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IN VITRO ANTIBACTERIAL AND ANTIFUNGAL ACTIVITY OF EXTRA AND INTRACELLULAR METABOLITES EXTRACTED FROM *ALISHEWANELLA FETALIS* KD167 AND *BACILLUS THURINGIENSIS* KD168 ISOLATED FROM COW DUNG

Deepanshu Rana and Kartikey Kumar Gupta*

Department of Botany and Microbiology, Gurukula Kangri University, Haridwar (UK), India

Abstract

Antibiotic resistance among human pathogens still remains a major concern for public health. The aim of this work is to investigate the bacterial isolates of cow dung for their antimicrobial properties. Twenty-seven bacterial isolates were preliminary screened and twelve isolates were selected for the production of antimicrobial agent in nutrient broth. Isolate KD167, KD168 and KD169 exhibited maximum bioactivity against *Bacillus subtilis*, *Enterococcus faecalis* and *Pseudomonas aeruginosa* with 15 mm of inhibition zone. In order to maximise the production and to improve the efficiency of antimicrobial agent six different medium were used and inhibition rate was more in the two medium i.e. Beef extract Peptone Broth (BPB) and Yeast extract Sucrose Glucose broth (YSG). Further the crude metabolites from both the isolates were extracted and intracellular metabolite of KD167 and KD168 showed maximum inhibition of 20mm and 15mm against *Proteus vulgaris* and *Enterococcus faecalis* while extracellular extract showed maximum inhibition of 15mm against *Listeria Monocytogenes*, *Proteus vulgaris* and *Enterococcus faecalis*. Hence results of present study evident that microflora associated with cow dung appear to have immense potential as a source of antibacterial compounds.

***Key words*:** Cow dung, Cross-streak, Extracellular metabolite, Intracellular metabolite and 16s rRNA.

Introduction

According to WHO, infectious diseases are the second most important cause of death globally (Ravnikar *et al*., 2015) and bacteria are the major agent causing 10-30% of diseases causing millions of death annually (Newell *et al*., 2010; Ganesan *et al.*, 2017). Control of disease causing organisms through the use of natural antagonistic has emerged as a promising alternative in the field of medical science (Gupta and Rana, 2017). Microbial secondary metabolites are the chemical compounds produced naturally by the microbes during there idiophase as one of the adaptation mechanisms (Gupta and Rana, 2018). Secondary metabolites are highly diverse in relation with structure and only some species can produce them (Karlovsky, 2008), representing an opulent source (Berdy, 2005) and played a key role in the discovery and development of many antibiotics such as Gramicidin and Rifampin (Mahajan and Balachandran, 2012; Gupta and Rana, 2017). About 4000 antibiotics discovered and approved for marketing nowadays are of microbial origin (Harvey, 2008; Butler *et al*., 2014; Gupta and Rana, 2016a). But overuse of these agents is the prime reason for the development of drug resistance which increases the substantial morbidity, mortality and also the cost of treatment (Al-Naiemi *et al*., 2006; Sikarwar and Batra 2011; El Shakour *et al*., 2012; Aly *et al*., 2012; Jeyasanta *et al*., 2012; Ullah *et al*., 2012; Desriac *et al*., 2013; Sharif *et al*., 2013; Kardos and Demain, 2011; Bii *et al*., 2017; Ling *et al*., 2015). Due to the development of resistance towards these drugs there is an alarming scarcity of new antibiotics (Singh *et al*., 2014; Gupta and Rana, 2016b).

*Corresponding author Email: kartikey77@gmail.com

Cow dung can be considered as gold mine of microorganisms as over 60 different bacterial species and 100 species of protozoa and yeast have been identified from cow dung. (Randhawa and Kullar, 2011). Antifungal and antibacterial agents inhibiting the growth of coprophilous fungi, *Candida sp., Escherichia coli, Pseudomonas sp.* and *Staphylococcus aureus* have successfully been extracted from cow dung (Muhammad and Amusa, 2003; Joseph and Sankarganesh, 2011; Shrivastava *et al*., 2014). Today researchers around the world are continuing their effort to isolate bacteria producing antimicrobial metabolites against microbes of medical importance. In view of this, the present study was undertaken to find potential bacterial isolate from cow dung having ability to produce bioactive compounds.

Materials and Methods

Collection of cow dung sample

Dung sample of cross breed cow was collected aseptically from Jatol, Deoband, Uttar Pradesh, India. The sample was analysed immediately after transporting to the laboratory (Gupta and Rana, 2016a).

Isolation of the bacterial strain

During the course of investigation for collected dung sample, strains were isolated by serial dilution method. Aliquots (0.2 ml) of each dilution were spread on Nutrient agar plate and incubated at 35±2°C for 24-48 hrs. Morphologically different bacterial colonies were selectively marked and propagated twice on NA plate to check the purity. The pure isolate was stored at 4°C for further investigation (Kadaikunnan *et al*., 2015; Hajare *et al*., 2016).

Preparation of 0.5 McFarland standard

McFarland standard was prepared by adding 0.5 mL of 0.048 mol/L $BaCl_2$ (1.17% w/v $BaCl_2.2H_2O$) in to 99.5 mL of 0.18 mol/L H_2SO_4 (1% w/v) with constant stirring. The equal volume of standard solution was distributed into same sized screw capped test tubes. These test tubes were tightly sealed and stored at room temperature to prevent from evaporation and light. Before use, turbidity standard was vigorously agitated by using vortex mixer (Gebreyohannesv *et al*., 2013).

Test pathogens

A total of 16 microorganisms namely, *Vibrio Cholera* MTCC 3904, *Salmonella typhi* MTCC 3216, *Staphylococcus aureus* (MTCC 7443), *Bacillus subtilis* (MTCC 441), *Bacillus cereus* (MTCC 6728), *Escherichia coli, Proteus vulgaris* (MTCC 426), *Enterococcus faecalis* (MTCC 439), *Pseudomonas aeruginosa* (MTCC 424), *Escherichria coli* (MTCC 118), *Shigella flexneri* (MTCC 1457), *Salmonella typhimurium* (MTCC 3231), *Streptoccus pyogenes* (MTCC 442), *Staphylocccus aureus* (MTCC 3160), *Listeria monocytogenes* (MTCC 657) and 1 fungal pathogen namely *Candida albicans* (MTCC 227) were used in this study as test organisms.

Preliminary screening of isolated strains by cross-streak method

The antagonistic activities of isolated strains were evaluated by cross-streak method against test pathogens. The isolates were streaked in the centre of plate and incubated at 35±2°C for 24 hrs. Later, targeted microorganisms were streaked perpendicularly to the margin of isolates and further incubated at 35±2°C for 18 h. The antagonistic activity was measured by distance of inhibition between the target microorganism and isolates (Gupta and Rana, 2016b).

Secondary screening of the selected strains

Isolates showing the antagonistic potential in primary screening were further studied for the production of antimicrobial substances in nutrient broth medium followed by secondary screening against test pathogen using agar well diffusion method. 5 ml of two days old culture was inoculated into 75 ml of liquid media contained in 150 ml flask and incubated at 35±2°C in a shaking incubator maintained at 150 rpm for 2 days. After incubation, culture broths were centrifuged at 5,000 rpm for 20 minutes at room temperature. On nutrient agar plates test pathogens were uniformly spread out with the help of sterile cotton swab. The cell free supernatant was administered to the wells separately and incubated at 35±2°C for 24 hrs. Antimicrobial activity was determined by measuring zone of inhibition (in mm). Un-inoculated liquid medium added to the wells were taken as negative control (Boottanun *et al*., 2017).

Morphological, cultural, physiological and biochemical characterisation of selected isolates

Morphological and cultural characteristics such as cell shape, Motility, Colony, Pigmentation, Opacity, Form of Growth, growth in NB and gram staining, of selected isolates were analysed using traditional methods reported in the Bergey's manual of determinative bacteriology (Holt *et al*., 1994). Carbohydrate utilization was determined by growth on carbon utilisation medium supplemented with 1% carbon source at 37°C. Different physiological characters such as temperatures range (25-50°C), pH (5-10) range and NaCl (1%, 3%, 5%, 7%, 8%, 9%) concentrations for growth was determined on NA medium. Hydrolysis of starch, Liquefaction of gelatine and other biochemical tests were also evaluated. All the results were recorded after 24-48 hrs of incubation.

Identification of bacterial strain

The isolates were also recognized by 16s rRNA gene sequencing method using the method describes by (Ganesan *et al*., 2016).

Genomic DNA isolation

The genomic DNA of active strains KD167 and KD168 was isolated as described by Ganesan *et al*., 2016.

Analysis of 16S rRNA

The primers 27F (50AGAGTTTGATCMTGGCTCAG30) and 1492R (50TACGGYTACCTTGTTACGACTT 30) were used to amplify 16S ribosomal sequence from genomic DNA in thermal cycler. The PCR products were confirmed by 1% agarose gel electrophoresis. The primers 785F 5' (GGA TTA GAT ACC CTG GTA) 3' and 907R 5' (CCG TCA ATT CMT TTR AGT TT) 3' were used as sequencing primer. The amplified DNA (~1400 bp) was sequenced at Humanizing Genomics Macrogen Inc. (Seoul, South Korea) (Kumar *et al*., 2012).

Construction of Phylogenetic Tree

The sequences were compared for similarity with the references species of bacteria using the NCBI BLAST tool. The DNA sequence was multiple aligned with CLUSTAL W program and phylogenetic tree was constructed based on bootstrap test of phylogeny with neighbour-joining method using MEGAX software. The 16s rRNA sequences were submitted to the GeneBank, NCBI, USA.40 (Ganesan *et al*., 2016).

Basal medium optimization

To maximize the production of bioactive compound, active isolates *i.e.* KD167 and KD168 were grown in six different media such as Nutrient Broth (NB), Leuri-Bertani Broth (LB), Beef extract Peptone Broth (BPB), Beef Extract Broth (BEB), Yeast extract malt extract broth (YMB) and Yeast extract Sucrose Glucose broth (YSG) and kept in incubator shaker at 150rpm for 2 days at 37°C. After the end of incubation period cell mass was removed by centrifugation and the supernatant was used for bioactive assay (Sharon *et al*., 2014).

Production of antimicrobial agent

Isolate KD167 and KD168 were subjected to submerged state fermentation in optimised mediums for production of crude extracts. Both isolates were cultivated in 2 litre of production medium at 35±2°C with a shaking speed of 150

rpm during an incubation period of 2 days. After incubation, culture was centrifuged at 5000 prm (Remi) for 20 min at room temperature and supernatant was collected, filtered with 0.45 µm Whatman filter (Sengupta *et al*., 2015).

Extraction and preparation of extracellular crude extracts

In order to choose the best solvent of extraction, liquid culture medium from KD167 and KD168 was extracted using two organic solvents *i.e.* Ethyl acetate and Chloroform. Culture supernatant was extracted by manual shaking thrice with equal volume of selected solvent (1:1) in a separating funnel. The organic layer was concentrated using the rotavapor. The resulting crude extract was recuperated in DMSO (Belghit *et al*., 2017).

Extraction and preparation of intracellular crude extracts

Cell pellet collected after completion of submerged fermentation was added with methanol for the extraction of intracellular extracts. The solvent added cell pellet was kept in a shaker for proper agitation. After 72 h the solvent phase was isolated by centrifugation (5000 rpm, 20 min) and the extracts were separated and stored at 4°C for further studies (Viju *et al*., 2017).

Quantitative bioassay of intra and extracellular crude extracts

Antimicrobial activity of intra and extracellular crude extract was carried out using well diffusion method. Petri plates were prepared and selected pathogens were swabbed on top of the solidified media. Agar wells (6×4 mm) were prepared with a sterile cork borer. 100 µL crude extracts was carefully dispensed into each well and allowed to diffuse for 2 h and incubated at 35±2°C for 24 h. After 24 h of incubation, zone of inhibition around each well was recorded. (Ganesan *et al*., 2016).

Results and Discussion

Isolation of bacterial species

Cow dung sample was collected from Jatol, Deoband (Latitude, 29°47'24.62"N; Longitude, 77°45'31.74"E; Altitude 263 m), Uttar Pradesh, India. Bacteria were isolated from dung sample by serial dilution method and total of 27 isolates namely KD164, KD165, KD166 and so on were obtained.

Preliminary screening of isolates

Out of 27 isolates only 12 (44.44%) isolates showed good antimicrobial activity against at least one of the tested pathogens in cross-streak method. Isolate KD165, KD167, KD168, KD169, KD170, KD172, KD175, KD178, KD179, KD180, KD181 and KD184 showed inhibition against *Pseudomonas aeruginosa*. Isolate KD175 also inhibit the growth of *Vibrio cholerae*, *Salmonella typhi*, *Salmonella typhimurium*, *Escherichia coli* (Table 1). These isolates were further selected for secondary screening.

Secondary screening of isolates

The cell-free supernatant of all the 12 positive isolates was evaluated for antimicrobial activity by agar well diffusion method against the test organisms. Out of 12 only 3 isolates (25%) were able to show the inhibitory activity. The maximum inhibition demonstrated by KD167 against *B. subtilis* (15mm), while isolate KD168 and KD169 shown the inhibition zone of 10mm against *E. fecalis* respectively. The minimum zone of inhibition of 5mm was shown by KD167 and KD168 against *P. aeruginosa* (Table 2).

Morphological, cultural, physiological and biochemical characterisation of selected isolates

The bacterial strains KD167 and KD168 were rods while isolate KD169 was cocci. Isolate KD168 and KD169 were gram positive while isolate KD167 was gram-negative. The colonies of KD167 and KD168 were white in colour while KD169 was showing golden-yellowish colouration on medium. The strain KD167 could grow up to 42°C and pH 9 with 8% NaCl concentration, KD168 showed growth upto 40°C, pH 9 with 7% NaCl tolerance and KD169 grow till 46°C, with maximum pH 9 with 9% NaCl tolerance. All the three isolates were not able to utilise lactose and manitol as carbon source while glucose and sucrose was solely utilised by isolate KD168. All the strains were catalase and nitrate positive. Detailed result of morphological, physiological and biochemical test are summarised in Table 3.

PCR amplification of KD 167 and KD 168 genomic DNA

The genomic DNA was isolated and was confirmed in 1% agarose gel stained with ethidium bromide. DNA was observed under UV Transilluminator showeing good yield. The PCR product was analysed in 1% agarose gel electrophoresis and the size (1458 bp and 1489 bp) was confirmed, sequenced and submitted in the GenBank (MN809397 and MN809398).

Molecular identification

The active strain KD167 (MN809397) showed 99.32% homology to *Alishewanella fetalis* strain CCUG 30811 (NR_025010.1) (Figure 1) while isolate KD168 (MN809398) showed 99.53% homology to *Bacillus thuringiensis* strain BAPE1 (KT206229.1) (Figure 2). The DNA sequence was aligned and phylogenetic tree was constructed by using MEGAX.

Basal medium optimization

Media composition significantly influenced metabolite production by strains KD167 and KD168. A total of six Culture media were used for the production of desired metabolite from *Alishewanella fetalis* KD167 and *Bacillus thuringiensis* KD168. The ability of microbes to form antibiotics is not a fixed property but can be greatly increased or completely lost under different conditions of nutrients or cultivation (Sharon *et al*., 2014). Maximum production of antimicrobial agent by KD167 was found in YSG medium after 24 h by showing 11 mm zone of inhibition against *Candida albicans*. It also inhibits *Bacillus cereus* and *Listeria*

monocytogenes by showing 10 mm zone of inhibition. Therefore YSG was confirmed (Figure 3) to be the effective culture media for the production of metabolite. Isolate KD168 produce maximum antimicrobial agent in BPB medium (Figure 4) showing 14 mm zone of inhibition against *Proteus vulgaris* and *Pseudomonas aeruginosa*.

Antimicrobial activity of intracellular and extracellular crude extracts

The extracellular and intracellular extracts from both the isolates *i.e. Alishewanella fetalis* KD167 and *Bacillus thuringiensis* KD168 showed antibacterial and antifungal activity against tested pathogens. Ethyl acetate extract of *Alishewanella fetalis* KD167 inhibit 5 test organisms with maximum inhibition (15 mm) against *L. monocytogenes*, *B. cereus* and minimum (11 mm) against *C. albicans*. While Chloroform extract also showed inhibition against 5 test organisms out of 14 with maximum inhibition (12 mm) against *L. monocytogenes* and minimum (11 mm) against *B. cereus, P. vulgaris, E. fecalis* and *C. albicans*. Ethyl acetate extract of *Bacillus thuringiensis* KD168 inhibit 4 test organisms with maximum inhibition (13 mm) against *E. fecalis* and minimum (10 mm) against *C. albicans*. While Chloroform extract showed inhibition against 3 test organisms out of 14 with maximum inhibition (11 mm) against *P. vulgaris* and *E. fecalis* minimum (10 mm) against *C. albicans*. Ethyl acetate extract is more active than chloroform extracts both quantitative and qualitatively showing the effectiveness of ethyl acetate extract (Kadaikunnan *et al*., 2015). Intracellular extract of KD167 showed inhibition against 7 test pathogens with maximum of 20mm inhibition against *P. vulgaris, E. fecalis and S. typhimurium* while minimum of 13mm inhibition against *C. albicans.* While intracellular extract of KD168 only inhibit 5 test pathogens with maximum of 15 mm inhibition against *P. vulgaris, E. fecalis and* minimum of 10mm inhibition against *S. aureus.* Results inferred that intracellular extract of *Alishewanella fetalis* KD167 was more effective than that of *Bacillus thuringiensis* KD168 inhibiting 12% more test pathogens. Apart from this, during the secondary screening both the isolates were able to inhibit some pathogen which were not inhibited by the crude extracts of the same isolate which may be due to the disintegration of some metabolites during extraction process (Gebreyohannesv *et al*., 2013).

Genus *Alishewanella* was proposed by Vogel *et al*., 2000 belongs to the class Gammaproteobacteria. comprise of 8 species *i.e. A. aestuarii* (Mogensen *et al*., 2005), *A. agri* (Kim *et al*., 2010), *A. fetalis* (Vogel *et al*., 2000), *A. jeotgali* (Kim *et al*., 2009), *A. solinquinati* (Kolekar, *et al*., 2013), *A. tabrizica* (Tarhriz, *et al*., 2012), *A. longhuensis* (Sisinthy *et al*., 2017) and *A. alkalitolerans* (Cantera *et al*., 2019), isolated from a different habitats ranging from human fetus, tidal flat sediment, landfill soil, and fermented food suggesting its great adaptability to diverse environments and unique genetic requirements for each habitat. Although (Cantera *et al*., 2019) isolated *Alishewanella* sp. strain RM1 from cow manure but as far as literature is considered present article is the first report where *Alishewanella fetalis* was isolated from cow dung and its secondary metabolites were explored for their antimicrobial potential.

Genus Bacillus was predominantly isolated from CD having biocontrol ability (Swain *et al*., 2008; Swain and Ray, 2009). De la Fuente-Salcido *et al*., 2008 studies the antimicrobial activities of bacteriocins produced by Mexican strains of *Bacillus thuringiensis* and stated that 5 bacteriocin were found active against *B. cereus, S. aureus, S. pyogenes, P. aeruginosa, P. vulgaris, S. xexneri* and *V. cholera*. The above presented data also confirmed the results of our study. But according to some reports *Bacillus* strains are most active against Gram-positive bacteria than Gram-negative bacteria (Oscariz *et al*., 1999; Aslim *et al*., 2002; Amin *et al*., 2015). But in our study we found that the isolated *B. thuringiensis* is not only active against gram-positive and gram-negative bacteria but also showed the promising antifungal activities.

Conclusion

This paper describes the synthesis of extracellular and intracellular metabolites extracted from two cow dung isolates *i.e.* KD167 and KD168 which were further characterized by molecular identification and through phylogenetic studies they were identified as *Alishewanella fetalis* and *Bacillus thuringiensis*. We have shown that crude extracts from both the isolates showed moderate broad spectrum antimicrobial activity against human pathogenic bacteria and fungal test pathogens. This is the first report on isolation of *Alishewanella fetalis* from cow dung and exploring its antibacterial and antifungal potential. Our future perspectives are purification and identification of active crude metabolites extracted from these isolates.

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Table 1: Primary screening of bacterial isolates by cross-streak method

Isolates/Test organisms	*V. cholera*	*S. typhi*	*E. coli*	*S. aureus*	*B. subtilis*	*B. cereus*	*P. vulgaris*	*E. fecalis*	*P. aeruginosa*	*E. coli*	*S. fexneri*	*S. typhimurium*	*S. pyogenes*	*S. aureus*	*Listeria monocytogenes*	*Candida albicans*
KD164	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
KD165	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-
KD166	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
KD167	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-
KD168	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-
KD169	-	-	-	-	-	-	-	-	++	-	-	-	-	-	-	-
KD170	-	-	-	-	-	-	-	-	++	-	-	-	-	-	-	-
KD171	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

KD172	-	-	-	-	-	-	-	-	++	-	-	-	-	-	-	-
KD173	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
KD174	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
KD175	+	+	++	-	-	-	-	-	+++	++	-	+	-	-	-	-
KD176	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
KD177	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
KD178	-	-	-	-	-	-	-	-	++	-	-	-	-	-	-	-
KD179	-	-	-	-	-	-	-	-	++	-	-	-	-	-	-	-
KD180	-	-	-	-	-	-	-	-	++	-	-	-	-	-	-	-
KD181	-	-	-	-	-	-	-	-	++	-	-	-	-	-	-	-
KD182	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
KD183	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
KD184	-	-	-	-	-	-	-	-	++	-	-	-	-	-	-	-
KD185	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
KD186	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
KD187	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
KD188	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
KD189	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
KD190	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

+++: Good activity; ++: Moderate activity; +: Weak activity; -: No activity.

Table 2: Zone of inhibition (mm) of Cell-free supernatant by agar well diffusion method

Isolates/Test organisms	***V. cholera***	***S. typhi***	***E. coli***	***S. aureus***	***B. subtilis***	***B. cereus***	***P. vulgaris***	***E. fecalis***	***P. aeruginosa***	***E. coli***	***S. fexneri***	***S. typhimurium***	***S. pyogenes***	***S. aureus***	***Listeria monocytogenes***	***Candida albicans***
KD165	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
KD167	-	-	-	-	15	-	-	-	5	-	-	-	-	-	-	-
KD168	-	-	-	-	-	-	-	10	5	-	-	-	-	-	-	-
KD169	-	-	-	-	-	-	-	10	10	-	-	-	-	-	-	-
KD170	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
KD172	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
KD175	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
KD178	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
KD179	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
KD180	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
KD181	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
KD184	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Table 3: Morphological, cultural, physiological and biochemical characterisation

Properties	**KD 167**	**KD 168**	**KD 169**
Morphological characters			
Cell shape	Rod	Rod	Cocci
Colony colour	White	White	Golden-yellowish
Opacity	Opaque	Opaque	Opaque
Margins	Smooth	Wrinkled	Smooth

Physiological characters			
Motility	Non-motile	Motile	Non-motile
Growth under anaerobic condition	Facultative	Facultative	Facultative
Temperature range for growth	25-42	25-40	25-46
Optimum temperature for growth	37	35	37
pH range for growth	6-9	6-9	6-9
Optimum pH for growth	7	7	7
NaCl tolerance	8%	7%	9%
Biochemical characters			
Gram staining	Negative	Positive	Positive
Glucose Fermentation	-	+	-
Lactose Fermentation	-	-	-
Sucrose Fermentation	-	+	-
Manitol Fermentation	-	-	-
Gelatine Liquefaction	+	-	-
Starch Hydrolysis	+	-	-
Indole	-	-	-
Methyl-Red	-	+	-
Vogeus -Proskeur	-	-	-
Citrate Utilisation	-	-	-
Catalase Production	+	+	+
Nitrate	+	+	+
Urease	-	-	+

Table 4: Antibacterial activity (in mm) of bacterial intra and extracellular extracts against test pathogens

Isolates/Test organisms	**KD 167**			**KD 168**		
	Extracellular			**Extracellular**		
	Chloroform	**Ethyl Acetate**	**Intracellular**	**Chloroform**	**Ethyl Acetate**	**Intracellular**
V. cholera	-	-	-	-	-	-
S. typhi	-	-	-	-	-	-
E. coli	-	-	-	-	-	-
S. aureus	-	-	-	-	-	-
B. subtilis	-	-	-	-	-	-
B. cereus	11	15	16			13
P. vulgaris	11	12	20	11		15
E. fecalis	11	14	20	11	13	15
P. aeruginosa	-	-	-	-	-	-
E. coli	-	-	-	-	-	-
S. fexneri	-	-	-	-	-	-
S. typhimurium	-	-	20	-	12	-
S. pyogenes	-	-	-	-	-	-
S. aureus	-	-	10	-	-	10
Listeria monocytogenes	12	15	15	-	12	-
Candida albicans	11	11	13	10	10	10

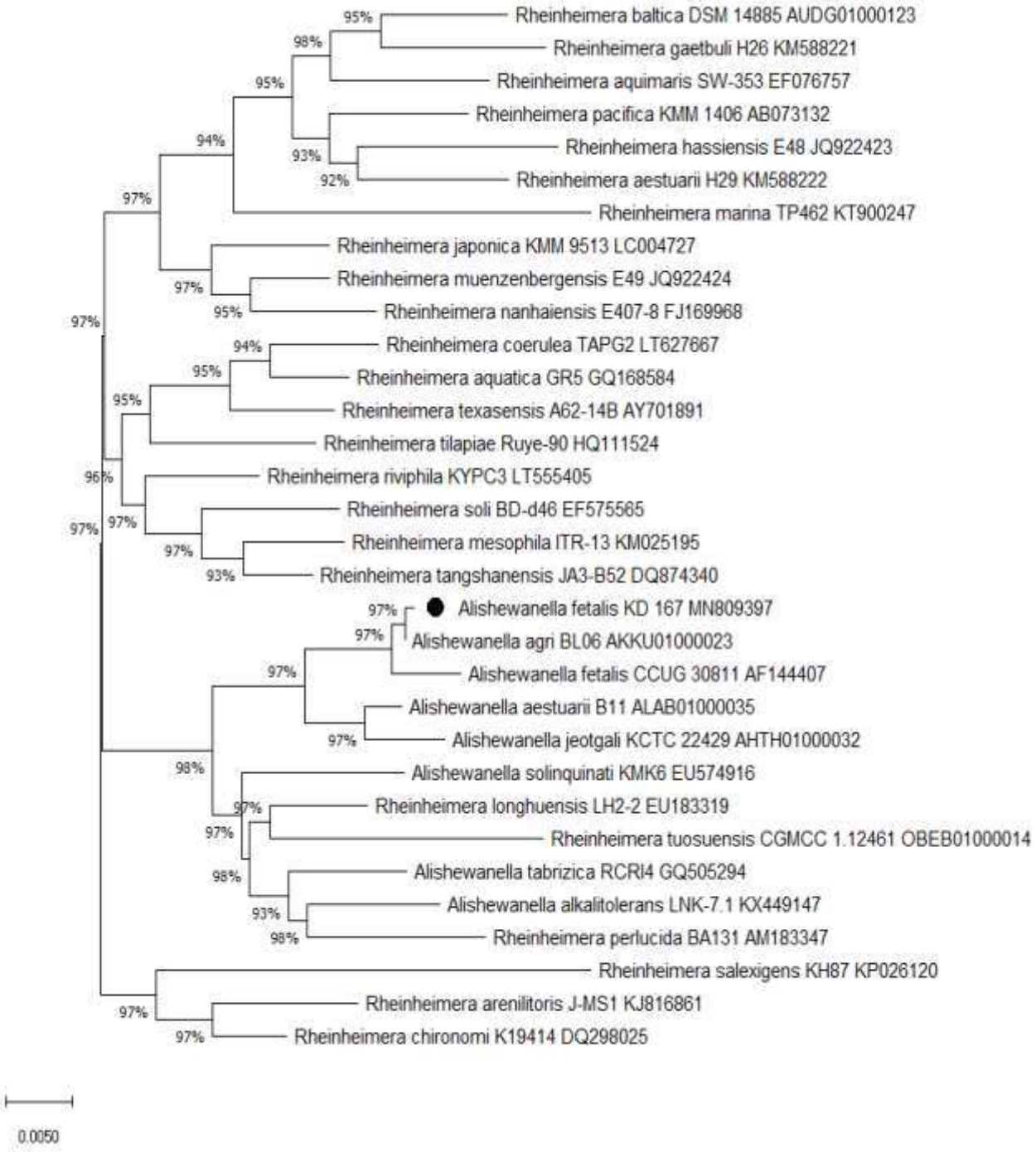

Figure 1: Evolutionary relationships of taxa [The evolutionary history was inferred using the Neighbor-Joining method. The optimal tree with the sum of branch length = 0.43007455 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The tree is drwan to scale with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the maximum composite Likelihood method and are in the units of the number of base substitutions per site. This analysis involved 32 nucleotide sequences. Codon positions included were $1^{st}+2^{nd}+3^{rd}$+Noncoding. All ambiguous positions were removed for each sequence pair (pairwise deletion option). There were a total of 1491 positions in the final dataset. Evolutionary analyses were conducted in MEGA X]

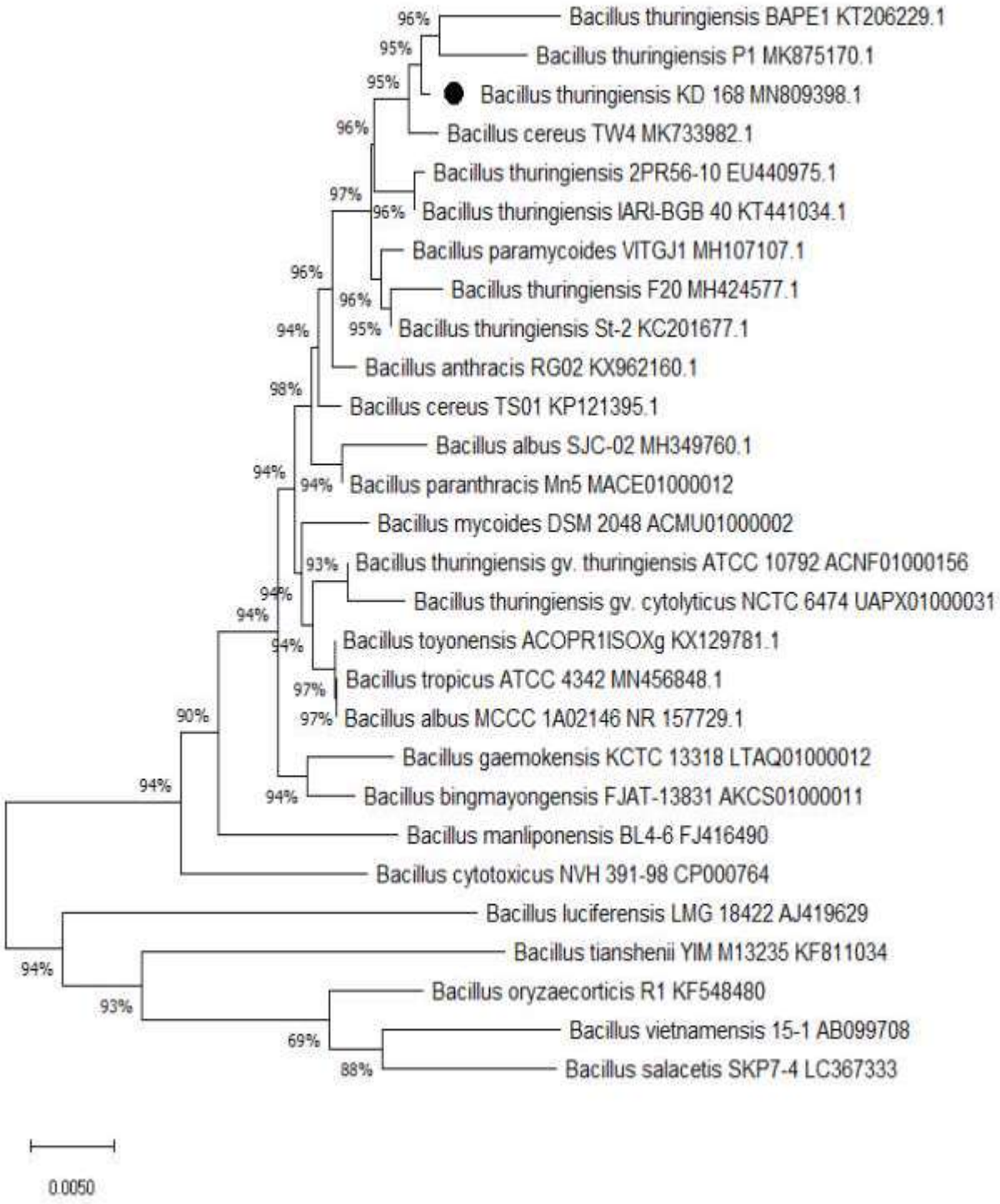

Figure 2: Evolutionary relationships of taxa [The evolutionary history was inferred using the Neighbor-Joining method. The optimal tree with the sum of branch length = 0.20131753 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The tree is drwan to scale with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the maximum composite Likelihood method and are in the units of the number of base substitutions per site. This analysis involved 28 nucleotide sequences. Codon positions included were $1^{st}+2^{nd}+3^{rd}$+Noncoding. All ambiguous positions were removed for each sequence pair (pairwise deletion option). There were a total of 1561 positions in the final dataset. Evolutionary analyses were conducted in MEGA X]

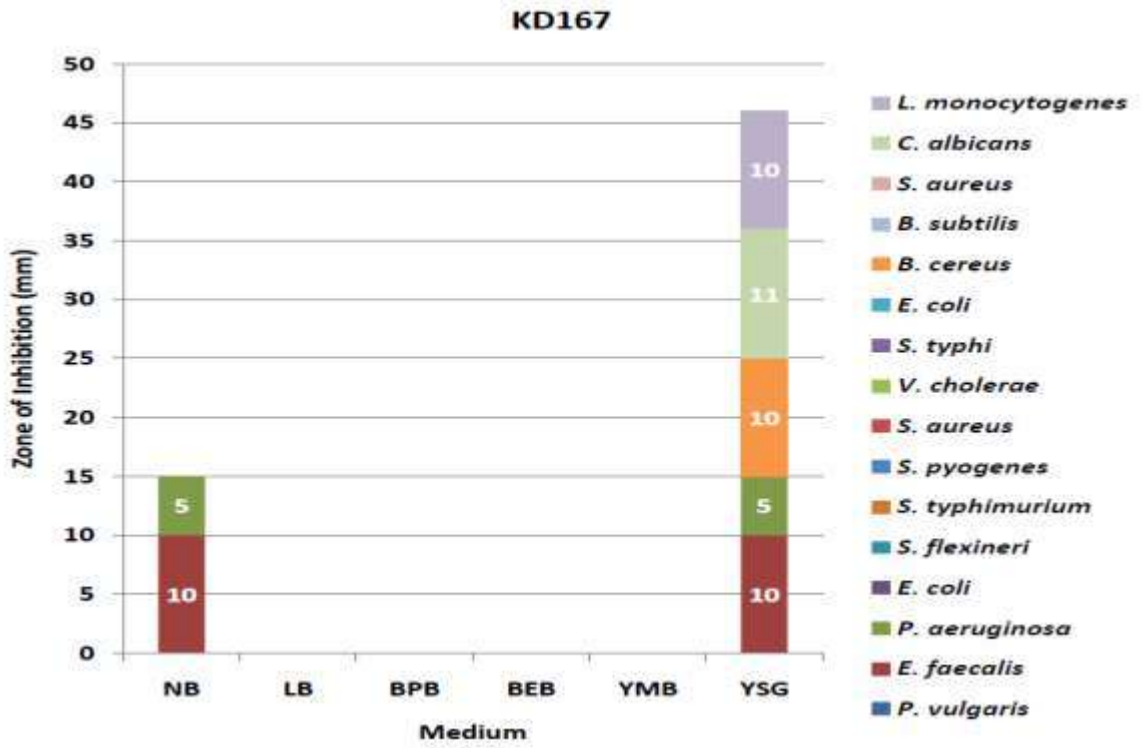

Figure 3: Medium optimization of KD167

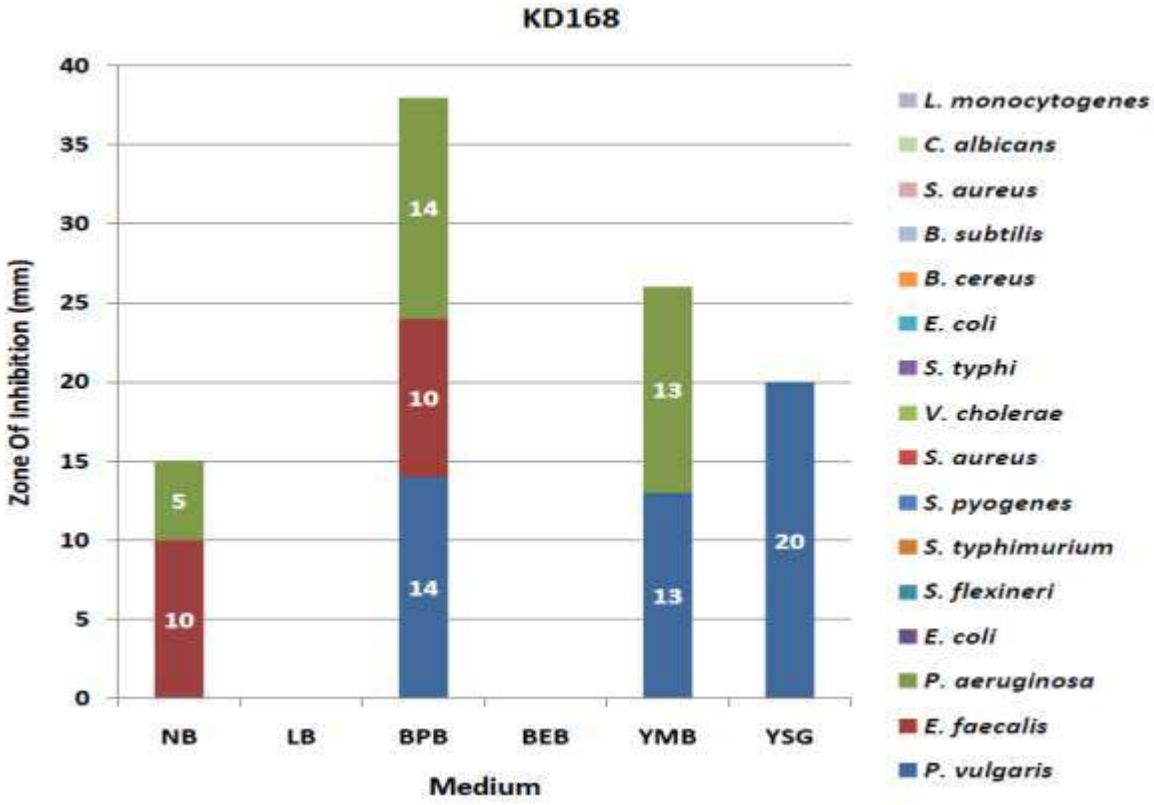

Figure 4: Medium optimization of KD168