ISSN No:-2456-2165

Evaluating the Oxalate Degrading Ability of Probiotic Isolates from Curd and Cow Dung Against Kidney Stone

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Abstract:- Probiotics are live microbial feed supplement that beneficially gets the host by provoking the immune response .The potential role of probiotics is accounted for its colonization, immune stimulation& antimicrobial activity. Probiotic organisms were isolated from curd & cow dung using selective medium [MRSA – De Man, Rogosa and Sharpe Agar]. Biochemical & molecular characterization were done to confirm the probiotic isolates. Based on the result, it was confirmed to be *Bacillus cereus & Bacillus sp*. The isolates were evaluated for its temperature range that supports the optimal growth. It was found that the isolates were able survive within the range of normal body temperature and evaluated for lactose utilization based on the color change. Both the isolates documented the production of lactic acid evidenced by the color change that confers for lactose intolerance. Then the isolates were screened for NaCl tolerance and found to exhibit tolerance upto 6% NaCl which is one of the major inhibitory and innate defense strategies against microbial growth. When the isolate was grown on Modified Barber's Medium, it was found to be capable of degrading/metabolizing oxalate. Further research on probiotic needs to be carried out to emerge it as an effective therapeutic tool against kidney stone disease.

Keywords:- *Probiotics, Modified Barber's Medium, MRSA*.

I. INTRODUCTION

Kidney stones are solid masses of crystals that originate and accumulates in the urinary system. The kidney stone formed is also known as renal calculi or crystal. Though it is not claiming lives at higher rate, the pain management and the other discomforts are challenges for the field of medicine. In medical terminology urinary calculi is termed as nephrolithiasis orurolithiasis: "Lith" meaning "a stone"[1].

Kidney stone or renal calculi is a most common human disorder and one of the major health issue. Nephrolithiasis is predominantly found in all geographical region throughout the world. The annual prevalence ranges between 3-5% and the life time prevalence approximates 15-25%. At the global level the incidence rate of renal calculi in adults is estimated higher in Western countries than that of the Eastern regions. Universally, people at higher risk of acute renal injury and epidemic of renal calculi prevail among all age groups including children of East Asia mainly Macau, Taiwan, Hong Kong and China due to use of different type of milk and milk product, melamine-tainted milk, cookies, candies and chocolate [2].

The word 'probiotic' comes from the Greek word 'pro bios' which means 'for life' and is antonyms to 'antibiotics'. In 1997, Barber et al., reported the presence of Oxalobacterformigenes, the oxalate degrading bacteria in the human intestine [3]Oxalobacterformigenesis an obligate anaerobe that is solely dependent on oxalate metabolism for energy [4]. The discovery of oxalate degrading bacteria within the human gastrointestinal tract has led the research regarding the role of probiotic in the treatment of kidney stones. Recent promising result shows a reduction of urinary oxalate with the administration of lactic acid [5]. Bacteria used as probiotic strains are tested for acid and bile tolerance because when ingested it joins with the food in its journey to the lower intestinal tract.

This study aims to isolate the Probiotic organisms from Dairy product (Curd) & Cow dung and screen the isolate for oxalate degrading ability.

II. MATERIALS AND METHODS

A. Sample collection:

Two different samples were collected. Sample I- Curd (Fresh dairy product); Sample II, Cow dung. One gram of the cow dung was mixed with 9 ml of the sterile distilled water. Similarly, one ml of curd was measured and mixed with 9 ml of sterile distilled water.

S.No	Source	Media	Organism
1.	Curd	MRS Agar	*Bacillus sp.*
2.	Cow dung	MRS Agar	*Bacillus cereus.*

Isolation, purification and evaluation of lactic acid bacteria from sample

10ml of each sample was dissolved into 90 ml of MRS broth. Then the homogenous mixture in the MRS broth was

ISSN No:-2456-2165

incubated at 37˚ C for 24h in aerobic condition. The sample was serially diluted up to $10^{-7}$and 1 ml of each dilution was added onto each plate of MRS agar using spread plate method. The plates were incubated at 37^0 C for the appearance of colonies and the number of colonies produced for different dilutions were recovered [6].The cultures were transferred to MRS agar slants and maintained in refrigerator at 4˚C for further analysis. Bacterial cultures were screened for temperature sensitivity, lactose utilization and NaCl tolerance.

III. MOLECULAR CHARACTERIZATION

A. DNA Isolation and Quantification

DNA was isolated from the samples by modified salting-out procedure. The isolated DNA was quantified by reading the absorbance at 260 & 280nm. The quality of the extracted DNA was checked on 0.8% agarose gel, by staining it with Ethidium bromide. The electrophoresis was performed at 70V for 20 min. Quantity of the extracted DNA was checked in UV spectrophotometer (SHIMADZHU, JAPAN) by reading the optical density (OD) at 260 nm and 280 nm.

B. DNA quality determination

- *Procedure*

0.24g of agarose powder was added in 30 mL of 1X TAE buffer and boiled until the preparation was clear. Then it was allowed to cool, down to approximately 50^0C. Then 1.5 µL of Ethidium bromide was added and mixed well. It was poured in gel casting plate with already adjusted gel comb and kept at room temperature for solidification. The gel was soaked in 1X TAE buffer in the electrophoresis tank. 3 µL of DNA with 3 µL of gel loading dye was loaded in the wells using micropipettes. It was electrophoresed at 70 V for 15 to 20 min. The orange color (DNA) bands were observed in UV illuminator.

C. Amplification and Sequencing of 16S rRNAgene

The PCR amplification was carried out in a PCR thermal cycler (GeneAmp PCR System 9700, Applied Biosystems) with the following cycling profile.

D. Sequencing

- *ExoSAP-IT Treatment*

Five micro litres of PCR product is mixed with 2 µl of ExoSAP-IT and incubated at 37ºC for 30 minutes followed by enzyme inactivation at 80ºC for 15 minutes.

E. Sequencing using BigDye Terminator v3.1

Sequencing reaction was done in a PCR thermal cycler (GeneAmp PCR System 9700, Applied Biosystems) using the BigDye Terminator v3.1 Cycle sequencing Kit (Applied Biosystems , USA) following manufactures protocol.

F. Screening for Oxalate Degrading Ability

The presumptive LAB was screened for oxalate utilization using agar well diffusion in calcium oxalate [4], [5]. Wells of 6mm diameter were prepared in calcium oxalate plate and each well was inoculated with 0.1 mL of overnight culture and incubated at 37ºC for 24 h. The oxalate utilizing bacteria can form clear zone around the well due to oxalate decomposition by the isolates. Zone diameter was measured and the isolated displaying 10mm of zone were subjected to quantitative determination of oxalate degradation.

G. Growth Kinetic Analysis of Oxalate Degrading Bacteria

Kinetic of growth and oxalate degradation of isolate were evaluated. The oxalate degrading strain was inoculated in MRS broth supplemented with 10mM potassium oxalate and sterile broth was used as control. The growth was monitored by reading absorbance at 600nm at 24 h time interval. The absorbance at A_{600nm} versus time curve was plotted to reveal growth kinetics. Similarly, the oxalate degrading ability was determined for every 24h. Quantifying oxalate in the growth was determined as per standard procedures [7].

IV. RESULTS

Gene	Direction	Sequence (5´ – 3´)
16S rRNA	Forward	AGAGTTTGATCMTGGCTCAG
	Reverse	TACGGYTACCTTGTTACGACTT

Table 1. Isolation of Probiotic Organism

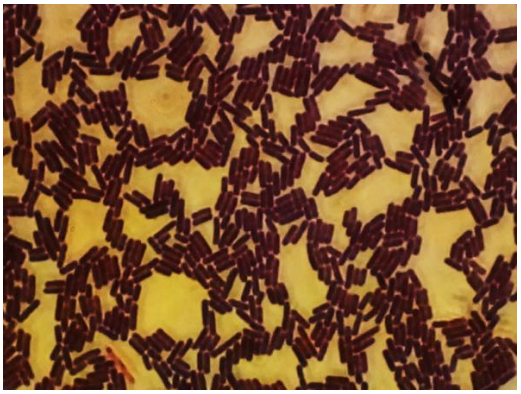

Fig 1:- Gram staining of isolated probiotic organism

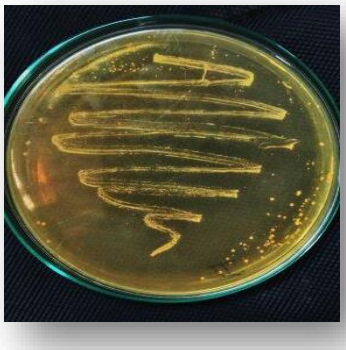

Fig 2:- Isolation of Probiotic Organism

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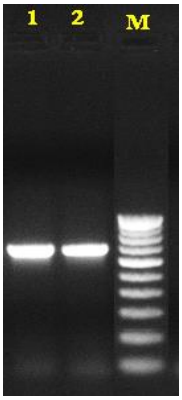

Fig 3:- PCR amplification of 16S rRNA gene

Table 2. Sequence and Phylogenetic tree for Probiotic Organisms

Analysis

Analysis	Phylogeny Reconstruction
Scope	All Selected Taxa
Statistical method	Neighbor-joining

Phylogeny Test

Test of Phylogeny	Bootstrap method
No. of Bootstrap Replication	1000

Substitution Model

Substitutions Type	Nucleotide
Model/Method	Tamura 3-parameter model
Substitutions to Include	d: Transitions + Transversions

Rates and Patterns

Rates among sites	Uniform rate
Pattern among lineages	Same (Homogeneous)

Data Subset to Use

Gaps/Missing Data Treatment	Complete deletion

A. Bacillus sp.

>GTAAGACTGGGATAACTCCGGGAAACCGGAGCTAA
TACCGGATAGTTCCTTGAACCGCATGGTTCAAGGATG
AAAGACGGTTTCGGCTGTCACTTACAGATGGACCCG
CGGCGCATTAGCTAGTTGGTGAGGTAACGGCTCACC
AAGGCGACGATGCGTAGCCGACCTGAGAGGGTGATC
GGCCACACTGGGACTGAGACACGGCCCAGACTCCTA
CGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACG
AAAGTCTGACGGAGCAACGCCGCGTGAGTGATGAAG
GTTTTCGGATCGTAAAGCTCTGTTGTTAGGGAAGAAC
AAGTGCAAGAGTAACTGCTTGCACCTTGACGGTACC
TAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGC
CGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGAAT
TATTGGGCGTAAGCGCGCGCAGGTGGTTTCTTAAGTC
TGATGTGAAAGCCCACGGCTCAACCGTGGAGGGTCA
TTGGAAACTGGGAAACTTGAGTGCAGAAGAGGAAAG
TGGAATTCCATGTGTAGCGGTGAAATGCGTAGAGAT
ATGGAGGAACACCAGTGGCGAAGGCGACTTTCTGGT
CTGTAACT

Tree

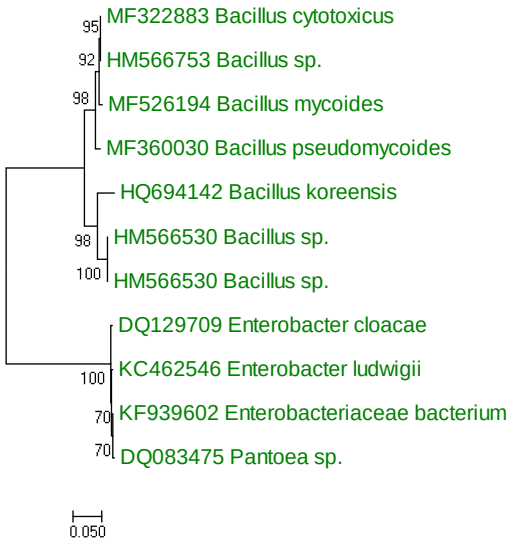

Fig 4:- Evolutionary relationships of taxa

By using Neighbor-Joining method, the evolutionary history was inferred (Saitou N. and Nei M. 1987). The optimal tree with the sum of branch length = 0.44069154 is shown. The percentage of replicate trees in which he associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. To infer the phylogenetic tree, the tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used. The evolutionary distances were calculated using the Tamura 3-parameter method and units of the number of base substitutions per site. The analysis involved 11 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 537 positions in the final dataset. Evolutionary analyses were done usingMEGA7.

B. Bacillus cereus

>TGAAGTTAGCGGCGGACGGGTGAGTAACACGTGGG
TAACCTGCCCATAAGACTGGGATAACTCCGGGAAAC
CGGGGCTAATACCGGATAACATTTTGAACCGCATGG
TTCGAAATTGAAAGGCGGCTTCGGCTGTCACTTATGG
ATGGACCCGCGTCGCATTAGCTAGTTGGTGAGGTAA
CGGCTCACCAAGGCAACGATGCGTAGCCGACCTGAG
AGGGTGATCGGCCACACTGGGACTGAGACACGGCCC
AGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCG
CAATGGACGAAAGTCTGACGGAGCAACGCCGCGTGA
GTGATGAAGGCTTTCGGGTCGTAAAACTCTGTTGTTA
GGGAAAAACAAGTGCTAGTTGAATAAGCTGGCACCT
TGACGGTACCTAACCAGAAATCCACGGCTAACTACG
TGCCACCAGCCGCGGT

ISSN No:-2456-2165

Tree

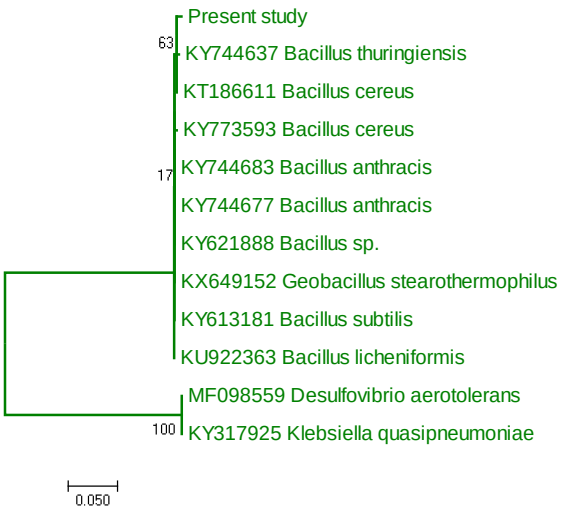

Fig 5:- Evolutionary relationships of taxa

The evolutionary history was evaluated using the Neighbor-Joining method. The ideal tree with the sum of branch length = 0.36229143 is shown. The percentage of replicate trees in which the closely related taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The phylogenetic tree is inferred using the tree drawn to scale, with branch lengths in the same units as those of the evolutionary distances. The evolutionary distances were computed using the Tamura 3-parameter method and are in the units of the number of base substitutions per site. The 12 nucleotide sequences were analyzed. All positions containing gaps and missing data were eliminated. There were a total of 437 positions in the final dataset. Evolutionary analyses were computed in MEGA7.

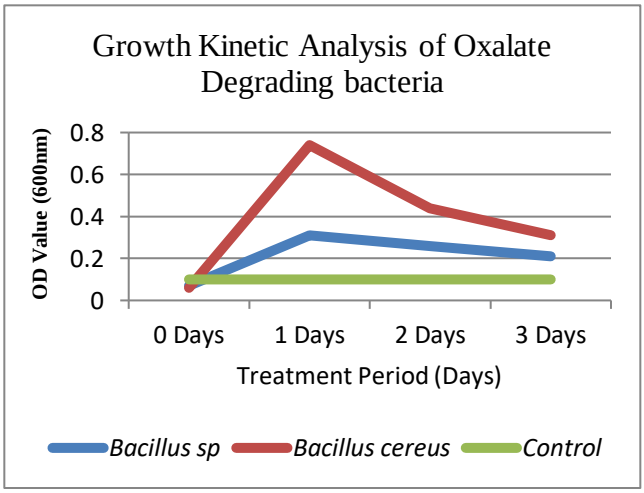

Fig 6:- Growth Kinetic Analysis of Oxalate Degrading bacteria

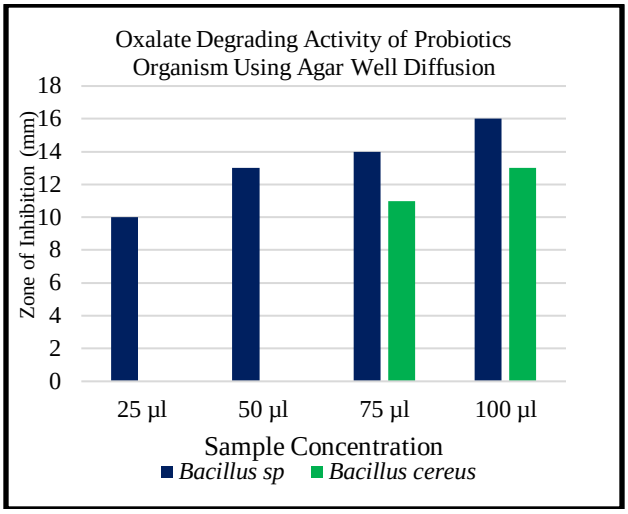

Fig 7:- Oxalate Degrading Activity of Probiotics Organism Using Agar Well Diffusion

V. DISCUSSION

In the medical arena probiotic organisms were found to be an alternative choice with demonstrable health prospects and have generally regarded as safe and proven low risk of inducing or being associates with etiology of diseases.

In the present study, we focused on characterizing the potential benefits of probiotic organism from dairy and animal source. In Dairy source: Curd is a common and regular food diet especially for south Indians. The lactic acid bacteria found in the curd have been well accepted as GRAS (Generally Recognized As Safe) for human consumption [8]. Apart from dairy source it has been documented that cow dung possess a good probiotic niche.

In the present study, the isolated probiotic organisms were able to survive at normal body temperature. The studies provide that the addition of certain starter cultures to milk products, allows the lactose intolerant people to consume those products without the usual rise of breath hydrogen or associated symptoms [9] [10] [11]. The lactic acid bacterial isolates were able to tolerate 1-6.5% NaCl concentration. NaCl is an inhibitory substance which may inhibit growth of certain types of bacteria.

Kidney stone formation is a worldwide complication with higher risk of developing chronic Kidney disease, sparing no cultural, or racial groups. To overcome this probiotic therapy induced to degrade kidney stone formation and also safe therapy for the patients. In probiotics, Oxalate is a strong oxalic acid act as chelator of cations and often found to be soluble potassium oxalate or precipitated as insoluble calcium oxalte. In present study, *Bacillus cereus* and *Bacillus* sp. were isolated from cow dung, curd and experimented degradation in modified barber medium to determine ability of oxalate degradation. The degrading ability is because of the oxalate degrading enzyme present in our isolates.

Thereby conclude that further study has to done to explore the best possibility of probiotic as a source to reduce the kidney stone formation and as an effective non-invasive therapeutic target against Kidney stone disease.

REFERENCES

[1]. Soni, A.B., 1999. A comparative study of PratisaraneeyaKshara in the management of Arshas. Ph.D. Thesis, Gujarat Ayurveda University, Jamnagar, India.
[2]. Bhalla, V., P.C. Grimm, G.M. Chertow and A.C. Pao, 2009. Melamine nephrotoxicity: An emerging epidemic in an era of globalization. Kidney Int., 75: 774-779.
[3]. Barber, H.M., and Gallimore, E.J. 1940. The metabolism of oxalic acid in the animal body. Biochem. J. 34: 144-148.
[4]. Alison, M.J., Dawson, K.A., Mayberry, W.R., and Foss, J.G. "*OxalobacterFormigenes* gen. nov., sp. Nov.: oxalate-degrading anaerobes that inhabit the gastrointestinal tract," Archives of Microbiology, vol. 141, no. 1, pp. 1-7, 1985.
[5]. Campieri, C., Campieri, M., Bertuzzi, V. et al., "Reduction of oxaluria after an oral course of lactic acid bacteria at high concentration," Kidney International, vol. 60, no. 3, pp. 1097-1105, 2001.
[6]. Hoque, M. Z., Akter, F., Hossain, K. M., Rahman, M. S. M., Billah, M. M. and Islam, K. M. D. Isolation, Identification and Analysis of Probiotic Properties of *Lactobacillus Spp.* From Selective Regional Yoghurts. World Journal of Dairy & Food Sciences. 2010; 5 (1): 39-46.
[7]. Hodgkinson, A., and Williams, A. "An improved calorimetric procedure for urine oxalate," Clinica ChimicaActa, vol. 36, no. 1, pp. 127-132, 1972.
[8]. Shruthy VV, Pavithra M, Gowri S, GhoshAsit R*. Probiotic potentials among lactic acid bacteria isolated from curd. Centre for Infectious Diseases & Control, Division of Medical Biotechnology, School of Bio Sciences & Technology, VIT University, Vellore, India Received on: 03/01/2011 Revised on: 25/02/2011 Accepted on: 12/03/2011. ISSN 2229-3566.
[9]. Lin, M., Savaiano, D. And Harlender, S. Influence of nonfermented dairy products containing bacterial starter cultures on lactose maldigestion in humans. Journal of Dairy Science. 1991; 74: 87-95.
[10]. Scheinbach, S. 1998. Probiotics: Functionality and commercial status. Biotechnology Advances 16(3):581-608.
[11]. Fooks, L. J., Fuller, R., and Gibson, G. R. Prebiotics, probiotics and human gut microbiology. International Dairy Journal.1999; 9:53-61.