The International Journal of Engineering and Science (IJES)
|| Volume || 9 || Issue || 06 || Series I || Pages || PP 20-25 || 2020 ||
ISSN (e): 2319-1813 ISSN (p): 20-24-1805

Isolation and Identification of Oxalate Degrading Bacteria, And In Vitro Determination of Its Probiotic Potential

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----------ABSTRACT----------
High concentration of urinary oxalate may lead to the formation of calcium oxalate kidney stones. There are many medications and surgical treatments are available for the management of kidney stone disease. But they have their own benefits and drawbacks. Degradation of urinary oxalate by bacteria by using them as probiotics can be a new therapeutic tool for the management of kidney stone disease. Present study focuses on the isolation of oxalate degrading bacteria from various food sources by using ATCC 1352 medium. Identification of isolated bacteria was carried out based on Bergey's Manual of Determinative Bacteriology and molecular techniques. Quantitative determination of oxalate degradation was carried out by using Barber's medium and concentration of oxalate was determined by oxidation of KMnO4 by oxalic acid. One bacterial strain showed 19.2% oxalate degradation. Based on Bergey's Manual of Determinative Bacteriology and 16s rRNA sequencing it was found that the isolated organism was Bacillus subtilis. Reduction of urinary oxalate by isolated organism Bacillus subtilis can be a new therapeutic tool for the management of kidney stone diseases. We can use this bacterium as a probiotic tool for the management of kidney stone disease but further in vitro and in vivo testing is required for the determination of its probiotic potential, they must be safe for humans.
Keywords: *Oxalate, kidney stone, Bacillus subtilis, probiotics*

Date of Submission: 21-05-2020 Date of Acceptance: 07-06-2020

I. INTRODUCTION

oxalate is a toxic compound abundant in the plant domain and extensively utilize in normal diets as a component of, vegetables, fruits, nuts, and grains. The regular consumption of oxalate ranges from 70 to 920 mg but extensively rises in vegetarians (Murru *et al*., 2017). Approximately 60 to 80 % of all kidney stones are composed primarily of calcium oxalate. The major factor of calcium oxalate crystal formation is the supersaturation of urinary calcium oxalate (Afkari *et al*., 2019). Oxalate degrading enzymes are not present in humans (Gomathi *et al*., 2014). *Oxalobacter formigenes*, an anaerobic bacterium that inhabits in the colon and depends solely on oxalate as a source of carbon and energy (Liebman and Wahsh, 2011). *O. formigenes* have two genes *frc* encode formyl-coA transferase and *oxc* encode oxyl-coA responsible for oxalate degradation (Kullin *et al*., 2016). Probiotics are defined as live organisms that can be administered in an adequate amount confer the health benefit (Gomathi *et al*., 2014). Screening of *Lactobacilli* has been done from several fermented food and pharmaceutical preparations to detect the oxalate degrading ability and to demonstrate the functionality of *oxc* and *frc* in the catabolism of oxalate (Turroni *et al*., 2010). Apart from the *O. formigenes* candidate with the greatest potential for the treatment of kidney stone disease are oxalate degrading *Lactobacillus* and *Bifidobacterium* spp. Hence, a new therapeutic tool for the management of kidney stone disease can be explored. 24 to 34 % of urinary oxalate comes from the normal diet. Oxalate content in the diet can be reduced to 54 to 59 % by avoiding oxalate containing food but unfortunately, it is difficult to achieve and would probably be deficient in essential nutrients (Siva *et al*., 2009). As earlier mentioned, oxalate is the major constituent of kidney stone, probiotic organisms can be used for the management of calcium oxalate kidney stones.

II. MATERIAL AND METHODS

Isolation and of oxalate degrading microorganisms

Isolation was done from various food sources such as homemade curd, commercially available probiotic curd, yogurt. Because the tomato and spinach contain high oxalic acid they were crushed in fine pest and mixed with the homemade curd and allowed for the natural fermentation at room temperature for 48 hours for enrichment. After 48 hours sample was streak by four flame method on ATCC medium 1352 agar plates and incubated anaerobically at 37 ºC for 5 days. After incubation of 5 days, colonies showing zone of colour change

from purple to colourless were randomly selected. Bacteria showing zone of the colour change of resazurin was maintained in Modified Barber's medium (Hokama *et al*., 2000).

Identification of oxalate degrading microorganisms

The identification of selected strain was done according to Bergey's Manual of Determinative Bacteriology and based on 16s rRNA sequencing.

Quantitative determination of oxalate degradation

For the quantitative determination of oxalate degradation 1 ml culture of the selected organism was inoculated into 200 ml modified Barber's medium and incubated anaerobically at 37 ºC for 5 days. An uninoculated Barber medium was used as a control. After incubation medium was centrifuged at 5000 rpm to allow cells to settle down and the concentration of oxalate in the supernatant was determined by oxidation of KMnO4 by oxalic acid by using an oxalic acid standard curve (Naik *et al*., 2014).

In vitro determination of probiotic potential of selected strain

Antimicrobial activity

Antimicrobial activity test of isolated organism against various pathogens was done according to Baqqal *et al*., 2019 by using a cross-streak method. Pathogens used for testing antimicrobial activity were *Escherichia coli, Enterobacter aerogenes, Staphylococcus aureus, Streptococcus, Pseudomonas aeruginosa, Proteus vulgaris, Salmonella typhi, klebsiella pneumoniae.*

Antimicrobial sensitivity

Antimicrobial sensitivity testing of an isolated organism was done by using the disk diffusion method. Combi disk containing multiple antibiotics was purchased from PATHOTEQ BIOLOGICAL LABORATORIES, Umbergam, Gujarat, India. The concentration of antibiotics per disk was amoxycillin(10 µg), cefaclor(30 µg), chloramphenicol (30 µg), neomycin (30 µg), doxycycline (30 µg), vancomycin (30 µg), furazolidone (100 µg), clarithromycin (15 µg), oxacillin (1 µg), clindamycin (2 µg), penicillin-G (10 U), erythromycin (15 µg). Cell concentration approximately 10^8 CFU/ml was adjusted (using McFarland standard 0.5) and spread on MHA agar plate and then combi disk containing above antibiotics were placed on the surface of MHA agar plated previously inoculated with isolated organism and incubate at 37 ºC for 24 hours.

Low pH and Bile salt tolerance test

Low pH and Bile salt tolerance test of isolated oxalate degrading organism was performed according to Nami *et al*., 2018 with slight modifications. The isolated organism was grown in MRS broth at 37 ºC for 24 hours and resuspended in sterile normal saline than inoculated in MRS broth adjusted to pH 3.0 and incubated for 2 hours anaerobically at 37 ºC. For determination of bile salt tolerance, the isolated organism was grown in MRS broth at 37 ºC for 24 hours and resuspended in sterile normal saline than inoculated in MRS broth containing 0.5% ox gall and incubated for 4 hours anaerobically at 37 ºC. These conditions were chosen because this represents the time that bacteria require to passing through the gastrointestinal tract and pH value and bile salt concentration found in the stomach and intestine respectively. 0.1 ml culture was spread on MRS agar plates initially and after the end of incubation. Then plates were incubated anaerobically at 37 ºC for 48 hours. The survival of the organism was determined in terms of counting the number of colonies.

Adhesion test

The in vitro determination of the ability of isolated organism to adhere to the intestinal epithelial cells was done by performing yeast agglutination assay described by Mirelman *et al*., in 1980 with slight modifications. Yeast cells *Saccharomyces cerevisiae* were grown in 5 ml YPD broth for 24 hours and after incubation, the cells were harvested by centrifugation at 5000 rpm for 10 min. and resuspended in sterile saline. Then the yeast cells were stained with safranin by mixing the cells with 100µL of 0.25% safranin. A drop of heavy suspension of isolated bacterial culture was mixed with a drop of safranin stained yeast cells on a glass slide and observed for the agglutination. A drop of distilled water and safranin stained yeast cell mixed on another slide which was used as control. The microscopic examination of agglutination was carried out by using 40X magnification.

III. RESULTS

Isolation of oxalate degrading bacteria from various food sources

Among the various samples used for the isolation of oxalate degrading bacteria only from the sample containing a mixture of tomato pest and curd showed the colour change of resazurin dye from purple to

colourless on ATCC 1352 medium agar plates. only one type of colony was showed the maximum zone of the colour change of resazurin dye.

Quantitative determination of oxalate degradation

Quantitative degradation of oxalate was carried out using modified Barber's medium and by using an oxalic acid standard curve. 19.2% of oxalate degradation was observed by an isolated organism.

Antimicrobial activity

The isolated strain which showed maximum oxalate degradation was analysed for their ability to inhibit the growth of the various pathogens. It was found that the growth of pathogen *Salmonella typhi* was inhibited by the isolated strain.

Antimicrobial sensitivity testing

Antimicrobial susceptibility testing was done by using combi disk and the isolated oxalate degrading bacterium was showed resistance to many antibiotics as described in table 2.

Low pH and Bile salt tolerance test

When isolated oxalate degrading bacterium was exposed in MRS broth pH 3 for 2 hours, it was observed that many numbers of colonies appeared on MRS agar plate after 2 hours of incubation it indicates that the bacterium has the ability to survive at low pH. When this bacterium was inoculated in MRS broth containing 0.5% ox gall for 4 hours, it was found that many colonies were obtained on MRS agar plate after 4 hours of exposure in ox gall powder it indicates that this bacterium has the ability to survive in the presence of bile salt.

Yeast agglutination assay

After performing yeast agglutination assay it was observed that the isolated bacterial strain shows strong agglutination of the yeast cell.

16s rRNA sequencing analysis

After performing 16s rRNA sequencing and bioinformatic analysis it was found that the isolated oxalate degrading organism was *Bacillus subtilis* based on of nucleotide homology and phylogenetic analysis

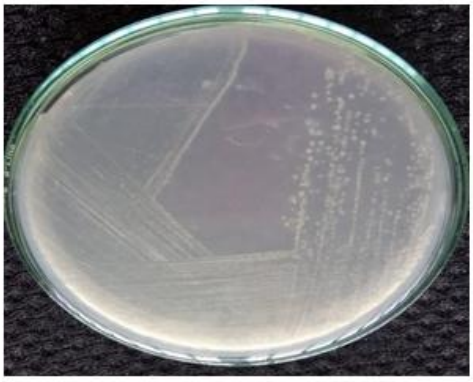

Figure 1. Oxalate degrading bacterial isolates showing colour change of resazurin from purple to colourless on ATCC 1352 medium

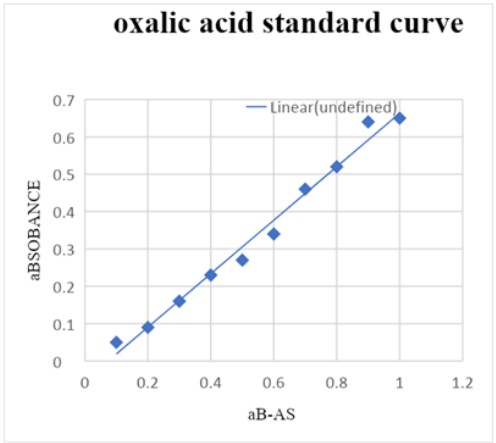

Figure 2. Oxalic acid standard curve

Table 1. morphological and biochemical characteristics of isolated strain

Characteristics	Isolated strain
Colony characteristics	Intermediate, Round, Smooth, Convex, Translucent, White, colonies
Motility	-
Gram staining	Gram-positive rods
Endospore staining	+
Starch hydrolysis test	+
Voges Proskauer test	+
Citrate utilization test	+

+ = positive, - = Negative

Table 2. Antibiotic sensitivity testing of isolated oxalate degrading bacteria

Antibiotics	Result	Diameter of zone of inhibition
Amoxycillin	Sensitive	11mm
Cefaclor	Sensitive	10mm
Chloramphenicol	Sensitive	32mm
Neomycin	Sensitive	23mm
Doxycycline	Sensitive	12mm
Vancomycin	Resistance	-
Furazolidone	Sensitive	24mm
Clarithromycin	Sensitive	12mm
Oxacillin	Resistance	-
Clindamycin	Resistance	-
Penicillin-g	Resistance	-
Erythromycin	Resistance	-

Table 3. Determination of no. of colonies at 0 min. and after 2 hours

Organism	pH of MRS broth	No. of colonies at 0 min.	No. of colonies after 2 hours
Isolated bacterium	3	190	101

Table 4. Determination of no. of colonies at 0 min. and after 4 hours

Organism	Concentration of ox gall powder in MRS broth	No. of colonies at 0 min.	No. of colonies after 4 hours
Isolated bacterium	0.5%	178	97

IV. DISCUSSION

In the present study, oxalate degrading bacterium *Bacillus subtilis* isolated from food sources give 19.2% oxalate degradation. There is no evidence about percent of oxalate degradation by *B. subtilis* but several studies reported that *YrvK* gene found in *B. subtilis* that encode 44kDa oxalate decarboxylase (Tanner and Bornemann, 2000 and Lee *et al*., 2014). In contrast to this, several studies reported that the recombinant strain of *Lactobacillus plantarum* with oxalate degrading enzyme gene inserted from *B. subtilis* has more ability to degrade oxalate than wild type strain. Sasikumar *et al*., in 2013 reported that the recombinant strain *of L. plantarum* showed 30% to 50% oxalate degradation in medium containing 10 mM oxalate. *B. subtilis* can produce a verity of antimicrobial substances like a group of Lipopeptides such as Surfactins, Iturins, and Fengycins. They have a broad range of activities like anti-bacterial, anti-fungal, anti-viral, and anti-tumor (Kimelman and Shemesh, 2019). In the present study, it was found that the growth of pathogen *Salmonella typhi* was inhibited by the isolated strain. Similar kinds of results were also obtained in past by Adak *et al*., 2013 they also reported that the growth of other pathogens like *Escherichia, coli, Staphylococcus, aureus Clostridium*

perfringens, Shigella dysentery, Vibrio cholerae were also inhibited. The spore-forming nature of *Bacillus* spp. protect them during passing through the gastrointestinal tract especially from the acidic condition in stomach and bile concentration in the gut. The isolated oxalate degrading bacterium identified as *B. subtilis* showed significant tolerance to low pH and bile salt concentration. Kim *et al*., in 2019 reported that strain of *Bacillus subtilis* and other probiotic organisms exhibit significant tolerance to low pH and bile concentration. Mannose-containing polysaccharides or mannans are key constituents of the cell wall of baker's yeast, *Saccharomyces cerevisiae*, same kind of molecules also found on the surface of gut epithelial cells of humans. Some micro-organisms have adhesins on their surface specific for mannose-containing receptors and, therefore, are able to agglutinate yeast cells in a mannose sensitive manner (Tallon *et al*., 2007). The isolated oxalate degrading bacterium showed maximum agglutination of safranin stained yeast cells. There is no any result found in past about agglutination of yeast cells by *B. subtilis*. therefore, it is assumed that the bacteria may possess lectins on their surface that specific for mannose containing receptors but the further confirmatory test is required to determine whether the lectins are specific for mannose receptor or not. *Bacillus subtilis* is not cause any harm to humans and animals and also it is commercially important as they produce certain antimicrobial substances, heterologous proteins, vaccines, enzymes, and antigen. Food and Drug Administration approved this organism Generally Regarded as Safe (GRAS). For example, natto, a common Japanese dish made from fermented soybeans by way of the bacterium *Bacillus subtilis*, contains oxalate dehydrogenase and is commonly consumed by humans with no apparent adversative effects (Cowley *et al*., 2010). Hence, *B. subtilis* could be considered as a multifunctional probiotic bacterium for humans and animals (Olmos and Paniagua, 2014).

V. CONCLUSION

In conclusion, the isolated oxalate degrading bacterium which is identified as *Bacillus subtilis* showed maximum (19.2%) oxalate degradation. In addition to oxalate degradation ability, this organism also has properties like other probiotic organisms. This bacterium has the ability to produce an antimicrobial substance that inhibits the growth of pathogenic *S. typhi*. The bacterium also showed resistance to many antibiotics that protect the bacterium into the gut during any drug treatment. This bacterium also tolerates low pH and bile salt concentration and also has the ability to agglutinate the yeast cell which helps the bacterium to survive in the human digestive system. Because of the above properties, we can use this organism as a probiotic tool for the treatment of kidney stone disease. Because the present study is only in vitro basis so, further in vivo and some animal testing is required to confirm the safety of bacterium to use as probiotic.

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Vaibhavi Mandanka, et. al. "Isolation and Identification of Oxalate Degrading Bacteria, And In Vitro Determination of Its Probiotic Potential." *The International Journal of Engineering and Science (IJES)*, 9(6), (2020): pp. 20-25.