ORIGINAL ARTICLE

Isolation and Characterization of Potential Microorganism from Dhanyamla - An Ayurvedic Formulation with Therapeutic Properties

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Abstract

Objectives: Ayurveda is one of the oldest codified medical knowledge systems with equal emphasis on curative, preventive, and promotive aspects of health. Ayurveda pharmaceutics was developed from the quest to administer plants, animals, or metals and minerals products in a palatable and longer shelf life modification. The study aims to evaluate and characterize the microbial sp. and their biochemical properties in extrapolating its use as a potent probiotic formulation with multifaceted use. **Materials and Methods:** The microbial diversity of the formulation was evaluated by isolation followed by its microbial characterization using Gram's staining, biochemical characterization using catalase assay, and molecular characterization by sequencing the internal transcribed spacer (ITS) region. **Results:** The study revealed that the bacteria isolated in the present study were Gram-positive, rod-shaped organism that exhibited catalase-positive test. Further, molecular characterization studies using the ITS sequence analysis revealed that the isolated organism showed similarity with that of *Bacillus* species. **Conclusion:** Therapeutic efficacy of any formulation depends on the process of its preparation, the kind of microflora that is established during aging or fermentation, and the kind of bioactive compounds released during fermentation. The present study identifies the microorganism that plays a pivotal role in this fermentation process and renders therapeutic properties to Dhanyamala formulation. This study can form the basis for further investigations on formulating this as a promising probiotic supplement.

Key words: Ayurveda, Dhanyamla, Evaluate, microbial and biochemical properties

INTRODUCTION

Ayurvedic pharmaceutics is formulated through the transference of active ingredients by different manufacturing processes.[1,2] Sandhana Kalpana (fermented preparations) is a unique form in which acidic and alcoholic fermented formulations are prepared.[3,4] The term Sandhana is used to denote the fermentation process. This processing method gained popularity since ancient times owing to its higher preservation, pharmaceutical, and therapeutic value. As per the information collected from Ayurveda texts and electronic sources, "Dhanyamla" is an acidic and alcoholic "'fermented cereal" (Suktakalpana) widely used in India for nutritional and therapeutic use.[5] According to Sushruta, Dhanyamla is a fermented product prepared from cereals such as rice, barley, and kodo millets.[6] Acharya Vagbhata mentioned that Dhanyamla is prepared by fermenting the water in which rice and such other grains and pulses have been slightly cooked or merely washed and kept for fermentation.

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[7] Sahasrayoga, a popular Ayurvedic text from Kerala describes the ingredients, procedure of preparation, and shelf life of Dhanyamla.[8] Dhanyamla is recommended internally for appetite, fatigue, exhaustion, obesity, urinary calculi, and fistula in ano. Dhanyamla is profusely used for Panchakarma and other external therapies, especially in inflammatory musculoskeletal disorders, muscular dystrophies, stroke, and headaches.[9] There is a paucity of studies to support that the process of fermentation imparts greater therapeutic potential to the formulation.[10,11] However, phytochemicals present in formulations at the time of fermentation are shown to promote the growth of probiotics. Triphala is found to promote the growth of Bifidobacteria and *Lactobacillus* species while inhibiting the growth of undesirable gut residents such as *Escherichia coli* owing to the presence of polyphenols such as quercetin and gallic acid.[9] Ranasinghe and Ediriweera 2015[12] reported constituents in Dhanyamla include starch, globulin, albumin, oryzagenin, vitamin B, trigonelline, trigonelline falvonoides, urease, glycosides, lenoleic acid, polyphenols, beta sitosterol, smino acids- glycine, alanine, cysteine, serine, isoflavones genistein, isoferririn, cumesterol, alkaloid, phenol, tannins, alkaloids, falvonoids, saponins zingerone, shogaol, camphene, phellandrene, zingiberene, cineol, borneol, gingerol, gingerin, resins, geraniol citric acid, malleic acid, phosphoric acid, volatile oil, hesperidin. This study aims to evaluate the type of microorganism is this formulation by microbial, biochemical, and molecular characterization to assess the potential of the formulation to be used as a probiotic supplement.[13,14]

MATERIALS AND METHODS

Preparation of dhanyamla

In a large earthen pot, water was filled in the required quantity [Table 1] and boiled. Meanwhile, all the aforesaid drugs [Table 1] were coarsely crushed and tied separately in nine bundles loosely tied in cotton cheesecloth bags. These bundles of drugs were immersed into the boiling water and the vessel loosely covered by an earthen lid. The boiling was stopped on the 1st day and on each consecutive day the earthen pot was subjected to heat only till the liquid started boiling for the next 6 days. On the eighth day, Dhanyamlawas filtered and collected in aseptic glass bottles.

Determination of pH

The pH of Dhanyamla was determined using the pH meter at 30ºC.

Isolation of probiotics by selective media DeManRogosa and Sharpe (MRS)

1 ml of sample was taken in 9 ml of saline, serial dilution tubes were prepared up to 10^{-8}, pour plate method was followed on MRS agar. Plates were incubated at 37°C for 24 h. Colony-forming Unit was enumerated. Procedure is repeated every week to understand microbial load.[15]

Gram staining

Gram staining test was performed for all isolated strains according to the standard procedure. A smear of single colony was prepared on a clean glass slide and the smear was allowed to air-dry and then heat fixed. The heat-fixed smear was flooded with crystal violet solution and after 1 min, it was washed with water and flooded with mordant Gram's iodine. The smear was decolorized with 95% ethyl alcohol and rinsed with water. Finally, safranin was used as counterstains for 60–80 s and washed with water, and examined under oil immersion (100×).

Catalase test

A drop of 3% hydrogen peroxide was added to a fresh culture on a sterile glass slide and mixed well. Producing bubble or froth indicated catalase-positive and no bubble or froth indicated catalase-negative.

Molecular characterization of bacteria

Genomic DNA extraction

Bacterial isolates were sub-cultured on MRS medium and incubated at 30°C for 48–72 h; The DNA of isolates was extracted and purified using the standard method.[16] Single colony was inoculated in nutrient broth and grown for overnight at 37°C. Cells were harvested from 5 ml of the culture and to this 100 µl of lysozyme was added and incubated at room temperature for 30 min, followed by the addition of 700 µl of cell lysis buffer (Guanidiumisothiocyanate, SDS, Tris-EDTA). The contents were mixed by inverting the

Table 1: Composition of dhanyamla

Identity of the ingredient	Part used	Quantity used
Tandula (*Oryza sativa*)	Seed	750 g
Pruthuka (*Oryza sativa*)	Pressed seed	750g
Kulattha (*Macrotyloma*)	Seed	200 g
Laja (*Oryza sativa*)	Puffed seed	200 g
Kangubeeja (*Panicum sumatrense*)	Seed	300 g
Kodrava (*Paspalum scrobiculatum*)	Seed	300 g
Nagara (*Zingiber officinalis*)	Rhizome	150 g
Nimbuka (*Citrus aurantifolia*)	Fruit	600 g
Deepyaka (*Trachyspermum involucratum*)	Seed	150 g
Jala	RO purified water	20 L

vial for 5 min with gentle mixing till the suspension looked transparent. 700 µl of isopropanol was added on top of the solution. The two layers were mixed gently till white strands of DNA were seen. The DNA extracted from the aqueous layer was ethanol precipitated. The DNA pellet was dried and dissolved in 50 µl of 1× TE buffer.

Amplification of 16S rDNA of bacterial isolates

Fragments of the 16S rRNA genes of each bacterial isolate were separately amplified using the universal primers 27F5'-AGAGTTTGATCCTGGCTCAG-3' and 1492R 5'-GGTTACCTTGTTACGACTT-3'. For amplification of 16S rDNA genes of each bacterial isolate, polymerase chain reaction (PCR) reaction mixtures1 µl DNA template (25 ng), 2 µl 10× reaction buffer, 0.5 µl MgCl2 (50 pM), 1 µl dNTPs mix (10 mM), 1 µl forward primer (10 pM), 1 µl reverse primer (10 pM), 0.5 µl Taq polymerase (5 U/pi) and the final volume 25µl will be adjusted with molecular grade water. The temperature program and the cycle of reactions were as initial denaturation step at 95°C for 2 min Final denaturation 95°C for 30 s. Annealing 50°C for 30 s Elongation 72°C for 1 min. Final Elongation 72°C for 10 min. Thermal cycle is executed for 30 cycles and finally held at 4°C until evaluated.

Sequencing of 16S rDNA bacteria

Sequence of the PCR amplified product was analyzed through Applied Biosystems 3130/3130 × 1 Genetic Analyzers. The bacterial 16S-rDNA sequences obtained were then aligned with known 16S-rDNA sequences in Genbank using the basic local alignment search tool (BLAST), National Center for Biotechnology Information, and percent homology scores were generated to identify bacteria, and get accession numbers shown in Tables 2 and 3. The deduced sequence was aligned using BLAST pairwise alignment using which a phylogenetic tree was drawn.

Ultraviolet (UV) spectroscopic analysis Spectra scan of Dhanyamla was determined using Systronics double beam spectra 2205 between range of 200 and 800 nm to identify bioactive compounds, analysis was carried out every week.[16]

RESULTS AND DISCUSSION

Dhanyamla is the fermentation of rice, millets, pulses, and few spices in water. There are several methods available for its preparation. Therapeutic values of Dhanyamla vary greatly depending upon combinations of ingredients used and method adopted for preparation. Presently Dhanyamla is a much preferred external application for Panchakarma therapies addressing ailments such as tender and painful joints, body ache, and obesity. The present study aims to find out the occurrence of any probiotics property of the formulation by assessing its microbial content.[17,18]

Dhanyamla is an acidic formulation that was proved with pH determination assay routine carried out. There was a gradual drop in pH from initial value of 4.7 to 3.6 over the weeks, probably due to mixed acid produced during fermentation. Ranasinghe and Ediriweera, 2015[12] in their study have reported the pH to be 4.13 for Dhanyamla at 30°C [Figure 1], which may be probably due to mixed acid produced during fermentation. MRS formulation is widely used for the isolation of probiotics and similar organisms. Serial dilution

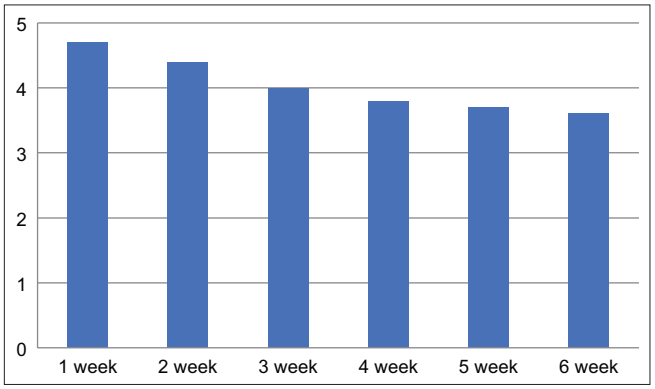

Figure 1: pH studies of Dhanyamla performed weekly

Table 2: Sequence similarity for amplified regions of primer 27F

Strain name	E-value	Percentage of similarity	Accession number
Bacillus subtilis strain GuanMX	$8e^{-108}$	96.33	MN473282.1
Bacillus subtilis strain OTPB4	$8e^{-108}$	97.07	KT265082.1
Bacillus subtilis strain M-1	$3e^{-107}$	96.68	MN538261.1
Bacillus subtilis strain N402	$3e^{-107}$	95.62	MK629804.1
Bacillus subtilis strain CTPRKVG 18-3	$3e^{-107}$	95.95	MK392043.1
Bacillus subtilis strain NA3	$3e^{-107}$	95.95	MH187647.1
Bacillus subtilis strain X502	$3e^{-107}$	95.62	KU240496.1
Uncultured *Bacillus* sp clone N98	$3e^{-107}$	95.60	KP704278.1
Bacillus *tequilensis* strain S521 B-7	$3e^{-107}$	95.95	HQ238440.1
Bacillus subtilis strain	$1e^{-106}$	95.93	MN84775.1

Table 3: Sequence similarity for amplified regions of 1492R

Strain name	E-value	Percentage of similarity	Accession number
Bacillus tequilensis strain NJ6	$1e^{-115}$	98.75	KT58868.1
Bacillus methylotrophicus PO7	$1e^{-115}$	98.75	JN700153.1
Bacillus subtilis strain NS6	$1e^{-115}$	97.98	HQ834863.1
Bacillus licheniformis CRN3	$5e^{-115}$	98.74	MK8635666.1
Bacillus velezensisi AAUBCT	$5e^{-115}$	98.74	MK801267.1
UnculturedDZGS22	$5e^{-115}$	98.74	MH267236.1
Bacillus sp strain 3BJ5	$5e^{-115}$	98.75	MG062834.1
Bacillus subtilis inaquosorum STRAIN YN32	$5e^{-115}$	98.74	KC511536.1
Bacterial environmental culture clone W141 (2011)	$5e^{-115}$	98.74	HQ731030.1

and pour plate method yielded confluent growth of creamish pink colony repeatedly [Figure 2].

Gram staining of confluent grown colonies yielded slender rods, purple in color, indicating Gram-positive reaction [Figure 3].

Catalase, an extracellular enzyme secreted by several microorganisms, helps in degradation of hydrogen peroxide produced during carbohydrates utilization by aerobic bacteria, thereby its presence or absence in a microbial cell can be used as a significant indicator that the isolates could belong to the *Bacillus* species that was further characterized through molecular means for confirmation [Figure 4].

Molecular approach is used in bringing about preferential amplification of 16srRNA genes by using universal primers 27F and 1492R [Figures 5 and 6]. The amplified segments are sequenced, sequence similarity assessed by BLAST and corresponding dendrogram was constructed. From the data, the isolate found in Dhanyamla had 98% similarity with *Bacillus* sp. There were reports of non-pathogenic mainly aerobic and mesophilic organisms and a few counts of *Staphylococcus aureus* that were reported previously (Ranasinghe and Ediriweera 2015).[10]

CONCLUSION

Ayurvedic formulation, resultant of aging is said to be rich in several bioactive compounds, some may be prebiotics or unusual amino acids having special therapeutic values. In our study, we could successfully isolate *Bacillus* sp. There are few species of *Bacillus cereus*, *Bacillus clausii*, and *Bacillus pumilus* currently being used as probiotics. The occurrence of *Bacillus* sp in Dhanyamla could be purely associated with the preparation method. Kumar *et al*. (2012)[19] reported the occurrence of Gallic acid, Ellagic acid, and its derivatives through. High-performance liquid chromatography–mass spectrometry analysis and presence of *Lactobacillus* sp., *Acinetobacter* sp., *Alcaligenes* sp., and *Methylobacterium* sp in Kutajarista through 16S rRNA gene clone library approach

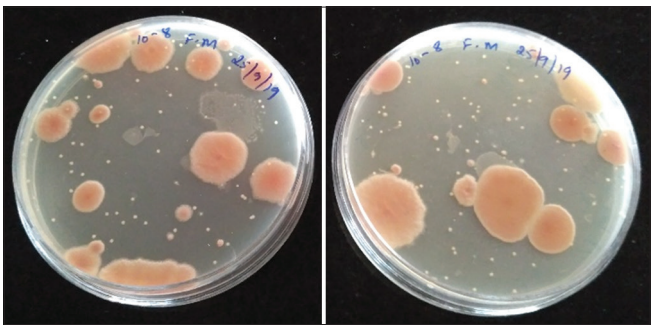

Figure 2: Isolation of microbes from Dhanyamala on DeManRogosa and Sharpe media

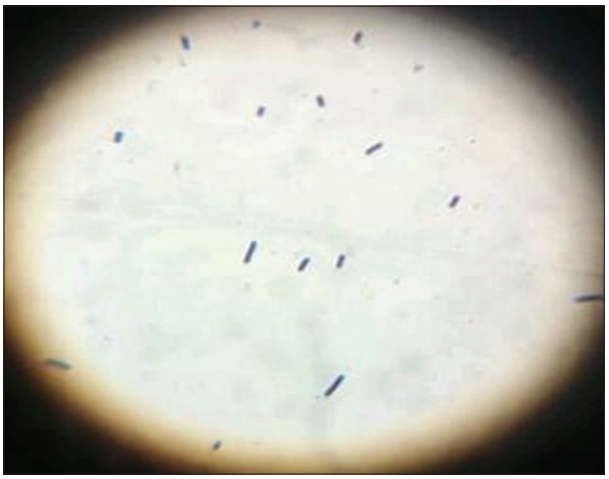

Figure 3: Gram positive rods

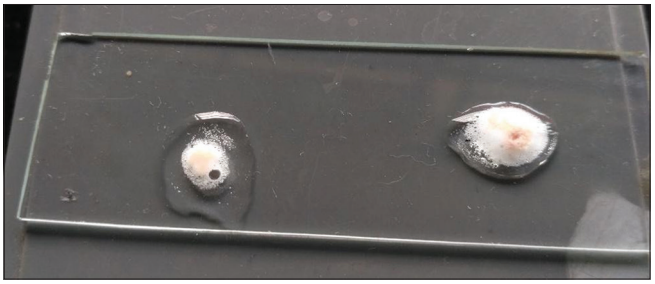

Figure 4: Catalase positive reaction

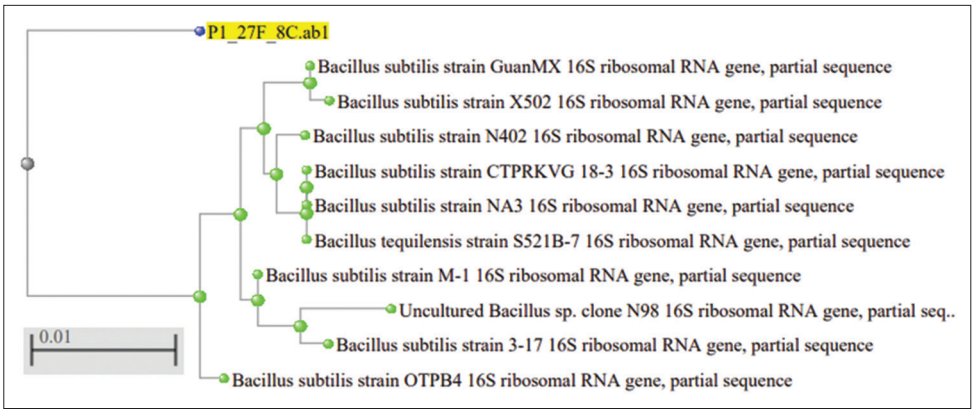

Figure 5: Dendrogram of nearest sequence similarity matching with amplified sequences for primer27F

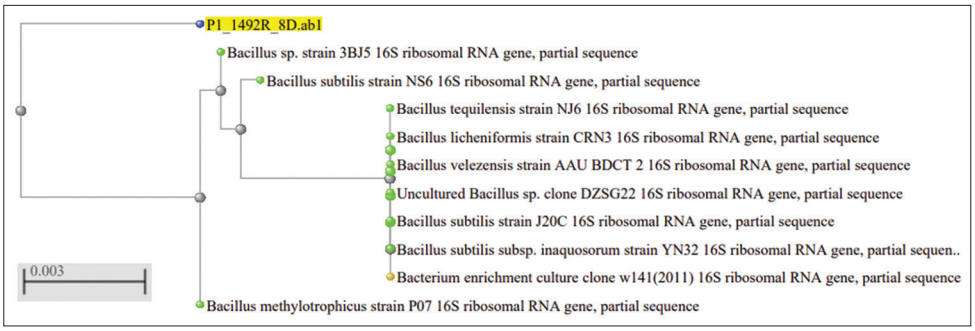

Figure 6: Dendrogram of nearest sequence similarity matching with amplified sequences for 1492R

in Kutajarista an Ayurvedic fermented herbal formulation prescribed for gastrointestinal disorders. The herbal formulation undergoes a gradual fermentative process and takes around 2 months for production with physicochemical changes in terms of alcohol percentage between 9% and 11% and a pH range from 3.6 to 3.8. A similar study was carried out by Ranasinghe and Ediriweera 2015 and reported Dhanyamla is to be light brown liquid with acidic odor and sour taste. Specific gravity and the pH of Dhanyamla was 1.0068 and 4.13 at 30°C. There was also reports of study was carried out by Elmahood and Doughari[20] for Kunun-zaki, an indigenous fermented non-alcoholic beverage which is widely consumed in Nigeria. The pH of the tested samples of Kunun-zaki was in a range of 3.34–4.42.

Although probiotics and prebiotics in isolation offer significant health benefits, multi-strain formulations boost their effectiveness combinatorically as the gut microbiota is a large dynamic system that requires intricate cross-communication to have significant clinical benefit. Hartmann *et al*., 2015 reported a group of more than 20 small water soluble compounds Mycosporine-like amino acids (MAAs) with absorption maxima between 309 and 360 nm primarily identified through UV spectralstudies and confirmed through hydrophilic liquid chromatography. Geraldes and Pinto 2021[21] suggested existence of MAAs are present especially in organisms that live in environments with high levels of UV radiation. Their composition varies according to the taxonomic group with the frequent coexistence of several MAAs with different absorption maxima allowing a more effective protective filter. Wittenberg (1960) isolated, for the 1st time, compounds with high UV absorption from a siphonophore, *Physalia physalis*.[22] In 1965, mycosporines were discovered in fungal sporulating mycelia.[23] A few years later, MAAs have also been detected in corals and cyanobacteria from the Great Barrier Reef.[24] Since then, MAAs have been identified in a wide variety of organisms, such as heterotrophic bacteria,[25] fungi,[26] cyanobacteria,[27] microalgae,[28,29] macroalgae,[30] lichens,[31] invertebrates (e.g., dinoflagellates, sponges, corals, sea urchins, and crustaceans) [32] and vertebrates (e.g., fishes).[33,34]

In our study, there was absorption maxima of 3.078 between 300 and 350 nm indicating the probability of MAAs as preliminary data this has to be validated further, strikingly this compound is neither produced by plant-based ingredient nor by micro-organisms alone but here the aging process during fermentation forces MAAs synthesis, which gradually develops moreover week confirmed by spectral analysis. So far MAAs are attributed as antioxidant, but they also act as immunomodulators. There needs more research to expedite further medicinal properties of Dhanyamla.

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