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Isolation and evaluation of cow urine micro-organisms for their antibacterial and antifungal potential

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Abstract
Cow urine has tremendous applications in agriculture. It is shown to possess inhibitory activity against many phytopathogenic fungi and bacteria. The present study was conducted to determine antibacterial and antifungal efficacy of cow urine. The well-plate assay showed marked growth of inhibition against *Aspergilus niger, Fusarium oxysporum, Xanthomonas auxonopodis, Psedomonas solancearum, Xanthomonas cucurbitae* by cow urine as well as microbs isolated from cow urine. The TLC and titrimetric analysis were showed presence of lipase enzyme in the cow urine. Moreover, sterilized cow urine can be utilized as natural growth medium for *Pseudomonas fluorescence, Bacillus subtilis,* mixture of fungi and yeast. The use of cow urine can be the cost-effective and eco-friendly approach for controlling various diseases of plants.

Keywords: Antimicrobial, Antifungal, Cow urine, Lipase

Introduction
Vegetable plants suffer from several diseases caused by various kinds of pathogens such as bacteria, fungi, viruses, nematodes, and mycoplasma (Dandve *et al.,* 2019) [1]. These pathogens considered as most aggressive pathogens causing qualitative and quantitative damage to crop plant and resulted in reduction of yield and economy. Some of these pathogens such as *Aspergilus niger, Fusarium oxysporum, Xanthomonas auxonopodis, Psedomonas solancearum* and *Xanthomonas cucurbitae* are causing disease to various crops such as fruit rot (Cherry; Thomidis and Exadaktylou, 2012) [2], rhizome rot (Ginger; Li *et al.,* 2014) [3], Bacterial wilt (Tomato and Eggplant; Yenare *et al.,* 2020) [4], t bacterial blight (Pomegranate*;* Sherkhane *et al.,* 2019) [5], leaf spots (Cucurbits worldwide; Maringoni and Leite, 1988) [6] respectively.

One of the most widely used strategies to control plant diseases is the use of chemical agents. However, overuse of these chemical agents causes environmental pollution and health hazardous effects to human. Also, it has some drawbacks such as high cost, toxicity to non target organisms, residual problem, and development of resistance in pathogens. This situation triggered interest in searching natural alternates for pant disease and pest control. In fact, organic agriculture is a holistic way of farming with an aim of conserving the natural resources through the agronomic practices and the use of locally available low cost inputs in order to maintain soil fertility and conserve the rich bio-diversity to provide safe clean water, air and to achieve economical sustainability. Considering these adverse effects of synthetic pesticides on environment and natural habitats and the promotion of environmentally sustainable and organic agriculture, fungicide alternatives such as the use of natural antagonistic microorganisms and natural bioagents such as biopesticides, panchagavya, cow urine, cow urine with plant extracts etc are the effective ways to controlling various plant disease (Basak *et al.,* 2002; Akhter *et al.,* 2006; Murugan *et al.,* 2012; Rakesh *et al.,* 2013 Pohare *et al.,* 2020) [7-11]. *Bacillus* and *Pseudomonas* were among the first bacterial isolates to show promising biocontrol characteristics and promoters of plant health and development and are known to survive in the rhizosphere, with the competence ability to tolerate a reasonable range of abiotic factors including temperature, pH and moisture. *Pseudomonas fluorescens* and *Bacillus subtilis* had been reported to manage several diseases caused by soil borne pathogens. In India cow urine is used by majority of rural population as folklore remedy in almost all the states. An ancient literature in Ayurveda states that cow urine is one of the best natural remedies to cure many bacterial and fungal diseases (Edwin *et al.,* 2008) [12].

It contains nitrogen, sulphur, phosphate, sodium, manganese, carbolic acid, iron, silicon, chlorine, magnesium, citric,titric, succenic, calcium salts, vitamins A, B, C, D and E, minerals, lactose, enzymes, creatinine, hormones, urea and gold acids (Virender, 2009) [13]. The cow urine has several biological activities such as antimicrobial, antidiabetic, antioxidant, antitumor, molluscicidal and others (Rakesh *et al.*, 2013) [10]. Cow urine has got several applications in agriculture. Cow urine is shown to control root knot nematode in tomato and melon aphids and pickle worms in watermelon cultivation (Burubai and Eribo, 2012) [14]. Cow urine, cow urine extracts of plants and cow urine in combination with plants were found to exhibit inhibitory activity against phytopathogenic fungi and bacteria (Basak *et al.*, 2002; Akhter *et al.*, 2006; Murugan *et al.*, 2012; Rakesh *et al.*, 2013) [7-10]. In the present study, we have determined the effect of cow urine and microorganisms isolated from cow urine against various phytopathogenic bacteria and fungi.

Material and methods

Antibacterial and antifungal activity cow urine:

Fresh urine was collected in a sterile air tight container from a local deshi cow at early morning 5:00-6:30 am, when cows micturated first time in the day. The urine was filtered through sterile whatman paper and stored in airtight sterile container.

The well-plate test was used for anti-bacterial and anti-fungal tests assay as described (Pawar *et al.*, 2019) [15]. In the well-plate technique, for anti-bacterial test, 50 µl of cow urine sample was micropipetted into wells within the nutrient agar, in autoclaved petridishes. These plates had already been pre-spread with a fine superficial layer of pure cultures of phytopathogenic bacteria and fugi scuh as *Fusarium oxysporum*, *Aspergillus niger*, *Xanthomonas compastris*, *Psedomonas solancearum* and *Xanthomonas cucurbitae.* The plates were incubated for 24 hours to observe the bacterial growth pattern (zone of inhibition).

Isolation of different types of microbes from cow urine

Isolation of bacteria, fungi and actinomycetes from cow urine were prepared by using Nutrient agar, PDA and Kuster's agar respectively. The serial dilution and standard plate count method was used for isolation of total bacteria, fungi, and actinomycetes on respective medium. The 10 ml of freshly collected cow urine sample was transferred to 90 ml sterile water and mixed well. One ml of this suspension was transferred to 9 ml sterile water and subsequent dilutions were prepared up to 10^{-4} in the same manner. 1 ml of suspension from each appropriate dilution was transferred aseptically to sterile petri plates on respective agar medium and incubated in an inverted position at 28^0C for 5 days and the colonies grown on respective plates were studied for cultural characteristics and sub cultured for further use.

Characterization of cow urine microorganism for antibacterial and antifungal activity

The isolates obtained were characterized for antibacterial and antifungal activity against *Xanthomosal oxanopodis* and *Fusarium oxysporum* respectively using the well plate technique.

The 24h old suspension culture of was used for the well plate technique. The 100 µl of suspension culture was micropipetted into wells within the nutrient agar and PDA, in autoclaved petridishes. These plates had already been pre-spread with a fine superficial layer of fresh sub-cultured *Xanthomosal oxanopodis* and *Fusarium oxysporum* from pure cultures and plates were incubated at 37^0C for 24h. After incubation, zone of inhibition (in mm) between bacterial/fungal strain and test organisms was measured and recorded.

Thin Layer Chromatography (TLC) Analysis of Cow's Urine for Enzyme Detection:

Silica Gel slides were prepared by introducing silica gel of thick sloth-like consistency, on clean slides and leaving the slides undisturbed for solidification. Cow's urine sample drop was spotted on one edge of each of the slides and then these slides were slightly immersed in the running solvent comprising chloroform to acetic acid in the proportion 8:2. The slides were incubated for 30 min and then ninhydrin solution were sprayed on the spotted areas within each slide and transfer it to hot air oven for drying. The development of pink color was observed according to the Shalaby *et al*, (1996) [16].

Lipase Assay –Titrimetric Analysis

Lipase activity was determined by incubating a reaction mixture containing 5 ml of olive oil emulsion, 5 ml of 0.1 M Tris-Hydrochloride buffer at pH 8.5 and 1.0 ml of the filtered urine sample at 30°C for 20 min and centrifuged at 120 rpm (Macedo *et al.*, 1997) [17]. After incubation, the reaction was stopped by the addition of 10ml of acetone and the liberated free fatty acids were titrated with 0.05N Sodium Hydroxide in the presence of phenolphthalein as an indicator (pale pink shade end point). The blank assays were performed by adding the extract just after the addition of the acetone solution to the flask. One unit of lipase activity was defined as the amount of enzyme that liberated 1µmol of fatty acids per minute under assay conditions. All experiments were carried out in duplicate, to ensure consistency in the titer values. The lipase activity was calculated using the standard formula below:

Lipase activity (in units) = [(Titre value of test solution (ml) - Titre value of blank solution (ml)) × Molarity of NaOH × 1000 ×2 × Dilution factor] / [Vol. of the Test Sample (ml)].

Note: Dilution factor value was calculated as 1.

Cow urine as a natural growth medium

The 4 beneficial microbes like *Pseudomonas fluorescence*, *Bacillus subtilis*, mixture of fungi (it contains *Tricoderma harzianum*, *Tricoderma viride*, *Pseudomonas flourescens* and *Bacillus subtilis*) and Yeast (*Saccharomyces cerevisiae*) were inoculated in cow urine. Cow urine was sterilized by heating and autoclaving for 65^0C and 150 psi for 15 min respectively, kept it to get cool at room temp. The test tubes containing 10 ml sterilized cow urine and 1 ml overnight grown culture of beneficial microbes were added in it (0 hrs OD was measured at this point), sterilized cow urine used as control. Tubes were placed for incubation at room temp for 72 h (72 h OD was measured at this point). Turbidity was observed and recorded by spectrophotometer at 600 nm, whereas sterilized cow urine used as blank.

To check the yeast growth in large quantity the 150 ml of heated and autoclaved urine sample was added in 500 ml flask congaing 1 gm of yeast (*Saccharomyces cerevisiea)* and incubation at room temp for 5-6 days. Viable count & sugar content was checked by microscopy & by refractometer respectively.

Result and Discussion

Collection of cow urine and their antibacterial and antifungal assay

Fresh urine was collected in a sterile air tight container from a local deshi cow at early morning when cows micturated first time in the day. The urine was filtered through sterile what man no. 1 and used to analyze the phytopathogenic activity.

The well-plate assay was used to analyze the antibacterial and antifungal property of cow urine. After 24 h of incubation the clear zone of inhibition against test samle was observed and recorded in milimeter (mm). The growth of inhibition against test samle such as *Aspergilus niger*, *Fusarium oxysporum*, *Xanthomonas auxonopodis*, *Psedomonas solancearum* and *Xanthomonas cucurbitae* were 9, 14, 12, 17 and 15 mm respectively (Fig.1). This result showed that cow urine has antibacterial and antifungal potential. Similarly, several previous studies showed cow urine alone or cow urine with some plant extracts showed antibacterial and antifungal activity. Kekuda *et al.,* (2010) [18] revealed that cow urine has antifungal activity against *A. niger*, *A. oryzae* and *A. flavus*. The cow urine prevents the development of antibacterial resistance by blocking the R-factor, a part of plasmid genome of bacteria. Also, cow urine contains phenolic acids (gallic, caffeic, ferulic, o-coumaric, cinnamic, and salicylic acids) which have antifungal characteristics (Singh *et al.*, 2012) [19]. Deshmukh *et al*, (2012) [20] found that germtube lengths of *Aspergillus* spp. and *Mucor* spp. were significantly reduced by treatment with cow urine distillates compared to the control. It has been found that cow urine has potential to control aphids and pickleworms in watermelon cultivation (Burbai and Eribo, 2012) [14]. It is observed that cow urine has inhibitory effect against several plant pathogens such as *Sclerotinia sclerotiorum*, *Fusarium solani* f. sp. *cucurbitae* (Basak *et al.*, 2002) [21], *Bipolaris sorokiniana* (Akhter *et al.*, 2006) [8] and *Xanthomonas oryzae* pv. *oryzae* (Murugan *et al.*,2012) [9]. Rakesh *et al.* (2013) [10] showed inhibitory activity of cow urine against *Fusarium oxysporum f. sp. zingiberi* isolated from rhizome rot specimen of ginger.

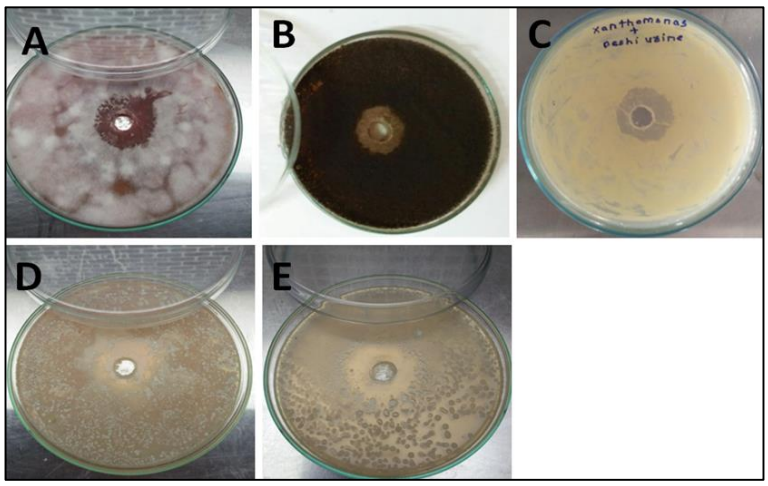

Fig 1: Antibacterial and antifungal activity of cow urine showing zone of inhibition. A) *Fusarium oxysporum B) Aspergillus niger C) Xanthomonas compastris D)* Psedomonas solancearum E) Xanthomonas cucurbitae

Isolation and characterization of different types of microbes from cow urine

To understand the antibacterial and antifungal potential, we isolated potential microorganisms from cow urine. A total of 21 isolates obtained, among that 9 isolates each belonged to bacteria, fungi and actinomycetes (Fig.2). These isolate were characterized for antibacterial and antifungal potential. Out of 21 isolates, 3 and 2 isolates were belonged to bacteria and actinomycetes respectively showed positive results of growth inhibition against *Xanthomonas oxynopodis* and *Fusarium oxysporium* (Fig.3). A bacterial isolate showed clear zone of inhibition against *Xanthomonas oxynopodis* and it is 30 mm. However the actenomycetes isolate did not showed clear zone of inhibition, it showed suppressed growth of *Fusarium oxysporium* (Fig.3). This result showed that bacterial and actinimycetes isolate isolated form cow urine can be used as a potential biopesticide to control the growth of disease causing microorganisms such as *Xanthomonas oxynopodis* and *Fusarium oxysporium* (Fig.3). Previous several study reports revealed that microorganisms were isolated from cow dung, cow urine, panchagavya, beejamruth and jeevamruth (Sharma and Singh, 2015; Teo and Teoh, 2011; Swain and Ray, 2006; Sreenivasa *et al.*, 2009a; Sreenivasa *et al.*, 2009b) [22-26]. The organic liquid manures *viz.*, Panchagavya, Beejamruth and Jeevamruth prepared by using cow products are known to contain beneficial microflora like *Azospirillum*, *Azotobacter*, *phosphobacteria*, *Pseudomonas*, lactic acid bacteria and Methylotrophs in abundant numbers and also contain some useful fungi and actinomyctes (Sreenivasa, 2009a; Somasundaram and Singaram, 2006; Maheshwari *et al.*, 2007; Singh, 1996; Pathak and Ram, 2007; Palekar, 2006; Salkinkop *et al.*, 2008) [26-32]. Lu *et al.*, (2014) [33] identified organisms from the *Alcaligenes*, *Bacillus*, *Proteus*, *Pseu domonas Staphylococcus and Microbacterium* genera from 219 bacterial strains isolated from cow dung. Xanthomonas axonopodis punicae is the causative agent of Bacterial blight of Pomegranate that is known to cause yield loss up to 70-90%. The disease management strategies are adopted includes use of chemical pesticides such as bromopal, zinc sulphate, magnesium sulphate, copper oxychloride, bordeaux paste with streptocycline antibiotic (Kumar *et al.*, 2009) [34]. The previous study results revealed that *Aspergillus niger*, *Trichoderma harzianum*, *Bacillus cereus* and *Bacillus subtilis* isolated from cow dung reduce the growth of *Sclerotium rolfsii*, *Fusarium oxysporum*, *Pythium aphanidermatum*, *Helminthosporium maydis* and *Rhizoctonia solani* with inhibitory zones of up to 58%. Furthermore, *B. subtilis* isolated from cow dung can enhance plant growth, sulphur oxidation, phosphorus solubilisation and was found to produce industrial enzymes such as amylase and cellulase (Swain and Ray, 2006) [20].

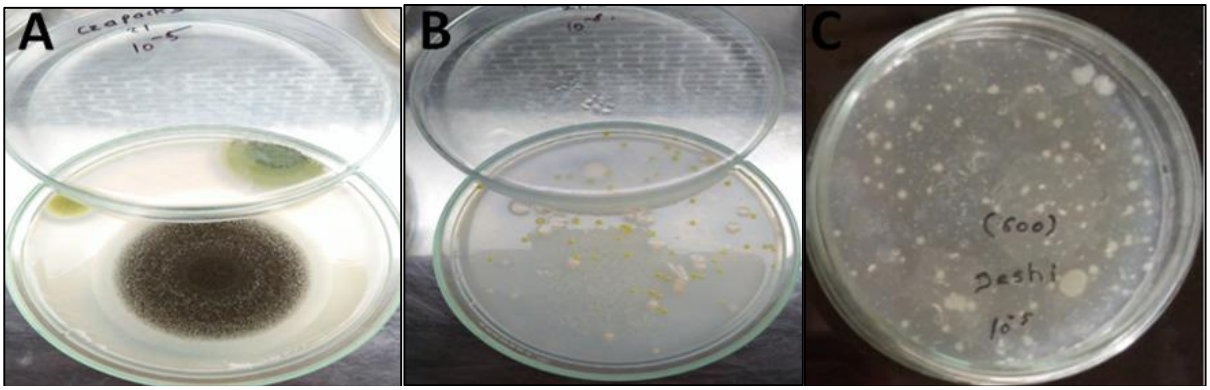

Fig 2: Various microorganisms isolated from cow urine on different medium. A) PDA B) Kuster's agar C) Nutrient agar

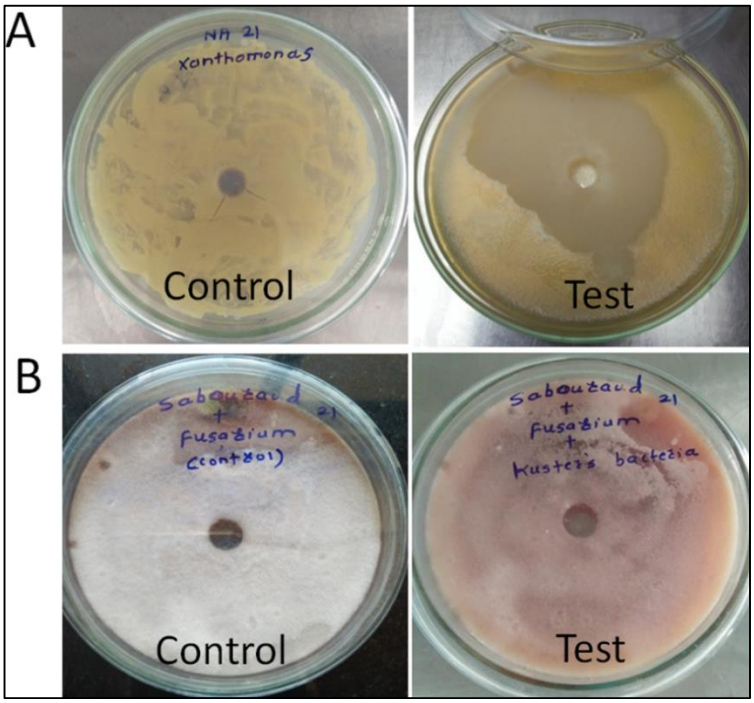

Fig 3: Phytopathogenic activity of microbes isolated form cow urine. A) Effect of cow urine bacteria against *Xanthomonas auxanpodis* B) Effect of cow urine actinomycetes against *Fusarim osysporum*

TLC for detection of enzyme in cow urines

The TLC assay was performed to understand the presence of enzyme in the cow urine. After spraying ninhydrin solution, pink color was observed on areas within the slides where the running solvent had diffused with the urine sample. According to Shalaby *et al*, (1996) [12] the development of pink color indicated the presence of amino-acids which confirmed the presence of an enzyme within the urine sample (Fig.4A). This result suggested that cow urine is a potential source of enzymes. The TLC assay of cow urine was previously reported and revealed the presence of an enzyme by kawle, (2016) [35].

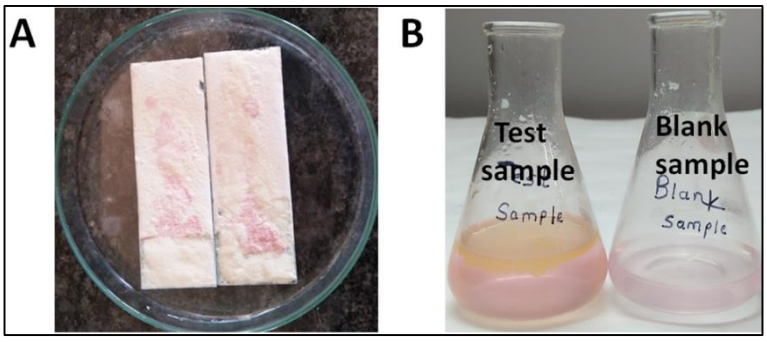

Fig 4: The enzyme assay using TLC and titrimetric analysis. A) Thin layer chromatography for detection of lipase enzyme, after sprayed with ninhydrin solution B) Sample visualized color change during titration for lipase enzyme

Lipase Assay –Titrimetric Analysis: The titration value showed the fact that there was lipase presence within the cow urine sample. This titration assay confirms the more dark pink color observed in test solution compared with blank solution (Fig. 4B). The titration value for test solution is 8.4 and for blank solution 6.9. The 150 units/ml lipase activity in cow

urine was recorded in test solution. Similarly, the lipase activity in cow urine was previously reported by Kawle, (2016) [35]. Cow urine has been found to possess lipase activity, which could be the key factor for it causing a reduction in the clostridium agar colony count from cow dung (Kumar *et al.*, 2004) [36]. This result showed that cow urine has a good source of lipase enzyme.

Cow urine as a natural growth medium for some beneficial microbes

To explore the utilization of cow urine as a natural medium for microbial growth, we inoculated beneficial microorganisms such as *Pseudomonas fluorescence*, *Bacillus subtilis*, mixture of fungi and yeast in the sterilized cow urine. After 72 h the turbidity of cultures in autoclaved cow urine were 2.4, 2.1, 2.4 and 2.1 for *Pseudomonas fluorescence*, *Bacillus subtilis*, mixture of fungi and yeast respectively. Moreover, in the heated cow urine medium the turbidity of cultures such as *Pseudomonas fluorescence*, *Bacillus subtilis*, mixture of fungi and yeast were recorded as 1.9, 1.6, 1.9 and 2.0 respectively (Fig.5). The turbidity of cultures at 0h was recorded as 01. All used microorganisms showed higher turbidity in sterilized cow urine at 72h compared with 0h. Further we confirm the growth of yeast by analyzing sugar content in cow urine and observed that collected cow urine contains 5% sugar. After 6 days growth of yeast in cow urine medium confirmed that reduction in sugar and it was 4% (Fig.5B). These results suggested that sterilized cow urine can be used as natural growth medium for growth of *Pseudomonas fluorescence*, *Bacillus subtilis*, mixture of fungi and yeast. The cow urine contents are water 95%, urea 2.5%, minerals, salt, hormones and enzymes 2.5%. It also contains iron, calcium, phosphorus, potassium, urea, uric acid, amino acids, cytokine and lactose etc (Bhadauria, 2002) [37]. Moreover, we first time reporting the presence of sugar in cow urine and this sugar might be responsible for growth of yeast.

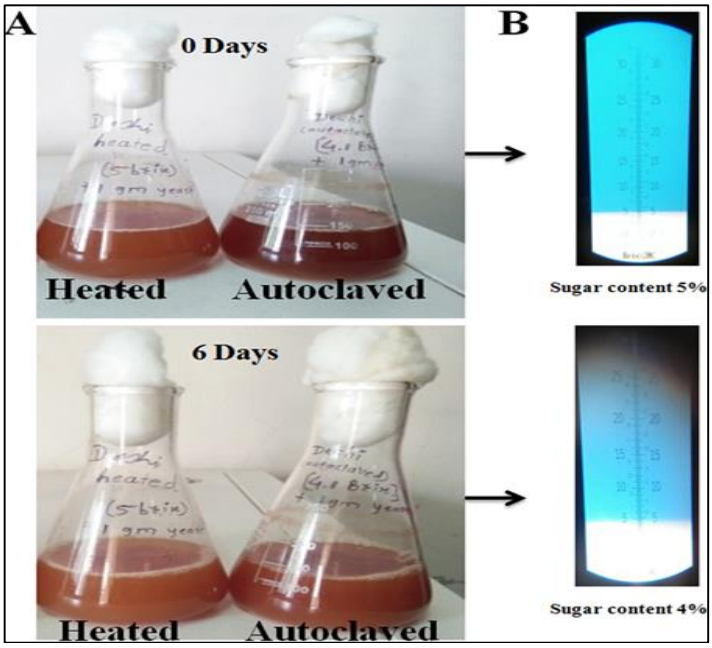

Fig 5: Yeast growth in sterilized cow urine showed the reduction in sugar content. A) Turbidity of heated and autoclaved cow urine inoculated with yeast at 0 and 6 days. B) Sugar content measured by refracto meter at 0 and 6 days after inoculation of yeast

Table 1: Antibacterial and antifungal activity of cow urine showing zone of inhibition in mm against various test organisms

Test organism	Zone of inhibition (mm)
Fusarium oxysporum	14
Aspergilus niger	09
Xanthomonas auxonopodis	12
Psedomonas solancearum	17
Xanthomonas cucurbitae	15

Table 2: The growth of various beneficial microbes in sterilized cow urine measured by spectrophotometer at 600nm wavelength

Test microbes	OD at 0 h	OD after 72 h
Autoclaved urine		
Pseudomonas fluorescence	1.0	2.4
Bacillus subtilis	1.0	2.1
Mixture of fungi	1.0	2.4
Yeast	1.0	2.1
Heated urine		
Pseudomonas fluorescence.	1.0	1.9
Bacillus subtilis	1.0	1.6
Mixture of fungi	1.0	1.9
Yeast	1.0	2.0

Conclusion

The cow urine alone and microorganisms isolated from cow urine has phytopathogenic activity. The TLC and titrimetric analysis were showed presence of lipase enzyme in the cow urine. Technological advances like CRISPR/Cas (Wagh *et al.*, 2019; Pohare *et al.,* 2019; Wagh *et al.,* 2020) [38-40] can be applied in this area of research in order to study the actual molecular mechanism involved in the process. Plug and play cloning techniques (Pohare *et al*, 2017) [41] can clone the different genes responsible for the production of antimicrobials from these microbes in urine can have commercial potential in the near future. The sterilized cow urine can be utilized as natural growth medium for *Pseudomonas fluorescence, Bacillus subtilis,* mixture of fungi and yeast. The use of cow urine can be the cost-effective and eco-friendly approach for controlling various diseases of plants.

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