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Optimization of antibacterial substances production from *Acinetobacter baumannii* strain LAN1, an isolate of buffalo milk

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Abstract
Raw milk has a high nutritional content and it supports a rich microbiota. The objective of this investigation was to isolate and identify the novel bacterial strain from raw milk of buffalo and to optimize distinct parameters for ensuring the maximum production of antibacterial substances. The antibacterial substance producing bacterium was identified as *Acinetobacter baumannii* strain LAN1 based upon morphological and molecular characterization. Process optimization was carried out using one factor at a time method. Bifidobacterium broth medium was observed to provide better bactericidal activity against the indicator pathogenic bacteria, including *Staphylococcus epidermidis* MTTC 3615, *Staphylococcus aureus* MTCC 96, *Shigella flexneri* MTCC 1457 and *Yersinia enterocolitica* MTCC 840. The maximum antibacterial substances production from strain LAN1 was recorded at pH 6.0 (141.2 ± 1.76 to 168.5 ± 2.62 AU/mL) and 30 ^{0}C (145.5±2.41 to 192.3±3.25 AU/mL). Likewise, lactose and ammonium chloride favored the maximum production of antibacterial substances from strain LAN1 with arbitrary units of 200.3±1.76 to 225.2±2.62 and 210.4±2.14 to 252.1±2.35 AU/mL, respectively. The isolate exhibited proteolytic activity and susceptibility towards conventional antibiotics too. In conclusion, *A. baumannii* strain LAN1, isolated from buffalo raw milk revealed antagonistic activity against human pathogens and suggested its further exploitation in varied industries based on its non-virulence characteristics.

Keywords: *Acinetobacter baumannii*, Antibacterial substances production, Buffalo milk, Human pathogens

1. Introduction
Microorganisms present in the animal's milk support a rich microbiota and can play various roles such as dairy fermentations, causing spoilage, promoting health, and/or causing disease depending on the nature of microorganism (Quigley *et al*., 2013) [17]. Milk being highly nutritious serves as an ideal environment for growth of many microorganisms. These nutrients are either directly available or are provided by metabolism of major components by specific microorganism resulting in the production of components or metabolites that are used by other microorganisms (Frank, 1997) [5].

The dominant population of bacteria in bovine, goat, sheep, and buffalo milk prior pasteurization belongs to the lactic acid bacteria group (Quigley *et al*., 2011) [16]. The psychrotrophic populations include *Pseudomonas* spp. and *Acinetobacter* spp. The consumption of raw milk contaminated with pathogens can have health related implications (Oliver *et al*., 2009) [15] whereas some microorganisms can contribute reducing the frequency of allergies, asthma and atopic diseases. The buffalo milk's dominant microorganisms are *Lactococcus* spp. (30%), *Pseudomonas* spp. (20%), and *Streptococcus* spp. (10%) (Ercolini *et al*., 2009) [4]. The presence of gram negative bacteria in dairy foods are considered as indicators of poor hygiene and can cause health risk if the species present is pathogenic (Delbes-Paus *et al*., 2011) [3]. One of such bacteria is of *Acinetobacter* species. These are ubiquitous and non fermenting coccobacilli (Al-Atrouni *et al*., 2016) [2]. It has evolved as global pathogen causing wide range of infection. *Acinetobacter baumannii* strains are present in milk (Gurung *et al*., 2013) [7] which proves the importance of examining milk for health concerns.

Molecular techniques like 16S rRNA polymerase subunit B (rpo B), DNA gyrase subunit B (gyr B) gene sequencing, DNA- DNA hybridization, and whole genome sequencing have been implemented for the identification of *Acinetobacter* species (Jung and Park, 2015) [8] *Acinetobacter* species can survive in dry conditions for long period and support desiccation

conditions, which may be due to over expression of proteins in the antimicrobial resistance, efflux pumps, down regulation of proteins in the cell cycle, transcription, and translation in order to enter dormant state (Gayoso, 2014) [6]. The present study was investigated to isolate new strain of *Acinetobacter* sp. from buffalo milk and to optimize medium components and abiotic factors for enhanced production of antibacterial substances against human pathogenic bacteria.

2. Material and methods

2.1 Collection of sample

Raw milk of buffalo was obtained in sterile bottles from milk farm of Chennai, India for the isolation of bacteria.

2.2 Isolation of bacteria

The milk sample was serially diluted (10^{-1} to 10^{-5}) and 1 mL of the suspension was poured onto the sterilized Bifidobacterium agar medium (g/L – peptone 22.2, corn starch 1.0, sodium chloride 4.8, glucose 2.0, lactulose 2.5, cysteine hydrochloride 0.5, riboflavin 0.01, agar 18.0) plates. Plates were incubated at 37°C for 48 h. The isolated colony was sub-cultured on Bifidobacterium-agar plate and pure culture was obtained using streak plate technique. The entire isolation process was performed in laminar air flow chamber.

2.3 Bacterial identification

The bacterium was identified using morphological and biochemical tests and further characterized using molecular tools. The genomic DNA of the potential isolate was isolated and purified using a QIAquick® kit (Qiagen Ltd., Crawley, UK). The amplicon sequencing was performed using universal primers 27F (5′ AGA GTT TGA TCG TGG CTC AG 3′) and 1492R (3′ GCT TAC CTT GTT ACG ACT T 5′). The 16S rRNA sequence of the isolate was subjected to BLAST, NCBI. Then, the sequence of the isolate was deposited into NCBI. GenBank, and an accession number was assigned. The isolate obtained was used for further experiments.

2.4 Bacteria of interest

Indicator human pathogens including Gram positive (*Staphylococcus epidermis* MTTC 3615, *Staphylococcus aureus* MTCC 96) and Gram negative (*Shigella flexneri* MTCC 1457, *Yersinia enterocolitica* MTCC 840) bacteria were used for the present experimental study. The indicator bacterial cultures were sub-cultured onto selective broth medium for further study. A 24 h old bacterial culture was used for further experiments.

2.5 Assay for antibacterial substance production

The isolated bacterium was screened individually for the production of antagonistic substances. The bacterium was inoculated into sterilized Bifidobacterium broth medium and incubated for 48 h at 30°C. The indicator microorganisms were inoculated into Mueller Hinton broth medium for 24 h at 37°C and swabbed onto Mueller Hinton agar plates. Agar plates were punched using a sterilized, flamed and alcohol-dipped cork borer, and 5 mm wells were created. The bacterium was centrifuged at 8000×*g* for 10 min, and the culture supernatant was subjected to membrane filtration (0.22 µm). The sterilized cell free supernatant was neutralized (pH 7.0) using 1 N NaOH in order to exclude the antibacterial effect of organic acids in the medium. The cell free neutralized supernatant (CFNS) was treated individually with catalase (Sigma, India; 1 mg/mL) and incubated at 37°C for 2 h in order to eliminate the inhibitory effect of hydrogen peroxide (Khusro *et al*., 2018) [13]. After catalase treatment, the CFNS obtained from isolate was then assayed for antibacterial assay against indicator bacteria using the agar well diffusion method. The growth inhibitory activity was expressed in arbitrary units (AU/mL). One AU was defined as the reciprocal of the highest level of dilution resulting in a clear zone of growth inhibition.

2.6 Media optimization

The isolate was inoculated individually into 250 mL conical flasks containing sterile production medium (50 mL) such as Nutrient broth, Mueller Hinton broth, Luria Bertani broth, Bifidobacterium broth, and Peptone broth to compare the production of antibacterial substances. The flasks were incubated at 30°C for 48 h in an orbital shaker. The CFNS was obtained, and the arbitrary units (AU/mL) were estimated as described above against the indicator bacteria.

2.7 Optimization of parameters using one factor at a time (OFAT) method

The suitable production medium was optimized using various culture conditions (pH and temperature) and medium components (carbon sources and nitrogen sources) utilizing the OFAT method after working out a series of experiments. The medium components were substituted one by one by keeping other factors constant in the production medium (Khusro, 2016) [10]. The antibacterial substance production by the isolate was examined by adjusting the pH (4, 5, 6, 7, and 8) of the production medium using 1 N HCl and 1 N NaOH. Similarly, the production of antagonistic substances from isolate was optimized by varying the incubation temperature (20–70°C). The various medium components such as carbon sources (maltose, fructose, sucrose, lactose, and xylose individually at 1.0 % w/v) and nitrogen sources (ammonium acetate, ammonium chloride, ammonium nitrate, ammonium sulphate, and sodium nitrate individually at 0.5 % w/v) were substituted in the production medium in order to achieve maximum production of antibacterial substances. An appropriate control medium was also maintained.

2.8 Partial purification of protein

Protein was partially purified using ammonium sulphate precipitation, dialysis, and SDS-PAGE. All the experiments for purification of protein were carried out using standard methodology.

2.9 Proteolytic activity

The protease activity of isolate was determined according to the method of Khusro (2015) [11]. One milliliter of overnight grown isolate was centrifuged at 6000 *g* for 15 min at 4°C. Agar plates containing % w/v - peptone 0.5, beef extract 0.3, skim milk 1.0, and agar 1.8 were prepared and allowed to cool. The agar medium was punched and wells were created with sterilized cork borer and bacterial supernatant was poured into it. The plate was kept at 30^0C for 48 h of incubation and observed for protease production in terms of clear zone surrounding the well.

2.10 Antibiotic susceptibility tests

Antibiotic susceptibility assay of the isolate was determined according to the method of Khusro and Aarti (2015) [11]. The disc diffusion method was used to depict susceptibility of

isolate against commercially available antibiotics. Hundred microliters of overnight grown isolate were spread onto Bifidobacterium agar plates and antibiotic discs were placed onto it. Plates were incubated at 37°C and zone of inhibition was measured after 24 h of incubation. The antibiotics used in this study were Ampicillin (10 µg), Gentamicin (10 µg), Kanamycin (2 µg), Penicillin G (10 µg), Streptomycin (10 µg), and Tetracycline (30 µg).

2.11 Statistical analyses
All the experiments were carried out in triplicate and data were represented as mean±SD.

3. Results
3.1 Isolation of bacterial strain
Colonies grown on the agar plates after performing plate technique were observed. A single colony was picked up and streaked on agar plates to obtain pure culture. Fig. 1 shows the colony plate and the purified culture of bacteria of interest.

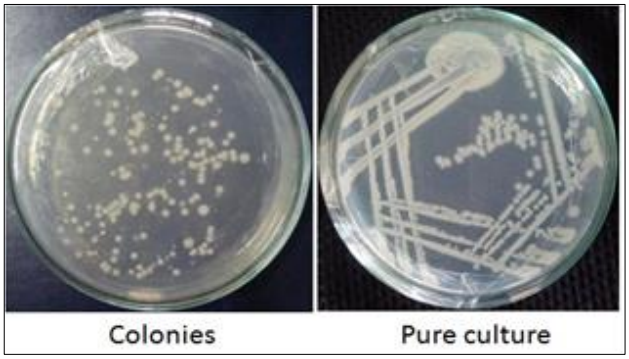

Fig 1: Bacterial colonies isolation from buffalo milk and pure culture preparation of strain LAN1

3.2 Identification of bacteria
The most potent bacterium underwent morphological identification, biochemical property characterization, and molecular characterization using 16S rRNA sequencing. An amplicon of 1519 bp was observed using PCR amplification (Fig. 2). The sequence was subjected to a multiple sequence alignment using the BLAST analysis of NCBI. The sequence was deposited in GenBank, maintained by NCBI, USA and the organism was identified as *Acinetobacter baumannii* strain LAN1 (Accession No: MF537043).

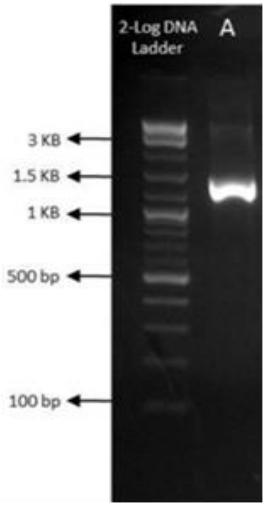

Fig 2: Amplicon of strain LAN1

3.3 Media optimization
Strain LAN1 was cultured in various media in order to ensure the maximum production of antibacterial substances. Bifidobacterium broth medium was found to be the most favourable medium for the maximal production of antagonistic substances (110.4±3.15 to 151.1±2.08 AU/mL). The other production media resulted in minimal yield of antibacterial constituents compared to bifidobacterium medium (Fig. 3). The antibacterial substance yield of various media was as follows: Bifidobacterium broth > Mueller Hinton broth > Nutrient broth > Luria Bertani broth > Peptone broth. Peptone broth was found to be the least effective medium for antibacterial substance production from strain LAN1, ranging from 18.3±1.36 to 30.4±2.33 AU/mL against the most susceptible indicator bacteria.

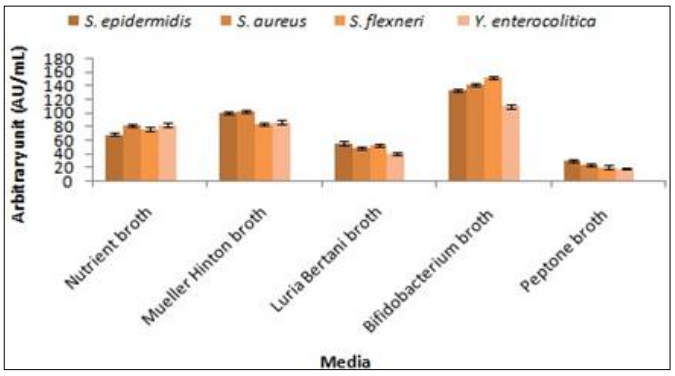

Fig 3: Antibacterial substances production from strain LAN1 in the presence of various media

3.4 Optimization of culture conditions and medium components
Subsequent investigation was carried out to optimize the production of antibacterial substances from strain LAN1 using the OFAT method. The culture conditions such as pH and temperature were optimized for maximum production of growth inhibitory substances. The production of antibacterial components was enhanced by adjusting the pH of the bifidobacterium broth medium. Among the tested pH, the maximum production in terms of antagonistic activity was recorded at pH 6.0 and ranged from 141.2±1.76 to 168.5±2.62 AU/mL (Fig. 4). However, a further decrease or increase of pH was found to mitigate the production of antibacterial substances significantly. The minimum production was recorded at pH 8.0 and ranged from 78.3±3.04 to 111.4±2.17 AU/mL against the control range of 100.4±2.51 to 150.1±2.08 AU/mL. Fig. 5 shows the effect of incubation temperature on antibacterial substance production from strain LAN1. The maximum production of 145.5±2.41 to 192.3±3.25 AU/mL was recorded at 30°C, and temperature lower or higher than 30°C markedly decreased the production of antibacterial substances. The minimum yield was within the range of 17.3±3.04 to 22.1±2.17 AU/mL at 70°C over the control. Strain LAN1 produced growth inhibitory components at a higher level (200.3±1.76– 225.2±2.62 AU/mL) when the carbon source of bifidobacterium medium was substituted with lactose. On the other hand, the minimum antagonistic activity (38.7±2.51 to 54.2±3.08 AU/mL) was observed in xylose supplied medium over the control (Fig. 6). Similar to the carbon source, the nitrogen source also favoured the optimal production of antagonistic substances from strain LAN1. The production of antibacterial substances from the isolate was higher (210.4±2.14 to 252.1±2.35 AU/mL) in the

presence of ammonium chloride. However, the minimum production (111.5±1.35 to 152.3±2.41 AU/mL) was obtained in ammonium nitrate supplied medium over the control range (166.3±3.05 to 220.3±3.08 AU/mL) (Fig. 7). The partially purified protein of strain LAN1 showed a single band of about 52KDa on SDS-PAGE (Figure not shown).

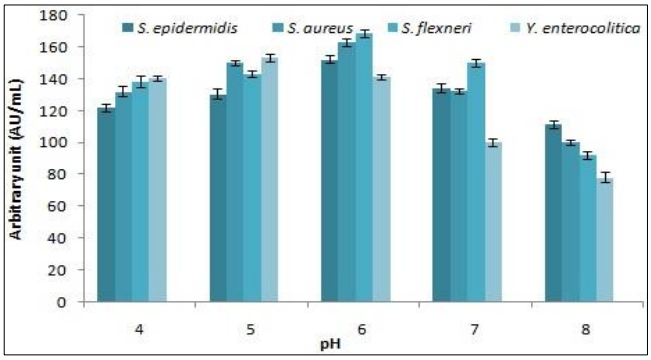

Fig 4: Antibacterial substances production from strain LAN1 at various pHs

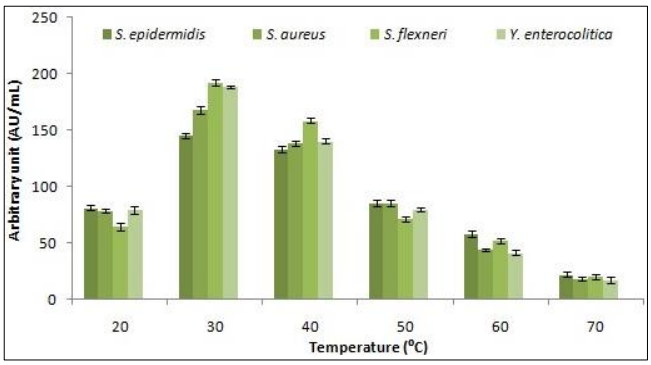

Fig 5: Antibacterial substances production from strain LAN1 at various temperatures

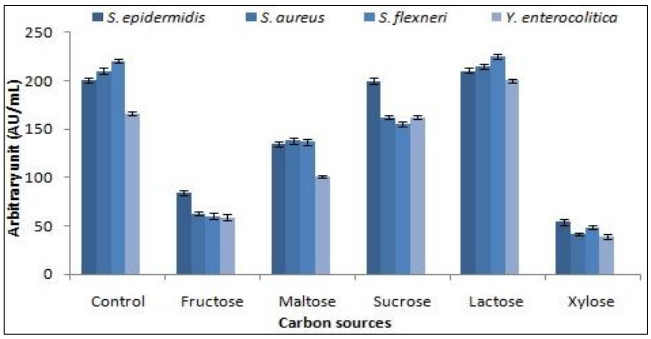

Fig 6: Antibacterial substances production from strain LAN1 in the presence of various carbon sources

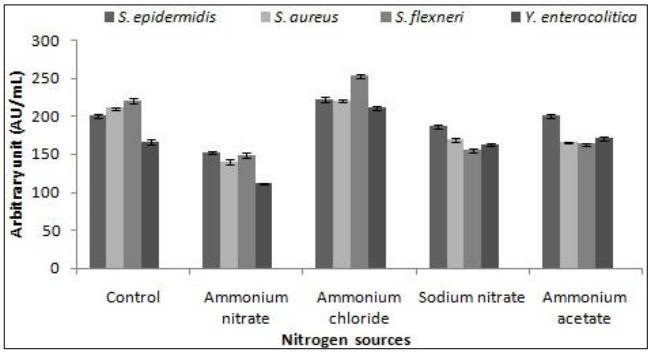

Fig 7: Antibacterial substances production from strain LAN1 in the presence of various nitrogen sources

3.5 Proteolytic activity and antibiotic susceptibility test

Strain LAN1 showed high proteolytic activity with zone of hydrolysis of 14.2±1.8 mm (Figure not shown). On the other hand, the antibiotic susceptibility property of strain LAN1 was determined against various antibiotics such as Streptomycin (S), Kanamycin (K), Nalidixic acid (N), Rifampicin (R), Chloramphenicol (C), Gentamicin (G), and Penicillin G (P). Results showed that strain LAN1 was susceptible to all the antibiotics tested except Nalidixic acid (Fig. 8).

Fig 8: Antibiotic susceptibility pattern of strain LAN1

4. Discussion

The isolate of the present context revealed a varying degree of antagonistic activity against indicator organisms by secreting different types of antibacterial substances. The growth inhibition of indicator bacteria by catalase-treated CFNS provided evidence that the antagonistic activity might be due to the production of antibacterial components (Aarti *et al.*, 2016) [1]. Media play a very important role in the successful isolation of bacteria. In the present context, strain LAN1 showed the maximum growth in bifidobacterium broth medium, since *Bifidobacterium* is part of microbiota of milk. Several selective and differential media are available for the isolation of *Acinetobacter* spp. But in the present study the strain *A. baumannii* was isolated from the bifidobacterium broth medium.

Previously, *A. baumanni* inhibited the growth of several phytopathogenic fungi such as *Cryphonectria parasitica*, *Glomerella glycines*, *Phytophthora capsici*, *Fusarium graminearum*, *Botrytis cinerea*, and *Rhizoctonia solani*. When the filtrate from the fermentation broth of strain *A. baumannii* was tested *in vitro* and *in vivo*, it showed strong growth inhibition against several phytopathogens including *P. capsici*, *F. graminearum*, and *R. solani*, indicating that suppression of the growth of fungi was due to the presence of antifungal compounds in the culture broth (Liu *et al.*, 2007) [14]. Moreover, the antifungal activity of the culture filtrate was significantly correlated with the growth of *A. baumannii*. The active metabolites in the filtrate were relatively thermo stable, but were sensitive to acidic conditions. Three antifungal compounds were isolated from the culture broth by absorption onto macropore resin, ethanol extraction, chromatography on silica gel or LH-20 columns, and crystallization. The structures of the bioactive compounds were identified by spectroscopic methods as isomers of iturin A, namely, iturin A2, iturin A3, and iturin A6. The study

suggested that the characterization of an unusual endophytic bacterial strain *A. baumannii* and its bioactive components may provide an alternative resource for the bio-control of plant diseases. In the present study, we reported the antagonistic activity of strain LAN1 against few human pathogenic bacteria. The optimized medium components showed significant enhancement in the antibacterial substances production, and thus exhibited high antagonistic activity against the human pathogens tested. However, the demonstration of the lack of virulence trait in this particular strain would be a significant step towards its further exploitation in varied industries. Moreover, strain LAN1 revealed susceptibility towards few conventional antibiotics, thereby suggesting its sensitivity properties against antibacterial drugs in order to avoid any kind of pathogenicity (Khusro *et al*., 2014) [12].

5. Conclusion

From the present investigation, it is clear that *A. baumannii* strain LAN1 isolated from buffalo milk produced antibacterial substances with growth inhibitory properties against human pathogens. The maximum production of antibacterial substances was obtained in bifidobacterium broth medium supplemented with varied components. The OFAT optimization data on antibacterial substance production provides strong basic information for further investigation of this bacterial strain. An extensive study needs to be performed to explore the potency of the organism in varied industries, considering its non-pathogenicity properties.

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