www.ThePharmaJournal.com

The Pharma Innovation

ISSN: 2277- 7695
TPI 2015; 3(11): 38-42

www.thepharmajournal.com
Received: 05-11-2014
Accepted: 15-12-2014

Jatindra Nath Mohanty
Centre of Biotechnology, Siksha O Anusandhan University Bhubaneswar, Odisha – 751003, India.

Pratyush Kumar Das
Centre of Biotechnology, Siksha O Anusandhan University Bhubaneswar, Odisha – 751003, India.

Sonali Nanda
Centre of Biotechnology, Siksha O Anusandhan University Bhubaneswar, Odisha – 751003, India.

Priyanka Nayak
Centre of Biotechnology, Siksha O Anusandhan University Bhubaneswar, Odisha – 751003, India.

Puspita Pradhan
Centre of Biotechnology, Siksha O Anusandhan University Bhubaneswar, Odisha – 751003, India.

Correspondence:
Pratyush Kumar Das
Centre of Biotechnology, Siksha O Anusandhan University Bhubaneswar, Odisha – 751003, India.

Comparative analysis of crude and pure lactic acid produced by *Lactobacillus fermentum* and its inhibitory effects on spoilage bacteria.

Jatindra Nath Mohanty, Pratyush Kumar Das, Sonali Nanda, Priyanka Nayak and Puspita Pradhan

Abstract
Lactic Acid is one of the most industrially important acids which have a wide spread application. It is mostly present in two forms i.e. L-form and D-form. In this work the aim was to produce lactic acid from *Lactobacillus fermentum* via three different production medium viz. MRS Broth, Whey Basal Broth and Skimmed Milk Broth and optimization of these mediums to maximize the production, followed by purification of the crude lactic acid by Ion exchange chromatography. Titrimetric assay was carried out at each & every step to determine the level of purity along with the checking of anti-bacterial activity. High purity as much as 85 percent was achieved. Finally the purified elutes were subjected to Thin layer Chromatography (TLC) and their Rf Values were determined.

Keywords: *Lactobacillus fermentum*, Lactic acid, Ion exchange chromatography, Antimicrobial activity, MRS Medium, Whey Basal Medium, Skimmed Milk Medium.

1. Introduction
Lactic acid, also called α-hydroxypropanoic acid or 2-hydroxypropanoic acid, has a wide range of application in different fields and in general in preservation of human food stuffs [1]. Discovered by Scheele in 1780 and was considered as a milk component and in 1789 was named as “acide lactique” by Lavoisier. Later in 1857, Louis Pasteur discovered that it was a fermentation metabolite released by certain microorganism rather than being a milk component [2]. When glycogen is broken down in muscle, lactic acid occurs in the blood in the form of salt called – “Lactates” and can be again be converted back to glycogen in the liver [3]. Lactic acid has two optical isomers: L(+)-lactic acid and D(-)-lactic acid (see figure -1 below). US FDA (Food and Drug Administration) has classified lactic acid as GRAS (Generally Recognized as Safe) for use as a food additive. But at times D(-)-lactic acid is harmful to humans and results in acidosis and decalcification of bones [4]. Although the racemic DL-lactic acid is produced by chemical synthesis from petrochemical resources, but still, optically pure L (+) and D (-) lactic acid can be obtained from microbial fermentation and can be polymerized to high crystalline Poly Lactic Acid (PLA) [5, 6]. Thus, the production of lactic acid by microbial fermentation provides a better alternative to the environmental pollution caused by the petrochemical industries during the production of the racemic one.

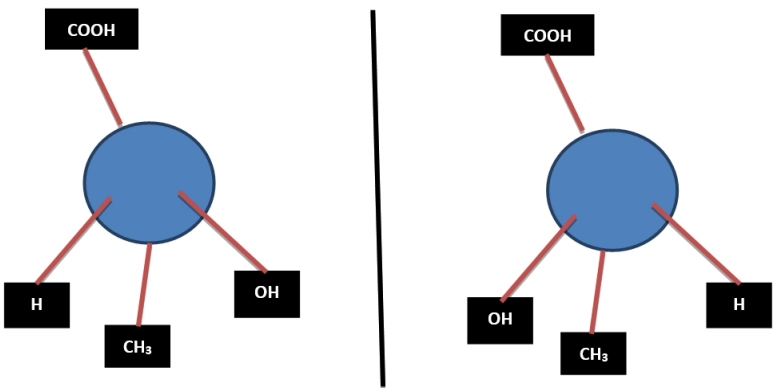

Fig 1: Stereoisomers of Lactic Acid

1.1 Chemistry of Lactic Acid

Lactic acid is a carboxylic acid with the chemical formula $C_3H_6O_3$, having a hydroxyl group adjacent to the carboxyl group, thus making it an α-hydroxy acid (AHA). Lactic acid in a solution can easily lose a proton from the acidic group, producing lactate ion. The chemical structure of lactic acid is elucidated below (see figure-2).

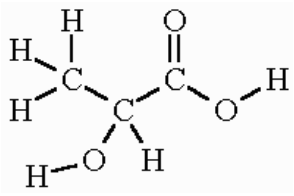

Fig 2: Chemical Structure of Lactic Acid

Chemical formula: $C_3H_6O_3$
Molecular Weight: 90.08
pKa: 3.86 at 25 ^{0}C
Melting Point: (52.7-52.8) ^{0}C for pure form and 16.4 ^{0}C for racemic mixture containing 50% of each isomer.

Description: Colourless, Syrupy liquid or white to yellow solid or powder.

Functional Uses: Acid, Acidifier.
Solubility: Liquid- Soluble in water and ethanol. Solid- Sparingly soluble in water, soluble in acetone.

1.2 Application of Lactic Acid

Lactic acid, also called α-hydroxypropanoic acid is considered as one of the most important and useful chemical. It has a wide range of application which includes its use as a preservative, acidulant and flavouring agent in the food processing industry. It is also used in the chemical industry as a raw material for the production of lactate ester, propanoic acid, acrylic acid, acetaldehyde and dilactide [7, 8]. It also plays an important role as a monomer in the production of biodegradable PLA, a well-known sustainable bioplastic material. Lactic acid is used as humectant or moisturizer in some cosmetics and as a mordant, a chemical that helps fabrics accept dyes, in textiles. It is used in the pharmaceutical industry as a starting material for other substances and involved in manufacturing of lacquers and inks [9]. It is also used in medicines as keratolytics and topically administered. It helps in increasing the moisture in the skin by softening the keratin that holds the top layer of the skin cells together. This in turn helps the dead skins to fall off and keep more water in the skin [10].

1.3 Lactic Acid Bacteria (LAB)

Lactic acid bacteria are gram positive microorganisms that are generally found in plants, meat and dairy products and can produce lactic acid as an anaerobic product. LAB are generally grouped into two classes i.e. Homo fermentative and Hetero fermentative, based on the fermentation end products. These bacteria can grow in the pH range of 3.5-10.0 and temperature of (5-45) ^{0}C, thus varying their optimal growth condition depending on the producers. These bacteria require some elements for growth such as carbon and nitrogen sources, in the form of carbohydrates, amino acids, vitamins and minerals [11, 12, 13]. The homo fermentative bacteria convert glucose directly into lactic acid, while the hetero fermentative bacteria catabolize glucose into ethanol, carbon dioxide and lactic acid [14]. Most of the lactic acid bacteria used for producing lactic acid belongs to the genus *Lactobacillus* [4].

1.4 Raw materials and Nutrients for Lactic Acid Production

A cheap raw material includes materials with high content of starch and cellulose, molasses and whey for the production of lactic acid [14]. Among these starchy and cellulosic materials are very cheap, abundant and renewable [8, 15, 16]. Starchy materials like sorghum [15, 17], wheat [8, 18, 19, 20], potato [21, 22], rice [20, 23], barley [20, 24, 25] and cellulosic materials like corn cob [26, 27], waste paper [28, 29] and wood [30, 31] have also been used. The best nutrient supplement for fermentation media for rapid lactic acid production is yeast extract, but is costly [32, 14]. Alternatively, corn-steep liquor obtained as a by-product from the corn-steeping process has also been used [20].

1.5 Antimicrobials from Lactic Acid Producing Bacteria

The term antimicrobial can be used to describe any substance produced by a microorganism that inhibits the growth of other microorganisms [33]. LAB bacteria show antagonism effect towards certain microorganism due to production of primary metabolites such as lactic acid, acetic acid, ethanol and carbon dioxide. Other antimicrobial compounds produced by LAB are formic acid, benzoic acid, hydrogen peroxide, acetoin and bacteriocins. LAB shows inhibitory activity towards gram positive microorganism due to the bactericidal effect of protease sensitive bacteriocins, however towards gram negative microorganism the antagonistic effect could be related to the production of organic acids and hydrogen peroxide.

2. Materials and methods

2.1 Isolation and confirmation of bacteria

The desired bacterial colonies were isolated from milk sample by serial dilution method and grown on De Man Rogosa Sharpe (MRS) media by spread plate technique. The bacterial species was confirmed by carrying out gram staining and a number of biochemical tests which includes catalase test, glucose fermentation and mannitol fermentation.

2.2 Production and Extraction of Crude Lactic Acid

Crude lactic acid was produced from 3 different fermentation broths viz. MRS broth, Skimmed Milk Broth and Whey Basal Broth, inoculated with the bacterial culture in three different conical flasks under incubation in an orbital shaker incubator for both 24 hours and 48 hours. After the desired incubation the fermentation broths were taken and centrifuged in a high speed cooling centrifuge at 6,000 rpm for 10 minutes and the supernatant (crude lactic acid) was collected and stored for further use.

2.3 Optimization of the production medium

The 3 different production medium were optimized by changing their carbon and nitrogen source for getting the optimum production of crude lactic acid.

2.3.1 Optimization of Carbon & Nitrogen Source for MRS Broth

In MRS broth sucrose and fructose were used as a carbon source in place of glucose and checked for optimum production. In the same manner Malt extract and Beef extract were used as a nitrogen source in place of Meat extract and were checked for optimum production.

2.3.2 Optimization of Carbon & Nitrogen Source for Skimmed Milk Medium

In Skimmed Milk Broth, sucrose and fructose is taken in place of glucose as the source of carbon to check for optimum production whereas Beef Extract and Malt Extract is taken as nitrogen source in place of Yeast extract and checked for optimum production.

2.3.3 Optimization of Nitrogen Source for Whey Basal Broth

In Whey Basal Broth, Meat extract and Malt extract is taken in place of Yeast extract to check for optimum production.

2.4 Purification of Lactic Acid

Lactic acid being a weak acid does not have highly negative charged molecules. As such purification is carried out through a weak anion exchanger in the form of DEAE cellulose.

2.5 Study of antimicrobial effect of crude and purified lactic acid

The antimicrobial effects of both the crude and purified lactic acid samples were tested upon cultures of *E.coli, Micrococcus luteus* and *Pseudomonas aeruginosa* by agar well-diffusion method. The zone of inhibitions obtained was measured.

2.6 Titrimetric Assay (Titratable Acidity) of Lactic Acid

This method takes into account the concentration of disassociated hydrogen molecules and un-disassociated hydrogen ions. Acidity is related with hydrogen in the solution so TA to measure the total acidity is a better indication of lactic acid levels. In this process the sample is titrated against 0.1N NaOH with the addition of 2-3 drops of Phenolphthalein indicator until the colour of the sample turns light pink. The percentage of purity of lactic acid is calculated using the given formula –

$$R = \frac{V\,(titr) \times C\,(titr) \times Mw. \times F \times 100}{1000 \times W\,(Smp)}$$

Where, R= % of Lactic Acid
V (titr) = Total volume of titrant needed to reach the end point in ml.
C (titr) = Concentration of titrant.
M.w = Molecular weight of lactic acid = 90.08
F = Dilution Factor
W (Smp) = Sample amount in either gram or ml.
100 is multiplied to obtain the percentage of lactic acid present.

2.7 Thin Layer Chromatography

Thin Layer Chromatography (TLC) is conducted to confirm the presence of lactic acid. In this process the sample is run on a glass slab containing a layer of silica gel in a TLC chamber containing the desired solvent. Later on the plates are dried and visualized in an UV-trans illuminator. Basing on the distance travelled by the solute and the solvent, Rf value is calculated.

3. Results

3.1 Physiological and Biochemical Characteristics of the isolated strain

The table below shows the physiological and biochemical characteristics that confirmed the isolated strain-

Table 1: Physiological and Biochemical Characteristics of the isolated strain

Physiological & Biochemical Tests	Result
Gram Stain	+
Catalase Test	-
Glucose Fermentation	+
Arginine Test	-
Mannitol Fermentation	+
Growth in 2% NaCl	+
Growth at pH 4.5-6.5	+
Citrate Hydrolysis	-

3.2 Estimation of Crude Lactic acid

Table 2: Estimation of crude lactic acid from un-optimized medium

Different Production Medium	% of L.A/100ml of sample
MRS Broth	8.1
Whey Basal Broth (24 Hrs.)	9.4
Whey Basal Broth (48 Hrs.)	8.4
Skimmed Milk Broth (24 Hrs.)	2.8
Skimmed Milk Broth (48 Hrs.)	4.2

Different nitrogen and carbon sources were taken for optimization of the entire three production medium. So in order to know the best Carbon and Nitrogen source in each case, estimation of lactic acid was done. The medium with a certain Carbon and Nitrogen source showing maximum percentage output of lactic acid will no doubt be considered as the best optimized medium and can be used for further experiments.

Table 3: Estimation of crude lactic acid from optimized MRS broth

(C – SOURCE)	% of L.A/100 ml of sample
1. Sucrose	8.1
2. Fructose	10.5
(N – SOURCE)	
1. Malt Extract	3.9
2. Beef Extract	4.9

As shown above Sucrose and Fructose were taken as C – Source instead of Glucose whereas Malt extract and Beef extract were taken as N – Source in the place of Meat extract.

Table 4: Estimation of crude lactic acid from optimized Whey Basal Broth

(N – SOURCE)	% of L.A/100 ml of sample
1. Meat extract (24 Hrs.)	26
2. Malt extract (24 Hrs.)	31
3. Meat extract (48 Hrs.)	32
4. Malt extract (48 Hrs.)	34

Table 5: Estimation of crude lactic acid from optimized skimmed milk broth

(C – SOURCE)	% of L.A/100 ml of sample
1. Sucrose (24 Hrs.)	2
2. Sucrose (48 Hrs.)	3.6
3. Fructose (24 Hrs.)	2
4. Fructose (48 Hrs.)	3.9
5. Sucrose+Fructose (24 Hrs.)	3
6. Sucrose+Fructose (48 Hrs.)	2.9
(N – SOURCE)	
1. Malt Extract (24 Hrs.)	2
2. Malt Extract (48 Hrs.)	3.3
3. Beef Extract (24 Hrs.)	3
4. Beef Extract (48 Hrs.)	2.5

In the above table Sucrose and Fructose were taken as individual carbon source and also in combination instead of Glucose and Malt Extract and Beef Extract were taken instead of Yeast extract as Nitrogen Source.

3.3 Estimation of Purified Lactic Acid

As the Whey Basal Medium (incubated for 48 Hrs.) gave the maximum output in terms of percentage of lactic acid, hence it was selected for further purification by Ion exchange chromatography using DEAE cellulose. 6 elutes collected from the ion-exchange column were hence estimated and the percentage of lactic acid was calculated as given below –

Table 6: Estimation of purified Lactic acid from optimized whey basal broth purified by Ion exchange chromatography

Ion Exchange Elutes	% of lactic acid/100 ml of solution
E1	85.0
E2	40.0
E3	35.0
E4	5.0
E5	4.5
E6	2.0

3.4 Antimicrobial activity of crude lactic acid

The table below gives an account of the antimicrobial activity of crude lactic acid extracted from different production medium on various test organisms –

Table 7: Antimicrobial activity of crude lactic acid (in mm) obtained from different production medium

Test Organisms	MRS (48 Hrs.)	Whey Basal (24 Hrs.)	Whey Basal (48 Hrs.)	Skimmed Milk (24 Hrs.)	Skimmed Milk (48 Hrs.)
E.coli	11.0	8.8	9.4	-	4.0
Micrococcus luteus	9.7	6.7	7.5	4.6	6.5
Pseudomonas aeruginosa	4.5	5.0	6.3	-	6.2

Table 8: Antimicrobial activity of crude lactic acid obtained from optimized whey basal medium (48 Hrs. incubation)

Test Organism	Zone of inhibition in mm
E.coli	28
Micrococcus luteus	29
Pseudomonas aeruginosa	29

The above table shows the antibacterial activity of crude lactic acid obtained from optimized whey basal medium after an incubation of 48 hrs. The zone of inhibitions thus formed are measured and noted in millimeter.

3.5 Antimicrobial activity of purified lactic acid

Lactic acid obtained from the optimized whey basal medium was purified through an ion exchange column containing DEAE cellulose as the matrix and then the antimicrobial activity of the purified lactic acid, thus obtained as ion exchange elutes was checked and the results are mentioned below in the table-

Table 9: Antimicrobial activity of lactic acid (in mm) purified by Ion exchange chromatography)

Test Organism	Elute 1	Elute 2	Elute 3	Elute 4	Elute 5	Elute 6
E.coli	31	0	0	0	0	0
Micrococcus luteus	20	0	0	0	0	0
Pseudomonas aeruginosa	21	0	0	0	0	0

3.6 Thin Layer Chromatography (TLC) and Calculation of Rf Value

The purified lactic acid (first two Ion exchange elutes) were allowed to run on TLC plates and the Rf value of each purified elute was calculated and matched with the standard Rf value of lactic acid. The table below shows the Rf value obtained of the two purified ion exchange elutes.

Table 10: Rf Value of Purified Lactic Acid

Elutes	Rf Value
1. Elute 1	0.75 (approx.)
2. Elute 2	0.66

4. Conclusion

The required bacterial colonies were isolated from milk sample by serial dilution method and then confirmed as *Lactobacillus fermentum* by gram staining, endospore staining and biochemical tests. The culture was inoculated into 3 different mediums viz. MRS broth, Whey Basal Broth and Skimmed Milk Broth and incubated in an orbital shaker incubator both for 24 & 48 hrs. respectively. Crude lactic acid was obtained from these broths and checked for its antimicrobial activity against spoilage microorganisms. To increase the production of lactic acid each media were optimized by changing their carbon and nitrogen sources. The lactic acid produced from the optimized mediums was checked for its antimicrobial activity. Estimation of lactic acid from these optimized medium was conducted to determine the percentage of lactic acid present per 100 ml of the solution. High amount of lactic acid was obtained from optimized Whey Basal Broth followed by MRS Broth and the least was obtained from Skimmed Milk Broth. For further purification through Ion exchange column lactic acid obtained from the optimized whey basal broth was preferred. Upon titrimetric estimation of the purified lactic acid, purity as high up to 85% was obtained. Finally, TLC of the best two elutes was conducted to determine their Rf value and the first elute or Elute 1 had a Rf Value approximately near about to 0.75 which in general is the Rf value of pure lactic acid. The Purified lactic acid had a pretty good antagonistic effect on target microorganisms forming inhibition zones of up to 31 mm or 3.1 cm. Lactic acid is a very useful industrial chemical that can be synthesized naturally from microorganisms in a lab scale.

5. References

1. Davison BE, Llanos RL, Cancilla MR, Redman NC, Hillier AJ. Current research on the genetics of lactic acid production in lactic acid bacteria. Int Dairy J 1995; 5:763–784.
2. Benninga H. A History of Lactic Acid Making, Kluwer Academic Publishers, Dordrecht, Netherlands, 1990, 1–61.
3. www.britannica.com/EBchecked/topic/327346/lactic-acid.
4. Datta R, Tsai SP, Bonsignore P, Moon SH, Frank JR. Technological and economic potential of poly(lactic acid) and lactic acid derivatives. FEMS Microbiol Rev 1995; 16:221–231.
5. Lunt J. Large-scale production, properties and commercial applications of polylactic acid polymers. Polym Degrad Stabil 1998; 59:145–152.
6. Södergård A, Stolt M. Properties of lactic acid based polymers and their correlation with composition. Prog Polym Sci 2002; 27:1123–1163.
7. Varadarajan S, Miller DJ. Catalytic upgrading of fermentation-derived organic acids. Biotechnol Progr 1999; 15:845–854.
8. Åkerberg C, Zacchi G. An economic evaluation of the fermentative production of lactic acid from wheat flour. Bioresour Technol 2000; 75:119–126.
9. Science.jrank.org/pages/3782/lactic-acid-uses-lactic acid.html.
10. www.webmd.com/drugs/2/drug-64136-762/lactic-acid-topical/keratolyticemollients-topical/details.
11. Axelsson LT. Lactic Acid Bacteria: Classification and Physiology. In: Lactic Acid Bacteria, S. Salminen, A. Wright (Eds.), Marcel Dekker, New York, USA, 1993, 1–63.
12. van Niel EWJ, Hahn-Hägerdal B. Nutrients requirements of lactococci in defined growth media. Appl Microbiol Biotechnol 1999; 52:617–627.
13. Amrane A. Evaluation of lactic acid bacteria autohydrolyzate for the supplementation of lactic acid bacteria fermentation. World J Microbiol Biotechnol 2000; 16:207–209.
14. Hofvendahl K, Hahn-Hägerdal B. Factors affecting the fermentative lactic acid production from renewable resources. Enzyme Microb Technol 2000; 26:87–107.
15. Richter K, Berthold C. Biotechnological conversion of sugar and starchy crops into lactic acid. J Agric Eng Res 1998; 71:181–191.
16. Venkatesh KV. Simultaneous saccharification and fermentation of cellulose to lactic acid. Bioresour Technol 1997; 62:91–98.
17. Richter K, Träger A. L(+)-lactic acid from sweet sorghum by submerged and solid-state fermentations. Acta Biotechnol 1994; 14:367–378.
18. Åkerberg C, Hofvendahl K, Zacchi G, Hahn-Hägerdal B. Modeling the influence of pH, temperature, glucose and lactic acid concentrations on the kinetic of lactic acid production by Lactococcus lactis ssp. lactis ATCC 19435 in whole-wheat flour. Appl Microbiol Biotechnol 1998; 49:682–690.
19. Hofvendahl K, Hahn-Hägerdal B. L-lactic acid production from whole wheat flour hydrolysate using strains of Lactobacilli and Lactococci. Enzyme Microb Technol 1997; 20:301–307.
20. Oh H, Wee YJ, Yun JS, Han SH, Jung S, Ryu HW. Lactic acid production from agricultural resources as cheap raw materials. Bioresour Technol 2005; 96:1492–1498.
21. Xiaodong W, Xuan G, Rakshit SK. Direct fermentative production of lactic acid on cassava and other starch substrates. Biotechnol Lett 1997; 19:841–843.
22. Chatterjee M, Chakrabarty SL, Chattopadhyay BD, Mandal RK. Production of lactic acid by direct fermentation of starchy wastes by an amylase-producing Lactobacillus. Biotechnol Lett 1997; 19:873–874.
23. Yun JS, Wee YJ, Kim JN, Ryu HW. Fermentative production of DL-lactic acid from amylase-treated rice and wheat brans hydrolyzate by a novel lactic acid bacterium, Lactobacillus sp. Biotechnol Lett 2004; 26:1613–1616.
24. Linko YY, Javanainen P. Simultaneous liquefaction, saccharification, and lactic acid fermentation on barley starch. Enzyme Microb Technol 1996; 19:118–123.
25. Vishnu C, Seenayya G, Reddy G. Direct fermentation of various pure and crude starchy substrates to L(+)-lactic acid using Lactobacillus amylophilus GV6. World J Microbiol Biotechnol 2002; 18:429–433.
26. Miura S, Arimura T, Itoda N, Dwiarti L, Feng JB, Bin CH *et al*. Production of L-lactic acid from corncob. J Biosci Bioeng 2004; 97:153–157.
27. Ruengruglikit C, Hang YD. L(+)-lactic acid production from corncobs by Rhizopus oryzae NRRL-395, Lebensm. Wiss. Technol Food Sci Technol 2003; 36:573–575.
28. Yáñez R, Alonso JL, Parajó JC. D-lactic acid production from waste cardboard. J Chem Technol Biotechnol 2005; 80:76–84.
29. Park EY, Anh PN, Okuda N. Bioconversion of waste office paper to L(+)-lactic acid by the filamentous fungus Rhizopus oryzae. Bioresour Technol 2004; 93:77–83.
30. Moldes AB, Alonso JL, Parajó JC. Strategies to improve the bioconversion of processed wood into lactic acid by simultaneous saccharification and fermentation. J Chem Technol Biotechnol 2001; 76:279–284.
31. Wee YJ, Yun JS, Park DH, Ryu HW. Biotechnological production of L(+)-lactic acid from wood hydrolyzate by batch fermentation of Enterococcus faecalis. Biotechnol Lett 2004; 26:71–74.
32. Vick Roy TB. Lactic Acid. In: Comprehensive Biotechnology, Vol. 3, M. Moo-Young (Ed.), Pergamon Press, New York, USA, 1985, 761–776.
33. Das PK, Das S, Sahoo D, Dalei J, Rao VM, Nayak S *et al*. Comparative Evaluation of Purification Methods for Production of Polypeptide Antibiotics – “Polymyxin B” and “Cerexin A” from Bacillus Species. Pharma Tutor 2014; 2(8):188-200.