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Research Article

Evaluation of *Bacillus Circulans* in Imparting Aerobic Stability to Silage

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Abstract

Silage is the fermented product obtained through a process chain that starts from cutting of forage with high moisture to produce a stable feed that resists further digestion in anaerobic storage. Silage making is one of the best ways to overcome feed requirements of dairy cattle during summer seasons and drought situations occurring in India. It involves preservation of green fodder in acidic conditions due to action of native microbes and silage inoculant cultures under anaerobic conditions. A major challenge in silage is its instability upon air exposure. Fungal infestation and toxin production lead to deterioration of silage quality. Thus, research on aerobic stability enhancement and toxin reduction through use of anti-fungal agents has gained impetus. This paper explains potential of *Bacillus circulans* and its anti-fungal properties to protect silage during extensive use by inhibiting silage deteriorating fungi.

Keywords: *Bacillus circulans;* Toxin; Silage; Anti-fungal.

Introduction

India is the largest producer of milk in the world with an estimated production of milk in 2011-12 as 127.9 million tonnes with milking cattle numbers reaching 150 million animals in 2011-12 [1]. With sustained increase in livestock sector and milk production, the requirement of both dry and green type fodders is huge [2]. On an average in a day, a healthy bovine needs, 40 kg of wet and dry feed followed by, water requirement of minimum 80 L/day. In India, grasses like alfalfa, napier, non-flowering sorghum, baby corn, and maize varieties are used as wet/green fodders. Green fodder is the natural diet of cattle responsible for enhanced milk production, and qualitative change in the milk. Despite this importance & due to growing pressure on land for food, fodder production gets less priority. The purpose & thereby the availability of quality feed and fodder for livestock is essential for sustaining the livestock productivity. To overcome this situation and survival and feeding of livestock in dry and drought season, practice of silage making is gaining roots in India [3,4].

Silage is a feed obtained through fermentation of green, high moisture containing crops stored in "Silos" under anaerobic conditions. When the green fodder is stored in an airtight silo its fermentation by microbes results in production of lactic acid, acetic acid and formic acid, which prevent decomposition and growth of unwanted spoilage organisms. Eventually, the acids kill most of the microbes and preserve the silage as long as anaerobic condition is maintained [4].

Crops having good percentage of sugar and appropriate (35-40% dry matter; 65-60% moisture.) moisture are used for silage making. In India, green fodders in preflowering to flowering stage are most suitable for silage making [5]. Fodder is chopped to an inch size to prevent trapping of air and spillage of silage. After chopping, the fodder is packed in layers along with sugar syrup or molasses (3.5-4% w/w of green fodder), salt (0.5-1.0% w/w) and silage inoculant microbial culture (105 cfu/g of green fodder) using method of layering. Further the silo is covered with plastic film and weight is put over so as to keep the fodder compact and to avoid moisture loss. Silage gets ready with 4-5 weeks of incubation. Post incubation silo can be opened from one side and feeding can be started. Good quality silage has green, brown or golden color. Black color and presence of molds indicates poor quality silage. Good silage smells like lactic and acetic acid (like curd and vinegar). & taste is pleasant and acidic [5]. This occurs due to different phases associated with changes

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that take place in the designated "ensiling period".

There are two main phases in the ensiling period. First one is aerobic phase, in which oxygen present in the forage, is consumed by the plant material through the process of respiration. Under aerobic conditions, plant enzymes and aerobic microorganisms consume oxygen and burn up plant water-soluble carbohydrates, producing carbon dioxide and heat. First phase is kept short to maintain the quality of the silage because, excessive aerobic respiration reduces the energy content of the silage due to sugar breakdown and may cause heat damage to proteins [2,5].

Second or anaerobic phase begins with depletion of oxygen due to aerobic microbes. Anaerobic bacteria then begin to multiply rapidly and the fermentation process begins [2]. For good silage production, usage of silage inoculant cultures is preferred. These cultures mainly are of homo or hetero-fermentative Lactobacillus species [6]. Lactic acid produced by these silage inoculant cultures lowers the pH of silage thus inhibiting further fermentation process. Fermentation completely ceases after three to four weeks of ensiling [7]. At this point, pH becomes so low (3.8 to 4.0) that all microbial growth is inhibited (Figure 1).

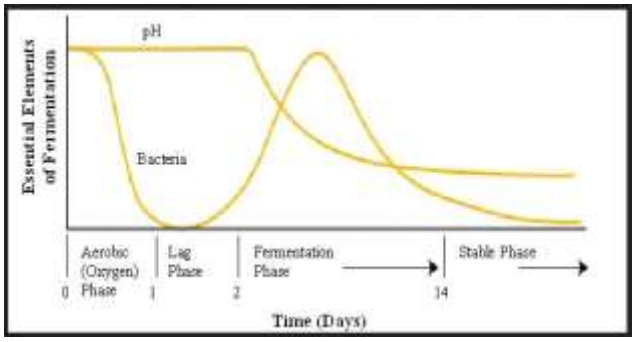

Figure 1. Phases in silage fermentation [7]

In any silage making process, main functions are to exclude air during the ensiling process and during storage. Limiting air present in the silage enhances feed quality and reduces spoilage during storage. In addition, whenever any silage is exposed to air due to frequent opening, there is a huge compromise on its quality. During such exposures, air facilitates growth of fungi that grow rampantly as, surface contaminants. High moisture, suitable temperature and air lead to rapid growth of fungi followed by spoilage of silage. This imparts bitter taste and enhances possibility of toxin production in silage by contaminant fungi [9,10].

This raises the challenge of aerobic stability of silage during its use. Most of the current commercial solutions for silage aerostability are centered on *Lactobacillus buchneri*. The present paper reports the capability of use of *Bacillus circulans* as robust alternative for aerostability of silage making in India. *B. circulans* is a gram positive, aerobic, sporulating, motile bacillus. It is a normal inhabitant of soil and well reported to produce antibacterial compounds of peptide origin [11-13]. Earlier it was being classified as *Bacillus subtilis*. But later reclassified as, *B. circulans* [14,15].

Materials and Methods

Ensiling protocol

The technique used for ensiling process was, drum silage. Evenly chopped maize and sugarcane tops were ensiled properly in 10 kg drums with spray of jiggery (3.5%, w/w) and salt (0.5%, w/w) water followed by spray of silage inoculants in concentration 105 cfu/g of maize and sugarcane tops. Silage drums were tightly packed and closed for 21 days. After 21 days, the silage was sampled from the drum and drums were resealed. Sampling was done after 45 and 60 days by following same sampling procedure. Frequent opening of drum silage revealed that, fungal contamination of silage was enhanced and could not be controlled. These contaminant fungi were then isolated for further study [16,17].

Protocol for fungal isolation and propagation

Contaminant fungi were isolated by suspending 1 g contaminated maize silage in 100 ml sterile physiological saline (0.8% NaCl solution) and 1 ml suspension was inoculated in potato dextrose broth (PDB) and Malt extract glucose yeast extract peptone broth (MGYPB) with pH 4.8 to 5.0 for enrichment at 30°C for 48-72 hrs at 150 rpm. Post incubation, loop-full of the enriched culture was streak inoculated on potato dextrose agar (PDA) and Malt extract glucose yeast extract peptone agar (MGYPA) with pH 4.8 to 5.0. Plates were incubated for at 30°C for 72-96 hrs.

For anti-fungal assay testing, predominant fungal isolate was grown and sub-cultured in MGYP agar and broth. The growth temperature used was 30°C and 150 rpm. For

assay, sporulated fungal culture was used. Spores were collected by harvesting fungal mycelia in sterile saline tween 80.

Identification of fungal isolates

Of the fungal isolates obtained only predominant isolate was further identified using ITS region gene sequencing method [18].

Protocol for growth and propagation Bacillus circulans culture

Bacillus circulans was obtained from MTCC. The culture was grown in 50 ml HiMedia nutrient broth (Composition: soya peptone 10 g/L, sodium chloride 5 g/L, beef extract 3 g/L, pH 7.2, sterilized at 121°C for 20 minutes) flasks of 250 ml capacity and incubated at 37°C for 18-24 hrs at 150 rpm. Post incubation, cell free extract (CFE) was obtained by harvesting the broth aseptically by centrifugation at 8000 rpm for 10 min. The supernatant was aseptically removed and stored at 4°C till further use [19-21].

Protocol for isolation of anti-fungal fraction from B. Circulans CFE

B. circulans being reported to produce anti-microbials mostly of proteinaceous origin, fractional ammonium sulphate precipitation technique was used for isolation of anti-fungal fraction from *B. circulans* CFE [5]. Centrifugation at 8000 rpm was used to obtain 500ml CFE from overnight grown culture of *B. circulans*. 100 mL CFE was subjected for precipitation using fractional ammonium sulphate precipitation protocol by, "Richard Simpson [5]. Then ammonium sulphate concentrations were increased gradually, till 60% saturation. Precipitate obtained through each step was then allowed to settle for overnight and next day the precipitate was recovered by centrifugation and re-suspended in minimum amount (1 mL) of physiological saline. This precipitate and supernatant were again tested for anti-fungal assay using anti-fungal assay protocol.

Protocol for S. monosporum growth and sporulation

Predominant fungal isolate (identified as, *S. monosporum)* was grown and sub-cultured in MGYP agar and broth. The growth temperature used was 30°C and 150 rpm. For assay, sporulated fungal culture was used. Spores were collected by harvesting fungal mycelia in sterile saline tween 80 using glass beads.

Protocol for anti-fungal activity assay

Malt extract glucose yeast extract agar (MGYP) medium plates with composition, Malt extract 3 g/L, yeast extract 3 g/L, Glucose 5 g/L, Peptone 3 g/L was used for all anti-fungal experimentation unless stated otherwise. 0.1 mL of *S. monosporum* spore suspension was spread inoculated over surface of MGYP agar plate. A well of 10 mm diameter was bored aseptically using sterile cork borer and 100 µL of CFE of *B. circulans* suspension was added to this well. Later these plates were kept in refrigerator at 4°C for 15 min to enhance CFE diffusion through agar. These plates were incubated at 30°C for 48-96 hrs. Post incubation the plates were observed for inhibition zones around the wells. The zone diameters were recorded. The re-suspended pellet & supernatant of fractional ammonium sulphate were evaluated similarly in anti-fungal assay. All the well diffusion experiments were performed in triplicates along with negative control of respective growth media.

Results and Discussion

Isolation and characterization of fungal isolates

Five fungal isolates were obtained from contaminated maize silage, using fungal enrichment and isolation protocol. Of these five fungal isolates, isolate no. 2 was predominantly present and thus was selected for further experimentation. Isolate was identified as, S*yncephalastrum monosporum* by ITS region gene sequencing technique and NCBI BLAST [18] with ITS region gene sequence as follows (Figure 2).

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>FI69 536 bp
ATCATTCCCAATTTTTTTTTCTCTCTTCATTGAGAGGAAAGAATTTGG
TATTCACCCAGTCTATTGCAACGATTCCTGGGTTAACAAAGAATGGATT
TTCAATTAAAAACATTTTTTTTTTAATTACCAATTGATTTTTAATTGAAT
TGAAGTATAAAAAAAAAAGAAAGAAGGCTTTCAATTGAGACTTTGGACTT
TTTTTAAACAACTTTAAGCAATGGATTTTTTGGCTCTCGCATCGATGAA
GAGCGTAGCAAATTGCGATAATTAGTGCGATTTGCATTTTGCGAATCAT
CGAGTTCTTGAACGCACCTTGCACCCTTTGGCTTGTCCTTGGGGTATGC
TTGTTTCAGTACAACTATAAACCCACAAATGACATTTTTTTATTGAAAT
GTCCATTGGGATTGGGATGTCAAAGGGAAACCTTTCATCCTGAAAATGA
GTCCATAGGATTAAAAATCAATTGAGGTTTTTTTTTTCCTTTGCATCAA
ATTTTTTTCAATTAGAAAAAAAGCAATTGGGAAAAAAGGATCCAAT.
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Isolate 2: Syncephalastrum monosporum

S. monosporum fungal colony represents characteristic white cottony structures with aerial hyphae protruding from colonies. Microscopy

reveals spores at the ends of aerial hyphae (Figure 3 and table 1).

2.0 **Technology: fungal** identification based on ITS region gene sequencing.

3.0 **Library: NCBI**

Library Comment
Molecule Type: DNA, Query length: 536 bases; Database Name: nr
Description:All GenBank+EMBL+DDBJ+PDB sequences (but no EST, STS, GSS,environmental samples or phase 0, 1 or 2 HTGS sequences) *Program: BLASTN 2.2.28+

4.0 **Hit List : First five hits in NCBI Blast record**

Sample	Gene bank entry	Max ident	Kingdom	Phylum	Order	Family	Genus	Species
FI-69	HM999975.1	100%	Fungi	Zygomycota	Mucorales	Syncephalastraceae	Syncephalastrum	monosporum
	HM999974.1	100%	Fungi	Zygomycota	Mucorales	Syncephalastraceae	Syncephalastrum	monosporum
	JN315030.1	100%	Fungi	Zygomycota	Mucorales	Syncephalastraceae	Syncephalastrum	racemosum
	KF225035.1	100%	Fungi	Zygomycota	Mucorales	Syncephalastraceae	Syncephalastrum	monosporum
	HM999977.1	100%	Fungi	Zygomycota	Mucorales	Syncephalastraceae	Syncephalastrum	monosporum

Figure 2. BLAST results

Table 1. Colony characteristics of *S. monosporum* of 48-72 hrs age

Colony Characters	Size	Shape	Margin	Elevation	Consistency	Opacity	Color
	2 mm	Circular	Irregular	Flat	Dry	Opaque	White

Partial purification and testing of anti-fungal fraction of Bacillus circulans CFE

Goal was to identify and isolate anti-fungal activity fraction from *B. circulans* CFE. Precipitation was carried out by the protocol mentioned in materials and methods. Peptide fractions precipitated and re-suspended were tested on *S. monosporum*. Table 2 and 3 indicate results of partial purification of CFE by fractional ammonium sulphate precipitation. Confirmation of anti-fungal assay results was done by keeping negative controls by testing the fungal inhibition against ammonium sulphate solution of particular saturation. From observation of table 3, it was observed that, CFE precipitate obtained after 50% ammonium sulphate saturation had highest potency (18 mm inhibition zone) (Figure 4), After which at 60% saturation no anti-fungal activity was left in remaining CFE. Fungal isolate no. 4 was most sensitive culture, whereas isolate no. 1 was least sensitive fungal culture with respect to zone of inhibition.

From this experimentation and literature search, it was evident that, *B. circulans* was being earlier known to be classified as, *B. subtilis. B. circulans* had been reported to have property of inhibiting various microbes through antimicrobial peptides production [14,15,22,25,26]. But its use as potent antifungal or fungal growth retardant was not evident in any of the searched research paper.

Figure 3. Predominant fungal isolate: Isolate 2: *Syncephalastrum monosporum*

Figure. 4. Anti-fungal assay with re-suspended pellet obtained through 50% ammonium sulphate precipitation of cell free extract

Table 2. Observations for ammonium sulphate precipitation for *B. circulans* CFE

Ammo. sulphate % saturation	Vol. post saturation (ml)	Vol. of supernatant (ml)	Re suspension vol. of pellet (ml)
0	100	100	No pellet
30	104	102	1
40	107	105	1
50	109	102	1
60	105	100	1

Remaining 60% supernatant could not inhibit any of the test fungus. Hence fractional precipitation was stopped at 60% ammonium sulphate concentration.

Table 3. Observations for ammonium sulphate precipitation followed by well diffusion assay of *B. circulans* CFE for *S. monosporum*

CFE salt saturation (%)	Isolate No.	Zone of inhibition in supernatant (mm)	Zone of inhibition in pellet (mm)
0	2	6	18
		6	18
30	2	4	16
		4	18
40	2	4	20
		4	18
50	2	2	18
	2	2	18
60	2	No zone	16
	2	No Zone	18

Conclusion

B. circulans was being earlier known to be classified as, *B. subtilis.* It was reclassified as, *B. circulans*. *B. circulans* has ability to produce anti-fungal products which may help in imparting aerobic stability to silage in Indian farm conditions. *B. circulans* can be checked for its use as silage inoculant for aerostability of silage. Anti-fungal properties of *B. circulans* have been exhibited in both CFE and 50% ammonium sulphate precipitated CFE pellet. But further research work is required for exploring the possibility of its usage as potent anti-fungal agent in various applications other than in silage making.

Conflicts of Interest

The authors hereby declare that they have no conflict of interest.

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