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# Isolation of biosurfactant producing *Bacillus* spp. from milk samples

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**Abstract**
Fresh raw milk, pasteurized milk and UHT milk samples obtained aseptically, after plating the fresh milk samples, and after the samples showed spoilage signs, enumerated to obtain biosurfactant producing *Bacillus* spp. by heating the samples to $80^0$C for 10 min. to select spores of *Bacillus* species using sterile butter fat agar medium. The colonies that showed clear zone were considered as biosurfactant species of *Bacillus*. Among the milk samples enumerated, pasteurized milk fresh as well as spoiled showed a higher viable count of 3.16 and 4.57 respectively. Biosurfactant producing *Bacillus* species in raw milk were nearly the same as both in fresh and spoiled samples (3.12 and 3.43 $\log_{10}$ cfu/ml). In Ultra High Temperature (UHT) milk, viable count of biosurfactant was absent in fresh sample and in spoiled showed a lower count of 2.24 $\log_{10}$ cfu /ml. A total of 23 isolates showing clear zone on butterfat agar from fresh and spoiled milk samples were obtained based on colony morphology of *Bacillus* spp. and coded from $B_1$ to $B_{23}$ continuously. The isolates obtained from pasteurized and raw spoiled milk samples were 10 and 6 numbers. Accounting for 43.47 and 26.08 respectively.

**Keywords:** Biosurfactants, *Bacillus* spp., Pasteurized milk, UHT milk, Spoiled

## Introduction
Saprophytic bacteria enter the raw milk from various sources and release biosurfactants and extracellular enzymes that lead to defects, lowering the shelf life. Biosurfactants or microbial surfactants are surface metabolites produced by bacteria having very different chemical structures and properties. Though biosurfactants produced from spoilage-causing bacteria cause the spoilage, they carry certain important functional properties. Many *Bacillus* spp. are major contaminating bacteria from soil and air involved in spoilage of milk, as they produce surfactants with many beneficial properties. They also survive heat treatment of milk like pasteurization, boiling, steaming and even in Ultra High Temperature (UHT) milk if initial aerobic spores are more. During storage after heat treatment of milk, the surviving spores may germinate extending the biosurfactants and extracellular enzymes (Nuneza *et al*., 2003) [6].
*Bacillus subtilis, Bacillus polymyxa, Bacillus licheniformis, Bacillus pumilus* are major biosurfactant producers among the genus of *Bacillus*. A large variety of *B. subtilis*, as well as *B. licheniformis* strains, produce lipopeptide biosurfactants which possess a high surfactant activity such as surface active properties and antibacterial activity having wide application in food and dairy industries (Yeh *et al*., 2005) [10].

## Materials and methods
### Enumeration of biosurfactant producing *Bacillus* spp.
Fresh milk samples of raw milk, pasteurized milk, and Ultra High Temperature (UHT) milk as well as the same samples after spoilage were subjected for enumeration of biosurfactant producing *Bacillus* spp. Milk samples were heated to 80ºC/10 min. for selection of spores of *Bacillus* spp. After heat treatment, the samples were cooled and serially diluted and pour plated using sterile butterfat agar and incubated at 37ºC/24-48 h. as per the procedure of Harrigan (1998) [2]. Colonies with clear zone were considered as biosurfactant producers (Sanjana *et al*., 2017) [7].

### Isolation and confirmation of biosurfactant producing *Bacillus* spp.
Isolates were purified by streaking 3 times onto poured nutrient agar plates. After the third streak, the discrete colonies were selected and maintained on sterile nutrient agar slants as stock cultures and subcultured once in a month. Working cultures were prepared by inoculating the isolate on the slant to sterile nutrient broth tubes and subcultured once in a

week (Meena & Kanwar, 2015) [4]. The isolates obtained from milk samples were subjected to preliminary tests like Gram's staining, bacterial spore staining, catalase, and oxidase tests to confirm the isolates as *Bacillus* spp as per the standard procedures of Harrigan (1998) [2].

## Result and Discussion

### Enumeration of surfactant producing *Bacillus* spp. from milk samples

Fresh milk samples such as raw milk, pasteurized milk, and UHT milk showed a viable count of surfactant producing *Bacillus* spp. ranging from 0 to 3.16 $\log_{10}$ cfu/ml. The fresh samples of raw milk and pasteurized showed around 3 log count of biosurfactant producers. The fresh milk samples when kept at room temperature showed spoilage. The same spoiled milk samples when plated, exhibited a log count of biosurfactant producing *Bacillus* spp. ranging from 2.24 to 4.57. Among the spoiled milk samples, pasteurized milk had 4.57 viable count of surfactant producing species of *Bacillus* followed 3.43 and UHT 2.24 of raw milk and UHT milk respectively. Pasteurized milk samples both fresh and spoiled showed more viable log count 3.16 and 4.57 of surfactant producing *Bacillus* spp. as clear zone formers around the colonies in sterile butter fat agar medium respectively compared to raw and UHT milk samples. When the milk samples were procured, fresh samples showed less count and UHT sample showed the absence of biosurfactant producers. But when the same milk sample was allowed to spoil and later enumerated for biosurfactant producers of *Bacillus* spp., the counts were observed and also more. This indicates that the contaminating bacteria enter through the soil, fodder, feed, air and utensils in a dairy farm and become active once they adjust to the environment, produce biosurfactants to utilize the milk constituents which predominate once the milk attains spoilage.

The literature regarding enumeration of milk borne surfactant producing *Bacillus* spp., are scanty. Isolation of species of *Bacillus* producing surfactant are available with respect to the soil sample, but no work on direct enumeration on selective medium for biosurfactant producers. The method adopted in this enumeration helps in the future to directly enumerate and isolate biosurfactant producing *Bacillus* species which has not been attempted so far with respect to milk samples. Hence, for enumeration of surfactant producing species of *Bacillus*, no supportive work has been traced. Nitschke and Pastore (2004) [5] proved that *Bacillus* spp. occurs mainly in soil and because of their spore forming ability; the spores have the ability to survive harsh conditions in the soil environment.

### Number of isolates and per cent occurrence of biosurfactant producing *Bacillus* spp. in milk samples

After the enumeration of surfactant producing *Bacillus* spp. obtained from milk samples, colonies with a clear zone of 23 numbers. Were selected based on colony morphology on butter fat agar medium. The isolates of *Bacillus* spp. were confirmed by preliminary tests like Gram's staining, spore staining, catalase and oxidase tests. Isolates from spoiled pasteurized milk sample was more ie., 10 numbers. (43.47%), followed by spoiled raw milk of 6 numbers. (26.08%) and only 1 no. from spoiled UHT milk sample. Only 4 isolates were picked from fresh pasteurized milk (17.39%) while 2 from fresh raw milk (8.72%) and none from fresh UHT milk samples.

It is clear from the present investigation that the fresh milk samples have a low incidence of biosurfactant producers compared to spoiled milk samples. Among spoiled milk samples, pasteurized spoiled milk showed more isolates as pasteurized milk might have helped in the selection of spores of *Bacillus* spp. due to heat treatment (80 ºC/10 min) provided. Raw milk compared to pasteurized milk had less number of isolates as sporulation in raw milk is less compared to pasteurized milk, due to the availability of nutrients. UHT kills vegetative cells as well as spores due to high heat treatment (135 ºC/1 to 2 sec) provided and hence less incidence of isolates of *Bacillus* spp. None of the literature exists with regard to the isolation of surfactant producing *Bacillus* spp. directly on butter fat agar medium. Authors have used crude oil or oil contaminated soil for isolation on nutrient agar and later screened for their biosurfactant ability. Chen *et al.* (2006) [1] showed 22.72% of *Bacillus* spp. isolated from crude oil samples in Iran on nutrient agar. High incident rate of *Bacillus* spp. of 22.73% was observed in domestic oil contaminated waste water when compared to other organisms isolated, may be a direct correlation to their ability to produce emulsifiers to degrade oil rather than just a coincidence (Tabatabaee *et al.*, 2005) [9], While Jaysree *et al.* (2011) [3] also found *Bacillus subtilis* and *Bacillus licheniformis* are most important biosurfactant producers among *Bacillus* spp. According to Singh (2012) [8], major species of *Bacillus* that produce biosurfactant are *Bacillus subtilis* and *Bacillus licheniformis*. The species were isolated from soil samples on nutrient agar but required screening tests for production of biosurfactant.

**Table 1:** Enumeration of surfactant producing *Bacillus* spp. from milk samples

| Sources of milk sample | Type | Sample codes | Viable count ($\log_{10}$ cfu/ml) of *Bacillus* spp. |
|---|---|---|---|
| Raw milk | Fresh | RMF | $3.12^{aA}$ |
| | Spoiled | RMS | $3.43^{bA}$ |
| Pasteurized milk | Fresh | PMF | $3.16^{aB}$ |
| | Spoiled | PMS | $4.57^{bB}$ |
| UHT milk (Ultra-High Temperature) | Fresh | UHF | $0.00^{aC}$ |
| | Spoiled | UHS | $2.24^{bC}$ |
| $CD_{source}$ ($p \leq 0.05$) | | | 0.056 |
| $CD_{type}$ ($p \leq 0.05$) | | | 0.039 |

**Note**

- Values are average of three trials
- Lower case alphabets as superscript indicate significant difference in type of milk.
- Upper case alphabets as superscript indicate significant difference in sources of milk.
- Samples heated to 80 ºC /10 min for selection of spores of *Bacillus* spp.
- Pour plated using Butter fat agar and incubated at 37 ºC/24- 48 h.
- Colonies with clear zone around the colonies were counted as biosurfactant producer.

**Table 2:** Number of isolates and per cent occurrence of biosurfactant producing *Bacillus* spp. in milk samples

| Sources of milk samples | | Code of Isolates | No. of Isolates | Percent occurrence |
|---|---|---|---|---|
| Raw milk | Fresh | $B_1$, $B_2$ | 02 | 8.72 |
| | Spoiled | $B_3$, $B_4$, $B_5$, $B_6$, $B_7$, $B_8$ | 06 | 26.08 |
| Pasteurized milk | Fresh | $B_9$, $B_{10}$, $B_{11}$, $B_{12}$ | 04 | 17.39 |
| | Spoiled | $B_{13}$, $B_{14}$, $B_{15}$, $B_{16}$, $B_{17}$, $B_{18}$, $B_{19}$, $B_{20}$, $B_{21}$, $B_{22}$ | 10 | 43.47 |
| UHT milk | Fresh | Nil | 00 | 0 |
| | Spoiled | $B_{23}$ | 01 | 4.34 |
| Total | | | 23 | 100 |

## Conclusion

Among the raw, pasteurized and UHT milk samples enumerated, pasteurized milk fresh as well as spoiled showed higher viable count. Biosurfactant producing *Bacillus* species in raw milk were nearly the same as both in fresh and spoiled samples. In UHT milk, viable count of biosurfactant was absent in fresh sample and in spoiled showed lower count. A total of 23 isolates showing clear zone on butter fat agar from fresh and spoiled milk samples were obtained based on colony morphology of *Bacillus* spp. and coded from $B_1$ to $B_{23}$ continuously. The isolates obtained from pasteurized and raw spoiled milk samples were 10 and 6 numbers accounting for 43.47 and 26.08 respectively.

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