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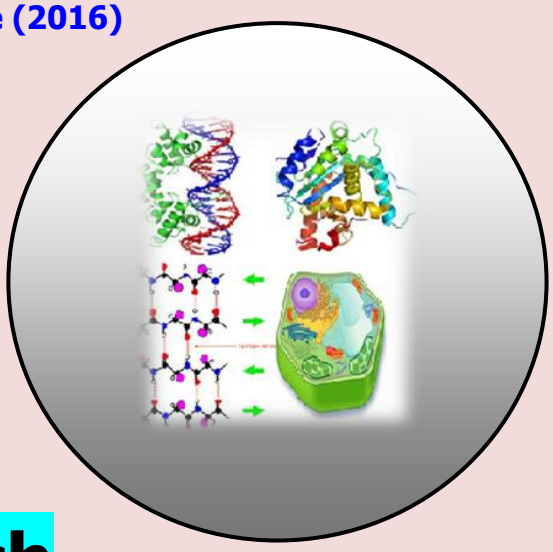

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Isolation and Identification of *Geobacillus kaustophilus* from Salbardi Hot Spring and Compared with *Geobacillus stearothermophilus* for Antibiotic Detection in Milk Samples by Agar Diffusion Method

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ABSTRACT

The presence of certain antibiotic residues in milk is dangerous for human consumption. These antibiotic residues are the potential risk for consumers'- health because it may be toxic and potentially help to develop antimicrobial resistance. Kundrat Agar is used for detection of antimicrobial residues in animal feed preparations and dairy products. The test is economically cheap, easy to perform and carried out using an Ampoule of Geobacillus stearothermophilus (LA840) as test microorganisms. Geobacillus stearothermophilus is an outstanding testing microorganism for its properties to grow rapidly at higher temperatures (64°C) and a high sensitivity to the β-lactam antibiotics. In the present investigation, a thermotolerant bacteria Geobacillus kaustophilus was isolated from Salbardi hot spring. Antibiotics detection in milk samples was performed in the form of an agar diffusion test. Antibiotics or inhibitors present in the samples showed inhibition zones more than 10mm detecting up to lowest concentrations of 1 ppm to 10ppm. And after considering all the screening tests along with results on agar diffusion test on Kundrat agar, Geobacillus kaustophilus 2HSBW2 was found to be a prominent replacement for commercially used Geobacillus stearothermophilus.

Keywords: Antibiotic detection, Geobacillus kaustophilus, Geobacillus stearothermophilus, Thermo-stable and Salbardi hot spring.

INTRODUCTION

Globally, attention is paying towards the evaluation of the risk of occurrence of veterinary drug residues in foodstuffs and foods of animal origin, to take appropriate measures to reduce this risk. The design and strategy involved in antibiotics detection in milk are the health safety of the milk regulated by the recent legislative regulations (toxicological safety) and ability to sell the milk depending on its quality (technological safety). Veterinary drug residues in milk and other food products represent a health risk for the consumer. (Navratilova, 2008) In lactating cows, antimicrobial agents are used mostly for the therapy of mastitis but also of other diseases (e.g. laminitis, respiratory diseases, metritis). Long-acting antimicrobials are commonly used in dry-cow therapy. (Honkanen-Buzalski T., 1997; Botsoglou N.A., 2001) Antimicrobial agents administered to cows in the course of lactation can pass to milk in various levels. Therefore a screening assay is required that

gives a reliable and accurate indication of the presence of antibiotic residues at unsafe or violative levels (MRLs). The assay should be developed with a detection level optimized below the MRLs so that a violative sample above these levels will be detected with the positive test result. (De La Haye, 1996; O' Rangers, 1993) There are some maximum residue limits (MRLs) or tolerances of approved veterinary drugs directed by Canada, European Union and United States for milk which are followed all over the world. (Code of Federal Regulations, 1994 (1 April); Europian Commission, 1990; Stephany, 1990)

Many human and animal health concerns have been expressed over the years in regard to the overuse of antibiotics in agricultural production as well as the presence of residues in the food chain. Such concerns include the potential for allergic reactions in sensitized individuals (penicillins), toxicity such as aplasia of the bone marrow (chloramphenicol), effects on the human gut microbial populations, the emergence of resistant bacteria within animals and the transfer of antibiotic resistance genes to human pathogens. (Government of Canada, 1994; Franco, 1990; Garrod, 1964; Huber, 1986) In addition, some compounds such as the nitrofurans have been found to be animal carcinogens and mutagens in genotoxic tests. The validity of any public health threat posed by these concerns has been debated in the scientific community for over 40 years. A few cases of minor allergic reactions (e.g., skin rashes) in previously sensitized individuals to penicillin G residues in milk and meat have been documented as well as strong evidence linking the widespread agricultural use of antibiotics to an increase in antibiotic resistance among animal and human pathogens. (Government of Canada, 1994; Garrod, 1964; Mitchell, 1995; Van Houweling, 1969; Dewdney, 1984; Burgat-Sacaze, 1986; Kindred, 1993; Ormerod, 1987; Vickers, 1964).

In 1993 a survey of all violative carcasses in the United States revealed that the drugs most frequently causing residues were penicillin (20%), streptomycin (10%), oxytetracycline (10%), SMZ (9%), tetracycline (4%), gentamicin (4%) and neomycin (3%). (Paige, 1994).

The earliest methods used for the detection of antimicrobial residues in foods and milk were based on the detection of growth inhibition of various sensitive bacterial strains. Such methods, originally developed for use in clinical medicine, were based on microbial agar diffusion tests or the inhibition of acid production or coagulation by starter organisms. Nowadays a large variety of detection methods are available ranging from a physicochemical analysis (e.g. LC/MS) or immunological detection (e.g. ELISA) to microbiological methods (e.g. growth inhibition tests) (Navratilova, 2008; Babington R., 2012), with some commercially available as test kits. Agar diffusion methods are even implemented into official national standard methods such as the three-plate-test which is using *Bacillus subtilis* BGA or the brilliant black reduction test (BRT) using Geobacillus stearothermophilus. (Clauβen. M., 2013) Most of the rapid detection test kits and growth inhibition tests (for example Delvotest®SP Mini-NT and BR-Test®AS Brilliant) which again use *Geobacillus stearothermophilus,* commercially available were specifically developed for the dairy sector to detect antibiotics in milk. However, these sophisticated methods need expensive technical apparatus and often specific sample preparations are costly and laborious. (Zvirdauskienė. R, 2007; Kneebone J., 2010; Perme T., 2010; Kantiani L., 2009) Kundrat-agar contains bromo-cresol purple as pH indicator which turns from purple to yellow due to the formation of acid from glucose because of bacterial metabolism. A lack of change in colour from purple to yellow indicates growth inhibition by a clear zone. *Geobacillus stearothermophilus* is a gram-positive, spore-forming bacteria able to bring about the change in colour of the pH indicator present in the medium from purple to yellow. Presence of chemotherapeutic agents is indicated by the formation of inhibition halos or zones around the sample. Nevertheless, the same test can be done using the other bacterial strains are nowadays topic of research.

Geothermal springs including hot springs are substantially higher in temperature than the air temperature of the surrounding region. It has been a rich source of thermo-tolerant microorganisms which survive in extreme condition of higher temperature. Salbardi thermal spring situated near Maru River, in central India is known for its medically important hot spring water and its different chemical composition. Thermotolerant organisms often found in these hot springs produce thermo-stable biomolecules and enzymes which are economically important. (Dixit M., 2018).

The present study focuses on isolation and identification of thermotolerant bacteria *Geobacillus kaustophilus* from Salbardi hot spring and comparison between *Geobacillus stearothermophilus* and test isolate *Geobacillus kaustophilus* 2HSBW2 in accordance to observe the growth on Kundrat agar and detection of antibiotics mostly used in milk by microbial screening method.

MATERIALS AND METHODS

Sampling From Salbardi Hot Spring:

Soil and water samples were collected from salbardi hot spring region according to standard methods. Samples were collected from hot spring including other geological points between latitude and longitude 21° 25' 11.2" N, 78° 00' 41.4" E, in different weather conditions.

Isolation and identification:

Samples were processed in the laboratory within 24 hrs after collection, for isolation Luria-Bertani (LB) broth was used and for the thermo-tolerant organisms, selective media TT Medium (ATCC medium 6977) [Thermus medium] was used. Serial dilution and enrichment culture techniques were used to isolate pure colonies of bacteria at different incubation temperatures from mesophilic to the thermophilic range. Identification carried out using conventional biochemical tests (Cappuccino and Sherman, 1987) and by 16S rRNA sequencing.

Thermo-stable Bacterial Isolate:

Isolate 2HSBW2 was selected after it sustained the optimum growth for five subsequent sub-culturing on LB agar at 65°C. To check thermo-stability of bacterial isolate growth kinetics was observed at a higher temperature i.e., temperature range from 45 to 85°C in LB broth at 630 nm on UV – Visible spectrophotometer (Perkin Elmer).

Molecular Characterization

Total DNA isolation and extraction from bacterial cells for PCR analysis was done by Genomic CTAB protocol. PCR amplification of the 16S rRNA gene fragment was done by using 27forward (AGAGTTTGATCMTGGCTCAG) and1492reverse (ACGGYTACCTTGTTACGACTT) primers. The amplification mixture contains 32.0 µl nuclease free water, 5.0 µl PCR buffer 10x, 2.0 µl dNTP (10 mM), 4.0 µl forward primer (10 µM), 4.0 µl reverse primer (10 µM), 1.0µl Taq DNA polymerase enzyme (1U/ µl) and 200ng DNA template. PCR reaction was programmed as: Initial denaturation of 3 min. at 94ºC, denaturation of 1 min. at 94ºC, primer annealing for 1 min. at 54ºC, extension of 2 min. at 72°C, final extension for 5 min. at 72°C; total 30 cycles and stored at 4°C. Amplicon was sequenced and analyzed by BLAST to find the best-scored close homolog and further accession number of the isolate was obtained through NCBI genbank database. In a phylogenetic analysis, top five best homologs were aligned in CLUSTALW and later on designed for phylogram in MEGA5 software. (Eden P. A., 1991; Universal Bacterial Identification by PCR and DNA Sequencing of 16S rRNA Gene., 2010; Altschul S. F., 1997)

Antibiotic Susceptibility Test

Antimicrobial susceptibility test was done by Kirby Bauer Disc Diffusion Method on Mueller-Hinton Agar. (Bauer AW., 1966; Oyetibo GO., 2010) Commercially available antibiotic disks from HiMedia were used. The antibiotics discs were placed on Mueller-Hinton agar plates previously inoculated with cell suspension with a turbidity of 0.5 McFarland standards. The plates were incubated at 37°C for 24 h and observed for the zone of inhibition. Antibiotic sensitivity of 2HSBW2 was tested for 21 different antibiotics. The antibiotics used were ampicillin (10 mcg), augmentin (30 mcg), norfloxacin (10 mcg), co-trimoxazole (25 mcg), gentamicin (10 mcg), tobramycin (10 mcg), cefoxitin (30 mcg), cephalothin (30 mcg), chloramphenicol (30 mcg), tetracyclin (30 mcg), streptomycin (10 mcg), kanamycin (30 mcg), amikacin (30 mcg), ceftazidime (30 mcg), aztreonam (30 mcg), pipracillin (100 mcg), imipenem (10 mcg), ciprofloxacin (5 mcg), fusidic acid (30 mcg), linezolid (30 mcg) and rifampicin (5 mcg). The sensitivity and resistance profile was determined on the basis of the diameter of the zone of inhibition and evaluation done according to National Committee for Clinical laboratory standard's chart provided with the antibiotic kits by HiMedia.

Growth on KUNDRAT agar

2HSBW2 along with *G. stearothermophilus* was tested for the fast and optimum growth on Kundrat agar having components Casein peptone (7.8 gms/L), Meat peptone (7.8 gms/L), Starch (4.0 gms/L), Gelatin (4.0 gms/L), Sodium chloride (3.0 gms/L), Yeast extract (2.8 gms/L), Dextrose (1.0 gms/L), Bromocresol purple (0.016 gms/L), Agar (10.0 gms/L) but in present study Bromocresol purple was replaced by Bromothymol blue (0.016 gms/L) maintaining final pH at 25°C to 6.8±0.2. 1 ml broth culture of each isolate was seeded with Kundrat agar in petriplates for 6-8 hrs to observe the colour change from greenish-blue to yellow at 65°C. (Kundrat W., 1972; (Kundrat W., 1968)

Comparative study of *Geobacillus kaustophilus* 2HSBW2 with *Geobacillus stearothermophilus* for antibiotic detection in milk samples:

After phylogenetic analysis 2HSBW2 isolate identified as *Geobacillus kaustophilus* was compared with commercially used *Geobacillus stearothermophilus* for antibiotic detection in milk by agar diffusion method for microbial screening.

a) Preparation of Samples

1. 5 different antibiotics, Ampicillin- 500mg (Capsule), Erythromycin- 500mg (Tablet), Amoxycillin- 500mg (Capsule), Azithromycin- 500mg (Tablet), Gentamycin- 80mg/2ml (Injection) was taken and final dilution made to 50µg/1ml.

2. Milk samples were taken from the market: Raw milk and pasteurized milk i.e., Raw milk-1 (RM-1), Raw milk-2 (RM-2), Pasteurized milk-1 (PM-1), Pasteurized milk-2 (PM-2), Spiked milk with antibiotic -1 (SM-1), Spiked milk with antibiotic-2 (SM-2).

*SM-1 spiked with 1 ml working standard of Ampicillin (AMP i.e., 50µg/1ml) in 9ml raw milk-1

*SM-2 spiked with 1 ml working standard of Gentamicin (GEN i.e., 50µg/1ml) in 9ml raw milk-2

b) Assay

200 µl of each antibiotic working standard and 200 µl of each milk sample was added to the wells made in previously seeded petriplates of 20ml Kundrat agar with 1ml, 1 OD broth culture of *G. kaustophilus* 2HSBW2 and *G. stearothermophilus* and incubated for 6-8 hrs at 65°C.[33]

RESULTS

Salbardi Hot Spring water Chemical Characteristics

In Salbardi spring water, the concentration of sulphate was found higher i.e. 103 mg/kg and water is Na-Cl-SO_4 type against the Ca-Mg-HCO_3 type regional groundwater and also approximately same concentration and water type recorded in the study of Ball J. W., 1991 and Pitale U.L., 1980.

Isolation of Thermo-tolerant Bacterial Isolates

Total 79 bacterial isolates were isolated from water and soil samples at various incubation temperatures. Out of 79 bacterial isolates very few were able to tolerate thermophilic temperature (above 45°C) and isolate 2HSBW2 was able to survive for five subsequent sub-culturing on LB agar at 65°C and not lose the ability to tolerate high temperature whereas, other isolates lose this ability to grow and sustain at high temperature. Morphological and biochemical features of the isolate 2HSBW2 are showcased in Table 1.

Table 1. Morphological and Biochemical Characteristics Isolate 2HSBW2.

Sr. No.	Tests	2HSBW2
1	Morphology	Rods
2	Gram Staining	Positive
3	Motility test	Negative
4	Temperature range for growth (°C)	40-75°C
5	Catalase test	Positive
6	Oxidase Test	Negative
7	Indole Test	Negative
8	Methyl Red Test	Not Determined
9	Voges Proskauer Test	Negative
10	Citrate Utilization Test	Positive
11	H2S Production Test	Negative
	Acid produced from,	
12	Glucose	Positive
13	Fructose	Positive
14	Maltose	Positive
15	Lactose	Negative
16	Mannitol	Negative
17	Arabinose	Negative
18	Xylose	Positive

Selection of the Thermo-tolerant Bacterial Isolates:

On the basis of subsequent sub-culturing on Luria-Bertani (LB) agar at the thermophilic range (60-80°C), 2HSBW2 was selected and its growth kinetics was observed. 2HSBW2 was found to be showing a stable and optimum growth at 65°C, growth kinetics observed from 45-85°C at 630nm at the interval of 0 to 120 hrs is given in Figure-1.

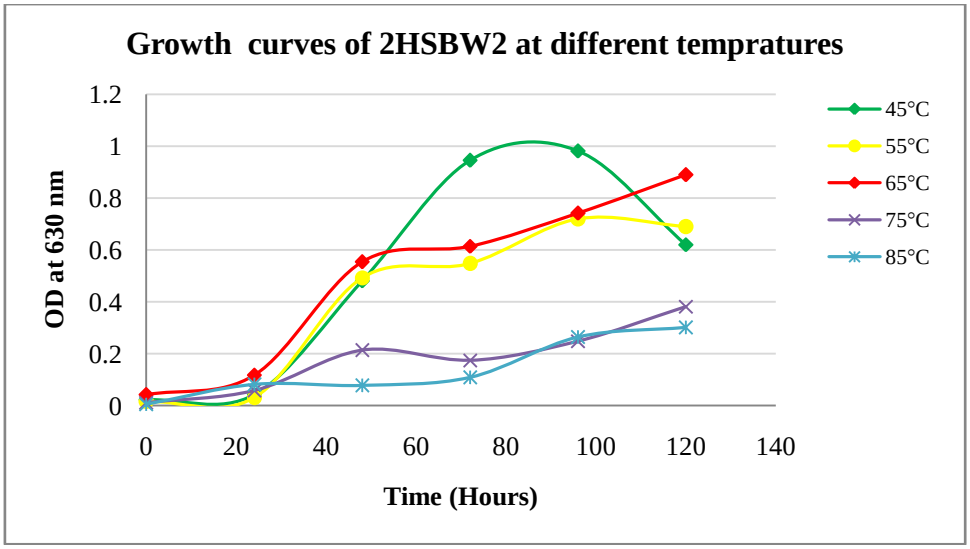


Figure 1. Graph representing growth of 2HSBW2 at different temperatures.

Phylogenetic Analysis by 16S rRNA Gene Sequencing:

Isolates 2HSBW2 was selected for the phylogenetic analysis and the criteria for selection was the organisms would be able to show antibiotic sensitivity to the maximum number of antibiotics and indicating similar growth pattern to that of *Geobacillus stearothermophilus*. After the phylogenetic study, the bacterial strain was identified and multiple sequence alignment of 2HSBW2 possessed 99% homology with *Geobacillus kaustophilus* strain N1216.

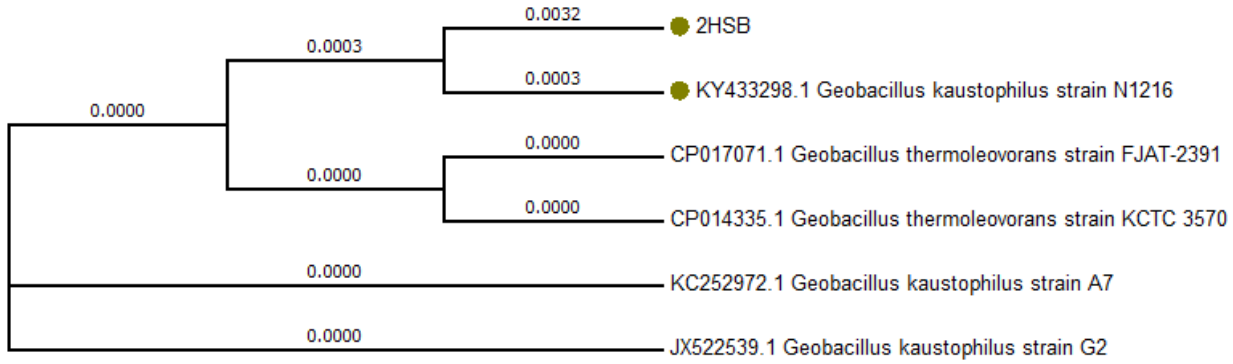


Figure 2. Phylogenetic tree for multiple sequence alignment of 2HSBW2.

Antibiotic Susceptibility Test

2HSBW2 was tested against 21 different antibiotics to identify the sensitivity to antibiotics with a higher zone of inhibition. In the present investigation out of susceptibility profile of 2HSBW2 was found to be highly sensitive to the maximum number of antibiotics which is shown in Figure - 3.

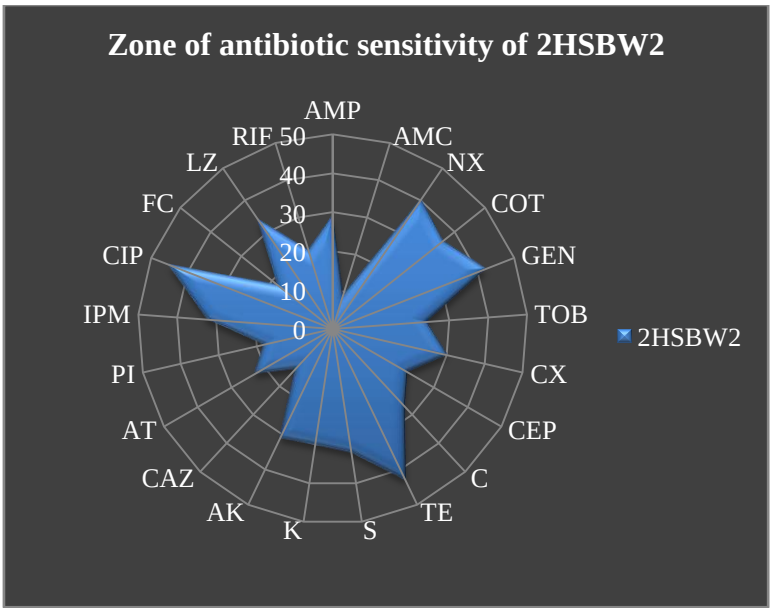
Growth pattern on Kundrat agar

Kundrat agar is generally used for antibiotic detection by microbial screening method. Isolates 2HSBW2 and *G. stearothermophilus* inoculated in this agar were observed for growth indicated by colour change of medium from greenish-blue to yellow due to acid formation within 6-8 hrs at 65°C. 2HSBW2 had given similar growth pattern like *G. stearothermophilus.*

Comparative study of *Geobacillus kaustophilus* 2HSBW2 with *Geobacillus stearothermophilus* for antibiotic detection in milk samples

Geobacillus kaustophilus i.e., 2HSBW2 subjected to a comparison with *Geobacillus stearothermophilus* for antibiotic detection in milk samples by microbial screening method on Kundrat agar.

Well diffusion method was used to test some antibiotics, raw milk sample, pasteurized milk sample and milk sample spiked with antibiotics in Kundrat agar previously seeded with 1 ml broth culture of both the organisms with an incubation time of 6-8 hrs at 65^0C, results are shown in Table-2. Colour change of medium from greenish-blue to yellow due to acid formation indicated the growth of organism while zone of inhibition measured for presence of antibiotic by measuring greenish-blue colour zone around sample well shown in Figure - 4.



[ampicillin (AMP), augmentin (AMC), norfloxacin (NX), co-trimoxazole (COT), gentamicin (GEN), tobramycin (TOB), cefoxitin (CX), cephalothin (CEP), chloramphenicol (C), tetracyclin (TE), streptomycin (S), kanamycin (K), amikacin (AK), ceftazidime (CAZ), aztreonam (AT), pipracillin (PI), imipenem (IPM), ciprofloxacin (CIP), fusidic acid (FC), linezolid (LZ) and rifampicin (RIF)]

Figure 3. Graphical representation of antibiotic sensitivity of 2HSBW2 against 21 different antibiotics.

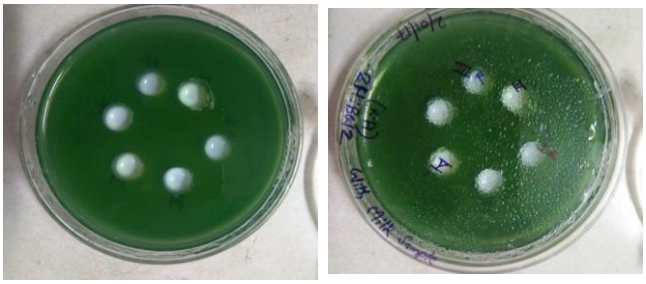
Table 2. Zone of inhibition for different samples on Kundrat agar with *Geobacillus kaustophilus* and *Geobacillus stearothermophilus*.

Sample	Zone of inhibition (mm)	
	G. kaustophilus 2HSBW2	G. stearothermophilus
AMP	19	11
ERY	0	0
AMX	20	0
AZM	0	0
GEN	25	13
RM-1	15	0
RM-2	13	0
PM-1	13	26
PM-2	18	31
SM-1	19	29
SM-2	22	17

AMP (Ampicillin-10mcg/ml), ERY (Erythromycin-10mcg/ml), AMX (Amoxicillin-10mcg/ml), AZM (Azithromycin-10mcg/ml), GEN (Gentamycin-10mcg/ml), RM-1 (Raw milk-1), RM-2 (Raw milk-2), PM-1 (Pasteurized milk-1), PM-2 (Pasteurized milk-2), SM-1(Spiked milk with ab-1), SM-2(Spiked milk with ab-2)

Milk samples in wells on KUNDRAT Agar plates seeded with broth cultures

***G. stearothermophilus* with milk samples** | ***G. kaustophilus* with milk samples**



Results after 6-8 hrs incubation at 65°C

Zone of inhibition on Kundrat agar of *G. stearothermophilus* | **Zone of inhibition on Kundrat agar of *G. kaustophilus* 2HSBW2**

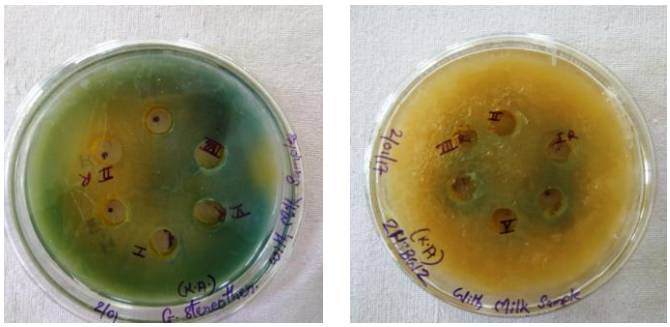


Figure 4. Zone of inhibition for different samples on Kundrat agar with *Geobacillus kaustophilus* and *Geobacillus stearothermophilus.*

Geobacillus kaustophilus 2HSBW2 had shown luxuriant growth on Kundrat agar even faster than *G. stearothermophilus* in given incubation time and also had shown sensitivity to maximum antibiotics and milk samples with lowest MRL (minimum residual limit) tolerances of approved veterinary drugs for milk.

DISCUSSION

Geobacillus stearothermophilus is a rod-shaped, gram-positive bacterium and a member of the division *Firmicutes*. The bacterium is a thermophile and is widely distributed in soil, hot springs, ocean sediment. According to Katz and Siewierski (1995), *Geobacillus stearothermophilus* is an outstanding testing microorganism for its properties from which the most important are: the ability of rapid growth at higher temperatures (64°C) and a high sensitivity to the β-lactam antibiotics. *Geobacillus stearothermophilus* is commercially used in antibiotic detection in milk and food products. Both *G. stearothermophilus* and *G. kaustophilus* belong to the division *Firmicutes* and same genus *Geobacillus* and almost have similar morphological and biochemical characteristics.

Similarly in the present investigation, *Geobacillus kaustophilus* had shown optimum growth at 65°C on Kundrat agar within 6-8 hrs incubation. Also, it had shown exponential growth curve at 65°C in spectrophotometric study at 630nm. It was sensitive to all the 21 antibiotics used i.e, ampicillin (10 mcg), augmentin (30 mcg), norfloxacin (10 mcg), co-trimoxazole (25 mcg), gentamicin (10 mcg), tobramycin (10 mcg), cefoxitin (30 mcg),

cephalothin (30 mcg), chloramphenicol (30 mcg), tetracyclin (30 mcg), streptomycin (10 mcg), kanamycin (30 mcg), amikacin (30 mcg), ceftazidime (30 mcg), aztreonam (30 mcg), pipracillin (100 mcg), imipenem (10 mcg), ciprofloxacin (5 mcg), fusidic acid (30 mcg), linezolid (30 mcg) and rifampicin (5 mcg) that includes β-lactam, tetracycline and other type of antibiotics.

Mitchell *et al*. in 1998 showed the advantage of agar diffusion method that it has a wide detection spectrum, it is simple to carry out, and it is not costly and can be used for the screening of a large number of samples.

Maike Clauβen et al in 2013 presented antibiotic detection in raw milk using *Bacillus pumilus*, the detection limit for tetracycline was 19 µg/l (water), 31 µg/l (milk, 1.5% fat) and 38 µg/l (milk, 3.5% fat) with the disk-based agar diffusion method. Of the strains tested in their study only *Bacillus pumilus* would be able to detect tetracycline at a concentration below the MRL of 100 µg/kg specified by the EU for milk. Similar results described by Navratilova P. et al in 2008 and Lina Kantiani et al in 2009.

The present investigation stated that test isolate 2HSBW2, *G. kaustophilus* had shown sensitivity to more antibiotics than *G. stearothermophilus* of lowest concentration i.e., from 0.1 µg/1mL to 1µg/1mL i.e. concentrations below the level specified in standards (MRL). Also, it had detected antibiotics present in raw milk samples, RM-1, RM-2 and amoxicillin (1 µg/1mL) which was not detected by *G. stearothermophilus.*

CONCLUSION

The entitled study was for the first time carried for Salbardi hot spring of Central India. The present study shows inhabitance of several thermo-tolerant bacterial species in this geological region.

A novel strain of *firmicutes, bacilli* i.e., gram positive, spore-forming bacteria *Geobacillus kaustophilus* was isolated with registered NCBI accession number LC368286. It is able to give optimum growth at a higher temperature range of 55-80°C. It had shown high antibiotic sensitivity to β-lactam antibiotics and tetracycline.

The present investigation can be concluded that for microbial screening test or detection of antibiotics in milk samples isolate *Geobacillus kaustophilus* can be used as an alternative to *G. stearothermophilus* because it had shown better results in comparison. 2HSBW2 had shown optimum rapid growth on Kundrat agar within 6-8 hrs incubation times and was sensitive to more number of antibiotics and milk samples with antibiotics. In the present study, raw milk samples and pasteurized milk samples detected with the presence of antibiotics with lowest MRLs by agar diffusion method using bacterial isolate *G. kaustophilus* and *G. stearothermophilus* in which *G. kaustophilus* had given better and promising results than *G. stearothermophilus*.

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REFERENCES

Altschul, S. F. M. T. (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res. 25,* , 3389-3402.

Babington, R. M. S. (2012). Current bioanalytical methods for the detection of penicillins. *Analytical and Bioanalytical Chemistry. 403* , 1549-1566.

Bauer, A.W., K. W. (1966). Antibiotic susceptibility testing by a standardized single disc method. *Am J Clin Pathol: 45* , 493-591.

Botsoglou, N.A. F. D. (2001). *Drug residues in foods, Pharmacology, Food safety, and Analysis.* New York: Marcel Dekker.

Burgat-Sacaze, V. A. (1986). *Toxicological significance of bound residue, p. 1-31.* Academic Press. Toronto, Ontorio: IN A. G. Rico (ed.), Drug residues in animals.

Clauβen, M. B. D. (2013). Detection of antibiotic residues in food - pitfalls and optimization of agar diffusion tests in comparison with commercial test kits. *Microbial pathogens and strategies for combating them: science, technology and education (A. Méndez-Vilas, Ed.)* , 359-366.

Code of Federal Regulations (1994 (1 April)). *21 CFR I, part 556.* U.S.: Department of Health and Human Services, Food and Drug Administration.

De La Haye, R. P. (1996). Monitoring for residues: a strategy for screening. *Proceedings of the Euro Residue III Conference,*, (pp. 6-8). Veldhoven, The Netherlands,.

Dewdney, J. M. (1984). Penicillin hypersensitivity - is milk a significant hazard ? A review. *J. R. Soc. Med. 77* , 866-877.

Dixit, M. I. A. (2018). "Screening & Isolation of Extracellular Thermo-Stable Enzymes Produced by Bacterial Isolates from Salbardi Hot Spring Region". *International Journal of Current Research and Modern Education, Volume 3, Issue 1* , 239-245.

Eden, P. A. S. T. (1991). Phylogenetic analysis of Aquaspirillum magnetotacticum using polymerase chain reaction - amplified 16S rRNA-specific DNA. *International Journal of Systematic Bacteriology. 41 (2).* , 324-325.

Europian Commission (1990). Council Regulation (EEC). 2377/90/EC. *Off. J. Eur. Commun. L224* , 1-8.

Franco, D. A. (1990). Antibiotic and sulfonamide residues in meat: implications for human health. *J. Food Prot. 53* , 178-185.

Garrod, L. P. (1964). Hazards of antibiotics in milk and other food products. *Proc. R. Soc. Med. 57* , 1087-1088.

Government of Canada (1994). *Food and Drugs Act and Regulations.* Canada: Supply and Services.

Honkanen-Buzalski, T. R. W. (1997). Antimicrobials, monographs on residues and contaminants in milk and milk products.IDF special issue 9701. *International Dairy Federation, Brussel.* , 26-33.

Huber, W. G. (1986). *Allergenicity of antibacterial drug residues, p. 33-49.* Ontorio: In A G. Rico (ed.), Drug residues in animals.Academic Press, Toronto,.

Kantiani, L. F. M. (2009). Analytical methodologies for the detection of b-lactam antibiotics in milk and feed samples. *Trends in Analytical Chemistry, Vol. 28, No. 6.*

Kindred, T. P. (1993). Residues prevention strategies in the United States. *J. Am. Vet. Med. Assoc. 202* , 46-49.

Kneebone, J. T. P. (2010). Short communication: Rapid antibiotic screening tests detect antibiotic residues in powdered milk products. *Journal of Dairy Science.93* , 3961-3964.

Kundrat, W. (1972). 45- Minuten - Schnellmethode zum mikrobiologischen Nachweis von Hemmstoffen in tierischen Produkten. . *Fleischwirtsch., 52* , 485-487.

Kundrat, W. (1968). Methoden zur Bestimmung von Antibiotika-Rückständen in tierischen Produkten. . *Z. Anal. Chem.* , 624-630.

Mitchell, M. B. (1995). Detection of beta-lactam antibiotics in bulk tank milk. *J. Food. Prot. 58* , 577-578.

Navratilova, P. (2008). Screening methods used for the detection of veterinary drug residues in raw milk- a review. *Czech J. Food Sci., 26* , 393-401.

O' Rangers, J. J. (1993). Utilization of new screening tests for animal drug residue detection. *Proceedings of the EuroResidue II Conference*, (pp. 3-5). Veldhoven, The Netherlands.

Ormerod, A. D. (1987). Penicillin in milk - its importance in urticaria. *Clin. Allergy 17* , 229-234.

Oyetibo, G.O. I. M. (2010). Bacteria with dual resistance to elevated concentrations of heavy metals and antibiotics in Nigeria in contaminated systems. *Environ Monit Assess; 168* , 305-308.

Paige, J. C. (1994). Analysis of tissue residues. *FDA Vet. 9(6)* , 4-6.

Perme T., B. M. (2010). Validation of TwinsensorBT, screening test for the detection of β-lactam and tetracyclines in milk and comparison to Delvotest® SP-NT. *Slovenian Veterinary Research. 47* , 97-106.

Stephany, R. W. (1990). Quality criteria in veterinary drug residue analysis. *Proceedings of the EuroResidue Conference*, (pp. 21-23). Noordwijkerhout.

Universal Bacterial Identification by PCR and DNA Sequencing of 16S rRNA Gene. (2010). *PCR for Clinical Microbiology. Part 3.* , 209-214.

Van Houweling, C. D. (1969). *Use of antibiotics in food animals: a review and status report.* FDA Pap. 3: 21-25.

Vickers, H. R. (1964). Dermatological hazards of the presence of penicillin in milk. *Proc. R. Soc. Med. 57* , 1091-1092.

Zvirdauskienė, R. S. J. (2007). An evaluation of different microbial and rapid tests for determining inhibitors in milk. *Food Control. 18* , 541-547.

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