clausii* spore suspensions were collected for the phenotypic characterization and biochemical analysis. The optimal viability assay conditions and the antibiotic susceptibility assay profiles are determined. The optimization of the key parameters is also presented. Sequence alignment was performed to check the evolutionary relationship of *Bacillus clausii* with organisms of different taxa. Statistical study was performed by using BEAST and a tree was constructed.

Keywords: Probiotics, *Bacillus clausii*, phenotypic, antibiotic susceptibility, sequence alignment, phylogenetic tree, and population genetics

Introduction

The Roman historians recommended the administration of formulated milk products for treating gastroenteritis in 76 B.C [1]. Oral bacteria therapy is generally marketed by using *Bacillus* species because of their ability to treat or prevent various gastrointestinal disorders. Probiotics are being developed commercially as novel foods or dietary supplements for human use and they are used in animal feeds for the prevention of gastrointestinal infections [2].

The market demand indicates that it is economically viable product [3]. In human gastrointestinal environment, *Bacillus* spores survive and can undergo germination and multiplication as vegetative forms [4]. It has been shown that spore mixture of *B. clausii* comprising of four strains (O/C, SIN, N/R and T) are resistant to wide range of antibiotics and are marketed as OTC medicinal supplement [5]. Spores are heat stable, capable of surviving the low pH and products made from them can

be stored at room temperature without any deleterious effect on viability [6, 7]. The phenotypic characterization of microbial strains acclimated as probiotics thus far represents the alone identification approach, although this alignment consistently leaves uncertainties and adversity of interpretation. Moreover, a number of papers have stated inaccuracies in phenotype based speciation of microbial strains [8]. BLAST is a quick sequence comparison tool that uses an investigative approach to construct alignments by enhancing a measure of local similarity. Since BLAST compares protein and nucleotide sequences considerably more rapidly than dynamic programming methods such as Smith-Waterman and Needleman-Wunsch, it is extensively used for database searches [9]. BEAST is a quick, adaptable software architecture for Bayesian analysis of molecular sequences related by an evolutionary tree. A huge number of popular stochastic models of sequence evolution are given and tree-based models appropriate for both within- and between-species sequence data are executed [10].

 In the present study, *Bacillus clausii* probiotic products were examined based upon the quality tests by microbial limit identification, biochemical analysis, phenotypic characterization and optimization of the key parameters for temperature, pH and alkaline tolerance. The quantity tests and different viable counts were also carried out. Local sequence alignment of the Tetracycline resistance protein for *Bacillus clausii* was done and analysed. Population genetics analysis has been done and a phylogenetic tree has been constructed.

Materials and Methods

Sample Collection:

Bacillus clausii spore suspension commercial samples have been collected from India and are named as: Sample1, Sample2 and Sample3. These strains acquired based upon their resistance to different antibiotic resistance markers: O/C (aggressive to Chloramphenicol), SIN (aggressive to Neomycin and Stre-ptomycin), N/R (aggressive to Novobiocin) and Rifampicin and T (aggressive to Tetracycline).

Culture conditions:

The strains were maintained on LB agar plates at 35°C. For biochemical studies, bacteria were inoculated with LB supplemented and growth performance was studied by using 250 ml flasks containing 100 ml LB broth at pH 8.0. The broth cultures were incubated at 35°C for 48 hours in an orbital shaker at 250 rpm. Optical density was observed at 600 nm. Culture medium containing cells were collected at stationary growth phase after 24 hr for further study. The experiment was performed in duplicates for each *Bacillus clausii* strain.

Screening of the antibiotic resistance strains:

The collected samples were analysed for microbial enumeration test by using XLDA (Xylose Lysine Deoxycholate Agar), MSA (Mannitol Salt Agar), Mc Agar (Mcconkey agar), CET(Cetrimide agar) SCDA (SoyabeanCaesin Digest Agar) and SDA (SabourdDextose Agar). Five fold dilution of samples were performed and 0.5 ml of the sample was taken into media plates (Trytone 10.0g, Yeast Extract 5.0g, NaCl 5.0g, DD H2O 1.0 L) containing 50 µg/ mL concentration of chloramphenicol, 100µg/ mL,Streptomycin, 100µg/ mL Tetracycline and 100µg/ mL rifampin.

The number of colonies were identified by using plate count method the initial number of viable organisms in the sample was calculated from the number of colonies formed multiplied by the dilution factor [11, 12]. Broth dilution technique was used for antibiotic resistance assay as the technique is relatively simple for manual testing of small numbers of cultures [13]. Phenotypic observations for the strain were studied according to Berry's manual of system bacteriology. Single colony isolates were stored in a glycerol stock solution at -80°C for further study.

Antibiotic susceptibility assays:

Minimum inhibitory concentration (MIC) for selected antibiotics was identified by using disc diffusion method with 30 µg/mL, 50 µg/mL and 100 µg/ mL concentration of Rifampicin, Chloramphenicol, Tetracycline and Streptomycin antibiotics individually for all the strains with both positive and negative controls.

Optimization parameters:

Optimization key parameters are temperature and pH were studied. The effect of temperature was studied from 35°C to 75°C and the effect of pH was studied from pH 5 - 11. Temperature is a critical parameter that has to be controlled and varied from organism to organism for maximum cell growth.

Alkaline tolerance study:

Tolerance for alkalinity for *Bacillus clausii* were studied by using 2 – 10% NaCl by culturing the bacterial cell cultures on LB agar buffered at pH 8.0. The cultures were then incubated at 35°C for 24 hr at 120 RPM.

Effect of Temperature for activation of spores:

The transition of every stage from spore to vegetative cell activation, germination and outgrowth has a characteristic response to the temperature. Optimal activation temperatures are generally considerably higher than the optima for germination and outgrowth [14]. The effect of the temperature on spores for the activation stage was studied by giving heat shock treatment to the spores from 35°C to 75°C. Sample was collected at 30 and 60 minutes from water bath incubator shaker (Royal scientific). Spotted 100uL of the above prepared dilutions on the agar plates and incubated for 48 hr for viable count.

Local sequence alignment:

The programme used for sequence alignment BLASTP, the Local search alignment tool for protein sequences often contains high scoring segment pairs between the query sequence and sequences in the database. For query sequence, we used Tetracycline resistance protein of *Bacillus clausii* having 647 amino acid query length with the non-redundant protein database of NCBI. For amino acid queries, the low complexity regions were selected by the SEG program and the filter was allowed in the search. The algorithm parameters were kept in default settings.

Population Genetics Studies:

Statistical inferences of the above-mentioned studies were performed by using the Bayesian Evolutionary Analysis Sampling Tree (BEAST). The XML file was generated by the BEAUTi, a graphical user interface. Tree model was selected as a random starting model for ease of interpretation of the results. Default settings were chosen for simplicity to record the sample states that Makarov chain has encountered. Two different file formats .log and. tree was generated for further analysis by the TRACER and FigTree respectively.

Results and Discussion

There is a growing surge of interest in the *Bacillus clausii* spore suspension products. Comprehensive studies of the *Bacillus clausii* products manufactured in India are virtually non-existent. The present study is the first attempt in this direction. Biochemical analysis and phenotypic characterization was carried out. Spectrophotometric quantification of the sample 0.8 OD was quantified and the

samples reach the viable count on the agar plate. Samples were analysed for microbial growth examination. Growth was observed in SCDA and SDA media plates and no growth was observed in XLDA, MSA, CET agar and MCA agar plates. In the following results (Table1) the symbols '+' indicates the result was positive and the symbol '-' indicates the result was negative. Phenotypical and biochemical examination for *Bacillus clausii* samples were as follows Table 2. The gram-positive, rod-shaped *Bacillus clausii* showed negative results for the hydrolysis of Tween 20 and Tween 80. Similar observations were corresponded with results of Nielsen et.al. 1995 [15] for phenotypic and biochemical properties of the strain *Bacillus clausii*. According to well-known spore biology, *Bacillus clausii* spores shows growth in the diluted sample containing antibiotic agar plates. Viable spores were observed when the spores were grown in plates supplemented with Tetracycline, Chloramphenicol, Streptomycin, and Rifampicin Table 3. The MIC was evaluated by Disc diffusion method Table 4. Positive control shows inhibition zones. No growth was observed in negative controls and tolerate acidity up to 10% were considered critical levels for tolerance, respectively for probiotics. The sample shows similar results of the *Bacillus clausii* aqueous suspension with literature where there is similarity in results between the samples [15].

Table 1. Microbial growth examination in specified media

Media	Sample1	Sample2	Sample3
XLDA	-	-	-
MSA	-	-	-
MCA	-	-	-
CET	-	-	-
SCDA	+	+	+
SDA	+	+	+

Table 2. Phenotypic and Biochemical characteristics of *B. clausii*

Characteristics	Observations
Colony Shape	Circular
Colony Size	0.8 - 1.0 mm
Form	Circular
Elevation	Slightly raised low convex
Colony margin	Filamentous
Surface	Shiny smooth
Color	Creamy white
Gram's stain	+
Cell shape	Rod shape
Endospores	+
Motility	+
Caesin hydrolysis	+
Starch hydrolysis	+
Gelatin hydrolysis	+
Tween hydrolysis 20	-
Tween hydrolysis 80	-
Oxidase test	+
Catalase test	+

Table 3. Viability count in media plates

Colonies Observed (10^{-6})	Sample1	Sample2	Sample3
Tetracycline	37	35	32
Chloramphenicol	36	28	33
Rimfampin	37	37	28
Streptomycin	32	35	32

Table 4. Minimum Inhibitory diameter

Minimum Inhibitory Concentration (mm)	Sample1	Sample2	Sample3
Tetracycline (30µg/ml)	10 ± 1	11 ± 0.8	11 ± 0.5
Tetracycline (50µg/ml)	18 ± 0.5	15 ± 1	16 ± 1
Tetracycline (100µg/ml)	25 ± 1	23 ± 0.8	24 ± 0.5
Chloramphenicol (30µg/ml)	16 ± 0.5	13	15 ± 0.5
Chloramphenicol (50µg/ml)	18 ± 0.5	18 ± 1	18 ± 0.5
Chloramphenicol (100µg/ml)	19 ± 0.5	19 ± 0.5	19 ± 0.5
Rimfampin(30µg/ml)	7 ± 0.8	7 ± 0.5	7 ± 0.8
Rimfampin (50µg/ml)	12± 0.8	12 ± 0.5	12 ± 0.5
Rimfampin (100µg/ml)	17 ± 0.8	14	14 ± 0.5
Streptomycin (30µg/ml)	18 ± 0.5	17 ± 0.8	17 ± 0.6
Streptomycin (50µg/ml)	20 ± 0.5	19 ± 1	19 ± 0.8
Streptomycin (100µg/ml)	22 ± 0.5	22 ± 1	23 ± 0.5

Table 5. NaCl tolerance Test for *B. Clausii* Spores

NaCl Concentration	Observation
2 % NaCl	+
4 % NaCl	+
6 % NaCl	+
8 % NaCl	+
10 % NaCl	+

Table 6. Effect of temperature

Temperature	Observation
35°C	+
40°C	+
45°C	+
50°C	+
55°C	+
60°C	+
65°C	+

All strains have shown growth at 8% NaCl and strains grew well in 2% - 10% NaCl after which the cell count was decrease drastically. The effect of temperature on Maximum Cell Growth was studied and the optimum growth was observed at 35°C. The temperature required for spore activation reveals that there may be an increase in spore germination with an increase in temperature. Maximum spore germination was observed to be at 65°C for 30 minutes and 60 minutes of heat treatment.

The strain had similar spore germination profiles with germination taking place between 30 - 60 minutes underlying the fact that they share common mechanism for activation. An apparent access in the sporulation ability was observed when temperature of incubation was shifted from 80°C (38 % efficiency) to 65°C (100 % efficiency) in case of *B. aerius*, advertence that the spores of *B. aerius*, are able to sustain a Temperature of 80°C, B. cereus, failed to show any sporulation at 80°C but could speculated efficiency at 65°C (100%) [16].

A range of environmental factors are crucial in heat activation and it must be carried out in the presence of water. *Bacillus* subtilis spore's maximum germination rate requires 75°C heat activation time of 15 min [17]. *Bacillus* cereus maximal temperatures are above 59°C and 80°C was found to be the highest temperature. Floating of a microbial culture for 30 min on distilled water at 60°C had no effect on the spore or on its germination [18]. Increasing the temperature of heat activation (aqueous suspension, 5ml) increases the germination ability of the spores. Activation as measured by way of extent germination, changed into most effective after heating at 45°C to 75°C and the rate of the spore activation was maximal afterheat activation at 45°C - 75°C (Figure1, Figure2 and Figure3). Increasing the temperature of activation above 45°C for one hour decreased the germination rate. Germination was passed off over an extensive range of temperatures, but turned into most suitable for aqueous suspension of the sample 35°C (figure 1).

Figure 1. Heat shock treatment for Sample-1

Figure 2. Heat shock treatment for Sample-2

Figure 3. Heat shock treatment for Sample-3
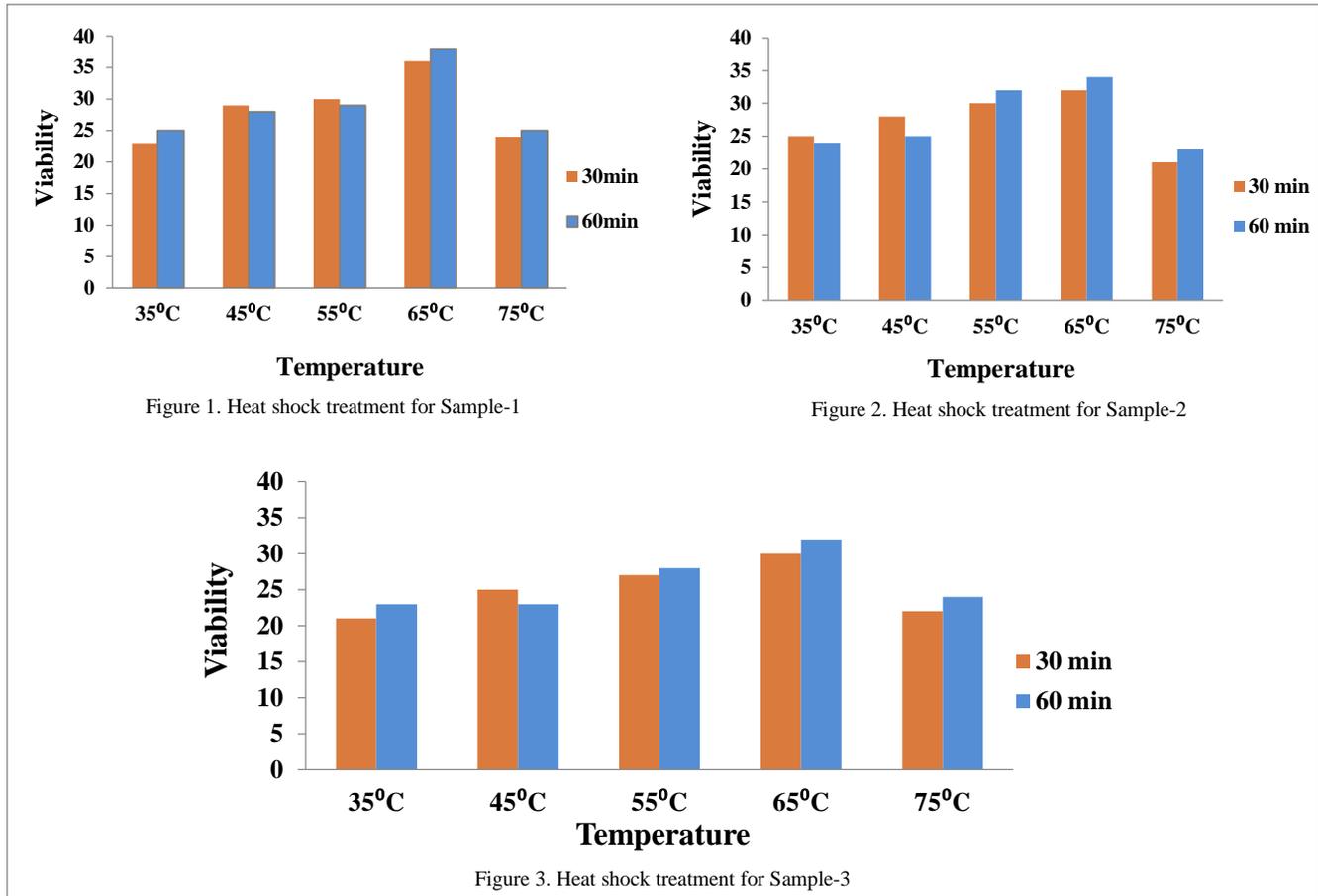
For assessment of the maximum cell increase, the cells were subjected to different ph. conditions which ranged from pH 5 - 11. *Bacillus clausii* suggests lush growth at pH 6, 7, 8, 9 and 10 suggests comparable growth. There was no increase in growth at pH 5 and pH 11. The unique species of spores seem to show off one of the kind levels of resistance to acid environment and thus entail individual characterization of each species. *Bacillus clausii* spores can survive the gastric pH, activate and reach the intestinal tract where they germinate to vegetative form [19, 20]. The pH of *Bacillus clausii* was sensitive to extreme acidic and basic environments. These observations can comply the *Bacillus clausii* products where in the presence of acidic or fundamental recipients can have an effect on the pH of the microenvironment. Cenci et.al. (2006) [21] suggested that no relevant diff-erences were found studying the growth at pH 8 and 10, whereas at pH 7 the yields obtained for O/C and SIN were higher than those obtained for N/R and T strains. Some of the suggested defensive strategies to deal with the pH instability are microencapsulation by enteric polymers like cellulose phthalate [22]. Coating of probiotics with lipidic excipients like waxes [23].

Mixing with resistant starch [24] and symbiotic microencapsulation by emulsion spray drying technique [25].

Table 7. Growth Observations on different pH

pH	Observation
pH 5	-
pH 6	+
pH 7	+
pH 8	+
pH 9	+
pH 10	+
pH 11	-

Local Sequence alignment

BLAST is a sequence alignment tool which optimizes a measure of the local similarity, the maximal segment pair for a given set of sequences. Similarity among the sequences was observed for the tetracycline resistance protein of *Bacillus clausii* (PAF09740.1). Ten sequences were selected based on the expected value (E-Value), the maximal score and the identity value. The lesser the E value a search, the more significant it is showing there was less background noise in each hit. Low complexity regions showed conserved domains responsible for the function of the protein in the study *Bacillus clausii* (PAF09740.1). Ten sequences were selected based on the expected value (E-Value), the maximal score and the identity value. The lesser the E value a search, the more significant it is showing there was less background noise in each hit. Low complexity regions showed conserved domains responsible for the function of the protein in the study. By applying the filter in the BLASTP search one can expect to find out the domains which are similar to other species.
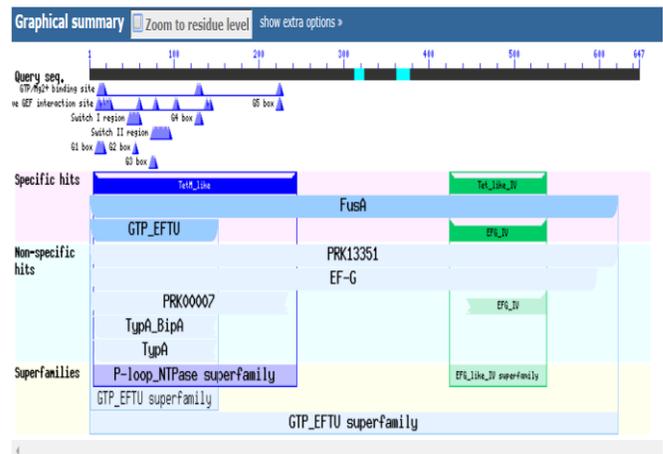

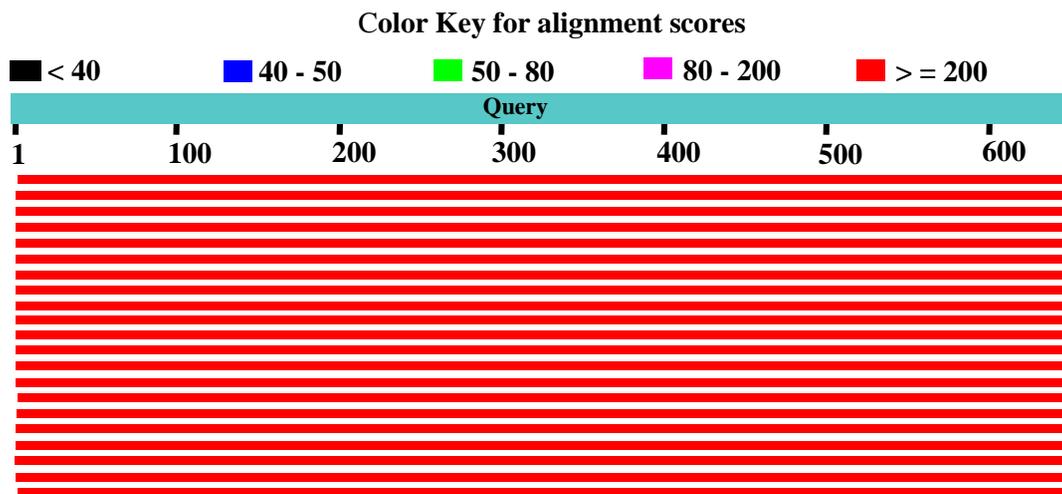Figure 4. Low Complexity regions filters depict the low conserved domains

Figure 5. Low Complexity regions filters depict the low conserved domains

Sequence Producing significant alignment
Select   All   None   Selected 0

Alignments   Download   Genpept   Graphics   Distance tree of result   Multiple Alignment

Description	Max Score	Total Score	Query Cover	E Value	Ident	Accesssion
GTP-binding. protein[Bacillus clausii]	1263	1263	100%	0.0	100%	WP_011248562.1
GTP-binding. protein[Bacillus rhizosphaerae]	1261	1261	100%	0.0	99%	WP_073304529.1
GTP-binding. protein[Bacillus clausii]	1259	1259	100%	0.0	99%	WP_095236425.1
GTP-binding. protein[Bacillus clausii]	1258	1258	100%	0.0	99%	WP_094424885.1
GTP-binding. protein[Bacillus clausii]	1257	1257	100%	0.0	99%	WP_095326647.1
GTP-binding. protein[Bacillus clausii]	1255	1255	100%	0.0	99%	WP_095294276.1
GTP-binding. protein[Bacillus clausii]	1253	1253	100%	0.0	99%	WP_035206202.1
GTP-binding. protein[Bacillus clausii]	1250	1250	100%	0.0	99%	WP_0952544726.1
GTP-binding. protein[Bacillus clausii]	1192	1192	100%	0.0	94%	WP_062745233.1
GTP-binding. protein[Paenibacillus sp. EZ-k15]	697	697	99%	0.0	55%	WP_098743630.1
GTP-binding. protein[Paenibacillus latus]	695	695	99%	0.0	55%	WP_076326053.1
GTP-binding. protein[Paenibacillus sp.LC231]	694	694	100%	0.0	55%	WP_071224254.1
MULTISPECIES: GTP-binding.protein[paenibacillus]	694	694	99%	0.0	56%	WP_036638349.1
GTP-binding. protein[paenibacillus vortex]	691	691	99%	0.0	56%	WP_006209881.1
GTP-binding. protein[paenibacillus glucanolyticus]	689	689	99%	0.0	55%	WP_076151083.1
GTP-binding. protein[Bacillus sp.FJAt-1809]	689	689	99%	0.0	54%	WP_053493716.1
GTP-binding. protein[paenibacillus sp.cl141a]	687	687	100%	0.0	55%	WP_090911413.1
GTP-binding. protein[paenibacillus sp.FSL H8-457]	685	685	100%	0.0	55%	WP_036663538.1

Figure 6. Putative hits in BLASTP having sequences with significant alignments with scores and accession number

This helps us in telling the protein of the interest is a homologous protein and its gene expression is evolutionary conserved. The results given by the BLASTP are distributions of the blast hits on the query sequence are shown in the following figure, the alignments are color coded ranging from black to red as indicated in the color label at the top of figure 5. The hits are colored according to the obtained aligned scores. Multiple sequence alignment is the skeletal frame for any tree construction software. A consensus sequence can be obtained after performing the multiple alignments which in turn helps invalidation of the tree. The BLASTP finds regions of local and global similarity between protein sequences. 100 % similarity was observed GTP-binding protein [*Bacillus clausii*] and very less similarity with WP_073304529.1 accession number in the NCBI database. It is important to search sequence databases to find homologous sequences to reach the better understanding of biological systems at the gene level.

Tree construction

Species selected from the BLAST were analyzed by the BEAST and tree was constructed by using Figtree. Differential homology was observed with different species in the rooted tree constructed with Bacillus rizosphareae being the closest neighbor in speciation with branch 0.8333. For validating a tree, a bootstrap method is used which tells us that each branch length of a tree should be less than 0.90 value for it to be used in evolutionary studies. The current tree constructed correlates with the bootstrap values. Different colors indicate the different taxa with the speciation event separating the species. The branch length is indicated by the numerical value which is calculated by the NJ method of the tree construction. The tree is validated by seeing the branch length as a bootstrap parameter.
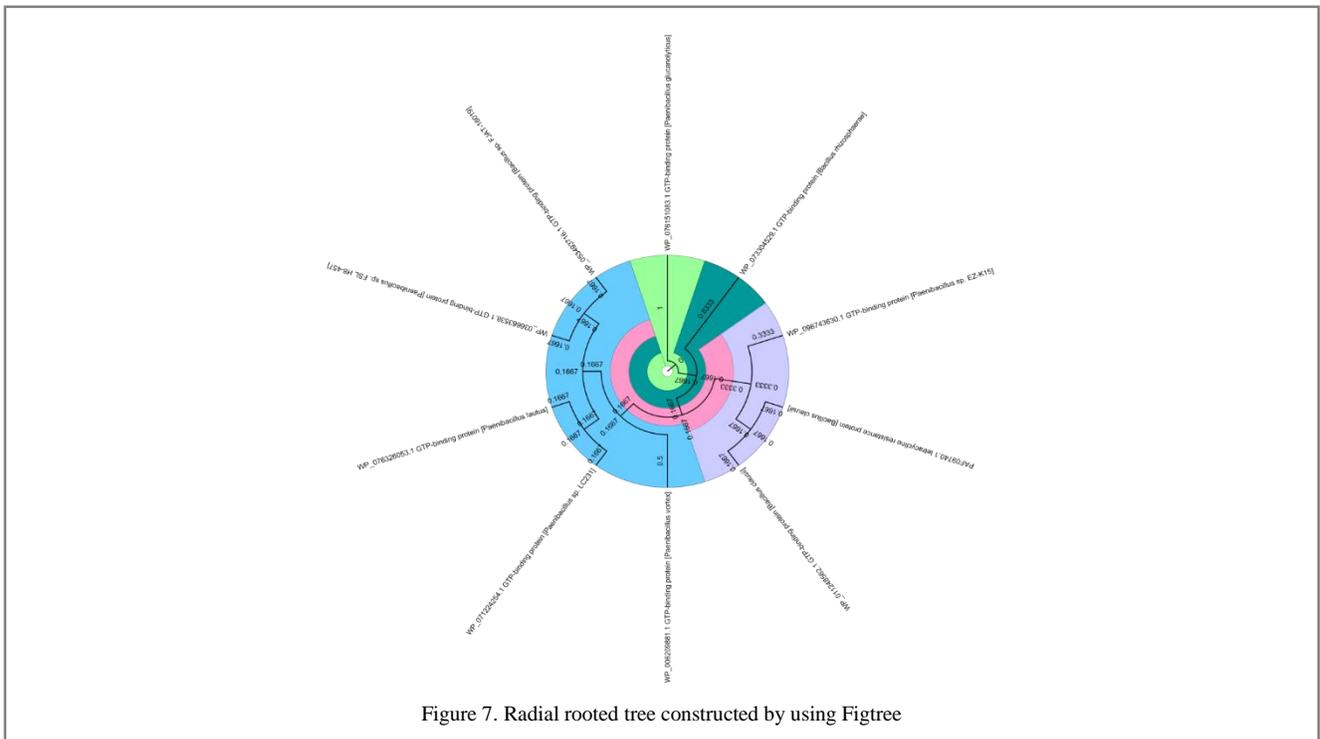

Figure 7. Radial rooted tree constructed by using Figtree

Statistical Inferences

Tracer is used to produce estimates of the parameters of interest like evolutionary rates, divergence times, population sizes and tree topologies. Trace selected for the study was treeModel.root because its provide the statistical data to infer the evolutionary distances between the selected species and gives an insight in the speciation event and lineage distribution. The effective sample size (ESS) was high which indicates the current analysis did yield a sufficient number of independent samples from the posterior distribution for that parameter. Autocorrelation time(ACT) indicate the number of states in the MCMC chain that two samples have to be from each other for them to be uncorrelated. Its high value indicates that the samples selected are not biased and selection of the samples is statistically correct.

By selecting the trace panel one can view the raw trace, that is, the sampled values against the step in the MCMC chain. Posterior probability is calculated by the Makarov chain estimates using the Bayesian algorithm for the effective sample size. Height ESS value indicates that the species selected under the study are unbiased and the tree generated is validated. When running a BEAST XML file, sometimes breakage happens because of the trace and log parameters 9 being large enough. The likelihood is captured and plotted against the log of how much tracer has repaired the breakage. This depicts the authenticity of the results. The current experimentation with insilico evaluation evidenced that the species under study matches with well-marked probiotics. The parameters under consideration were optimized to reach the level of excellence. Hence, the manipulated conditions for probiotics will help in the commercialization of the product to treat intestinal flora imbalance.
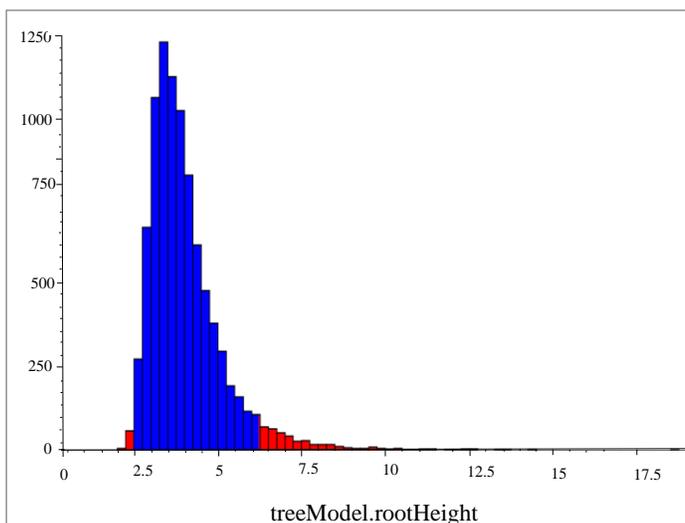
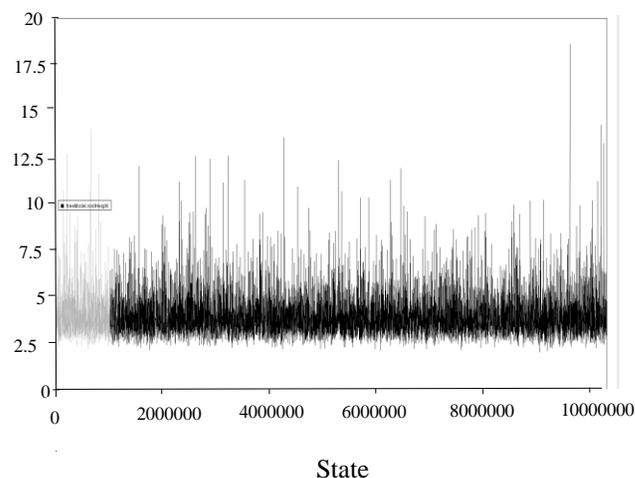Figure 8. Mean estimates of the tree likelihood from the TRACER

Figure 9. Trace result of all the breakage happened in the BEAST run on the Makarov chain

Conclusion

In conclusion Bacillus clausii spore suspension can be used as a source of improving the intenstinal imbalance of microbial floara, it helps in effective treatment of ill-health condtions such as diarrhea and other intestinal malfunctions. There are already few capsulated probiotics in market which is a method of choice of the treatment for the clinicians.

Acknowledgments

Conflict of interest

The authors declare that there is no conflict of interest.

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