

Biochemical Analysis Of Fermented Milk Products And Characterization Of Symbiotic Bacteria

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ABSTRACT

Symbiotic organisms growing in fermented milk is a rich source of vitamins and other nutritious minerals. Lactobacillus has reported as a major microbial growth in milk. Isolated lactobacilli were able to produce organic acid in skim milk which was determined by titrimetric method. The Lactobacillus spp. also did show good survival abilities in stimulated gastric juice at pH 2.22 and pH 6.6 (Control). Their susceptibility to selected nine antibiotics was determined in terms of minimum inhibition concentration (MIC). The MICs results showed that, Lactobacillus spp. isolated from Yoghurt were sensitive to amoxicillin, moderately sensitive to gentamycin, clindamicin, azithromycin and resistance to amoxicillin, tetracyclin, kanamycin, nalidixic acid, metronidazole, azithromycin and cefradine. In conclusion, most of the results from the present experiments showed that, there were variations in probiotic properties of the isolated Lactobacillus spp. from different regions.

KEYWORDS:

Regional yogurts, *Lactobacillus spp.*, Inhibitory substances, Simulated gastric juice, and MIC.

INTRODUCTION

Milk has been reported to contain all the basic nutrient requirements for the growth of all eukaryotic organisms. Lactobacillus probiotic bacteria exhibit high growth rate in milk. Bile tolerance, and gastric juice resistance are other important characteristics of probiotic lactic acid bacteria used as adjuncts because they enable them to survive, to grow and to perform their beneficial action in the gastrointestinal tract (GIT).[1-3] Although the degree of tolerance required for maximum growth in the GIT is not known, it seems reasonable that the most bile and acid-resistant species should be selected.

MATERIALS AND METHODS:**COLLECTIONS OF SAMPLES:**

Yoghurt samples were collected from chennai and kanchipuram districts of tamilnadu due to their wide acceptance among the consumers of tamilnadu. Immediately after collection the samples were stored aseptically in low temperature (-4°C) refrigerator to protect from contamination and deterioration. [4-12]In primarily investigations the yoghurt sample of chennai region were used as they contained sufficient amount of probiotic properties.

MEDIA:

The bacteria lactobacillus spp. were isolated from yoghurt samples by using modified MRS broth and MRS agar media. Additionally, 0.05% cysteine was added to MRS to improve the specificity of this medium for isolation of lactobacillus. The pH of the media was adjusted to 6.5

LACTOBACILLUS MRS AGAR:

Lactobacillus MRS agar is recommended for cultivation of all lactobacillus species.

DIRECTIONS:

Suspend 67.16 grams in 1000ml distilled water. Heat to boiling to dissolve the medium completely. Sterilize by autoclaving at 15lbs pressure (121°C) for 15 minutes. Mix well and pour into sterile Petri plates.



FIG:Growth of Lactobacillus in MS media.

ISOLATION OF BACTERIA:

Lactobacillus were isolated from yoghurts by using MRS medium. One gram of each sample was dissolved into 100ml of MRS broth at pH 6.5. After dissolving into MRS broth they were shaken homogeneously and were incubated at 37°C for 24hrs in aerobic conditions. The cultures were subjected to five subculture at 37°C under low pH (pH 4.5) and anaerobic conditions in presence of 10% CO₂ to remove unwanted bacteria. After seven subcultures, the bacteria culture was streak onto the MRS agar media at pH 4.8. Finally, [13-19] the single colony of lactobacillus was isolated by observing their colony, morphology and some biochemical tests (Gram staining, catalase, endospore and motility test) and the culture were maintained in MRS broth at pH 5.5

IDENTIFICATION:

The isolated bacteria were identified as lactobacillus spp by observing their morphological characteristics and by means Gram staining, motility test, catalase test, endospore test, milk coagulation activities, 0.4% bacteriostatic phenol tolerance test and 1-10% NaCl tolerance test. MRS broth containing inhibitory substances such as 0.4% phenol and 1-10% NaCl were inoculated with (v/v) 24 h active culture of lactobacillus and incubated (anaerobically) for 24 hours at 37°C in the presence of 10% CO₂.

DETERMINATION OF OPTICAL GROWTH AND pH:

For the determination of optical growth and pH of lactobacillus, 1% (v/v) fresh overnight culture of lactobacillus were inoculated into MRS broth by varying pH ranging from 2.5-8.5. The pH were adjusted with concentrated acetic acid (99%) and 5N Na-OH. The inoculated broths were incubated in aerobic condition 24 h at 37°C in the presence of 10% CO₂. [20-24] After 24 h incubation growth of the bacteria were measured using a spectrophotometer, reading the optical density at 560 nm (OD₅₆₀) against the uninoculated broth.

ASSAY OF NaCl TOLERANCE:

For the determination of NaCl tolerance of lactobacillus 10 test tube containing MRS broth were adjusted with different concentration (1-10% NaCl). After sterilization each test tube was inoculated with 1% (v/v) fresh overnight culture of lactobacillus and incubated at 37°C for 24 h. After 24 h of incubation their growth were determined by observing their turbidity. Maximum growth were incubated as double positive (++), normal growth as single positive sign (+) and no growth were indicated as negative sign (-).

QUANTIFICATION OF ORGANIC ACID AND DETERMINATION OF pH VALUE:

One percent (v/v) 24 h active culture of lactobacillus was used to inoculate 10% sterilized skim milk obtained from Chennai and initial pH (6.68) was determined by a digital electrode pH meter. The inoculated skim milk was incubated at 37°C for 72 h and samples were

collected in every 24 h, 48 h, and 72 h and liquids were coagulated milk were separated by filtration. pH of the separated liquid was recorded using a digital electrode pH meter and quantification of organic acid was performed through titration with 0.1N Na-OH.

ASSAY FOR BILE TOLERANCE:

Bile tolerances of isolated lactobacillus from yoghurts were determined using the protocol described by Graciela and Maria. Strains were grown in MRS broth at 37 °C overnight; saturated bile solution was prepared separately by dissolving powdered bile extract (Oxoid). Bile solution was then filter sterilized by 4 micron filter and was added to two of the cultures to achieve a final concentration of 0.3 % and the second culture with 0 % bile served as a control sample.[25-29] The cultures were incubated at 37 °C for 3 hours and then every hour for 3 hour. Viable counts of *Lactobacillus* strains were determined by pour plate counts of all the samples using 10-fold serial dilutions prepared in 0.1% peptone water. Simultaneously bacterial growth was monitored by measuring absorbance with a spectrophotometer (Nova Spec II, Pharmacia) at 600 nm. All the experiments were replicated twice.

ASSAY FOR GASTRIC JUICE RESISTANCE:

Gastric juice resistance of isolated lactobacillus from yoghurts were assayed using the protocol described by Graciela and Maria.

ASSAY FOR SENSITIVITY TO ANTIBIOTIC:

Sensitivity of lactobacillus to different antibiotics was determined by minimum inhibitory concentration (MIC) assay. The assay was done using the method described by Andrews[16]. According to this method, stock solutions 128 µg/ml of nine different selected antibiotics such as amoxicillin, gentamicin, tetracyclines, clindamycin, kanamycin, nalidixic acid, metronidazole, azithromycin and cefradine were prepared. Stock solutions of selected antibiotics were two-fold diluted in the range 0.25 µg/ml to 1024 µg/ml in MRS broths. For preparation of the test inoculums the 16 h active cultures of lactobacillus were adjusted to 0.5 McFarland standards (10^7 to 10^8 cfu/ml of bacteria) by adding sterile distilled water. 50 µl of test inoculums were inoculated into each dilution and incubated was done for 24 h at 37°C in the presence of 10% CO₂ in a CO₂ incubator.

RESULT

Bacteria isolated from different yoghurts were identified as lactobacillus spp. by observing their colony morphology, physiological and as well as some biochemical characteristics. Microscopically they were Gram-positive, rod shaped, non- motile, catalase negative and absence of Endospore. The isolates have the abilities to coagulate milk and were able to tolerate inhibitory substances such as 0.4% bacteriostatic phenol and showed growth in MRS broth containing 1-9% NaCl. Their characteristics have been shown in Table 1.

Table 1: Morphological, physiological and biochemical characteristics of isolated *Lactobacilli*

Biochemical and physiological characteristics	Isolate-1	Isolate-2	Isolate-3	Isolate-4
Colonies morphology	1.0 mm, white, rough, irregular and round	small 0.1-0.5 mm, rough, dull and brownish	white, shiny, smooth and 1.0 mm diameter	Small circular, white-creamy in colors and disc like
Gram stain	+	+	+	+
Motility test	Non motile	Non motile	Non motile	Non motile
Catalase test	-	-	-	-
Endospore test	-	-	-	-
0.4% phenol	+	+	+	+
Milk coagulation	+	+	+	+
NaCl tolerance	+	+	+	+

OPTIMAL GROWTH AND pH:

Maximum growth (OD= 2.054) of isolated lactobacilli from chennai yoghurts was observed at pH 5.0. and maximum growth of lactobacillus isolated from kanchipuram yogurt (OD= 1.93) was observed at pH 6.5. [30-34]The OD reading was the average value of two readings, where control OD was 0.152. The results are shown in the form of graph.

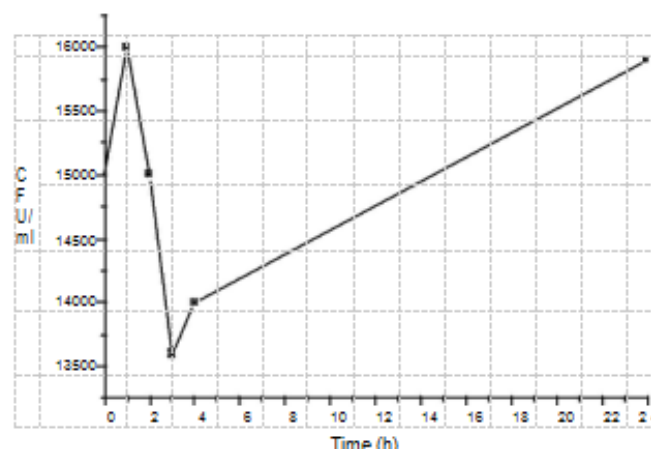


FIG: Survival abilities of lactobacillus species (Isolate-2)**in stimulated Gastric juice at pH 2.2****TOLERANCE OF NaCl:**

The isolated lactobacilli from yoghurts were able to tolerate 1-9% NaCl. The results are shown in Table 2.

Table 2: Tolerance to NaCl of isolated *Lactobacilli*

Concentration of NaCl (%)	<i>Lactobacilli</i> isolated from yoghurt of Kanchipuram region	<i>Lactobacilli</i> isolated from yoghurt of Chennai Region
1	+	++
2	+	+
3	+	+
4	+	+
5	+	+
6	+	+
7	+	+
8	+	+
9	+	+
10	-	-

QUANTIFICATION OF ORGANIC ACID AND DETERMINATION OF pH VALUE:

The results are shown in Table 3.

Table 3: Organic acids (%) and pH in skim milk produced by isolated *Lactobacillus* spp

Sources of Bacteria	Name of the bacteria	Incubation time (Hour)	Incubation temp. (°C)	Organic acid (%)	pH
Kanchipuram yoghurt	<i>Lactobacillus</i> spp.	24	37	2.290	5.09
		48	37	6.615	3.73
		72	37	6.530	3.65
Chennai yoghurt	<i>Lactobacillus</i> spp.	24	37	2.130	5.13
		48	37	3.920	4.50
		72	37	6.260	3.88

BILE TOLERANCE:

Isolated *Lactobacillus* spp. was able to survive in 0.05, 0.1, 0.15 and 0.3% bile acid. The isolated *Lactobacillus* spp. was also able to multiply in above mentioned concentrations of bile acid. In the Figure 5 the optical density values against incubation time are shown.[35-37]

GASTRIC JUICE RESISTANCE:

Isolated *Lactobacillus* spp. (Isolate - 2) was able to survive in gastric environment at low pH (2.2) but they were not able to multiply. In per ml artificial gastric juice (pH 2.2) the colony forming unit at 0 h, 1 h, 2 h, 3 h, 4 h, and 24h were 1.5×10^4 (CFU/ml), 1.6×10^4 (CFU/ml), 1.4×10^4 (CFU/ml), 1.36×10^4 (CFU/ml) and 1.59×10^4 (CFU/ml) respectively. On the other hand they were able to survive and had partial multiplication abilities at pH 6.60. In per ml gastric juice (pH 6.60) the colony forming unit at 0 h, 1 h, 2 h, 3 h, 4 h, and 24 hr were observed respectively. Results are discussed and as colony forming unit after 0,1,2,3,4 and after 24 hr incubation.

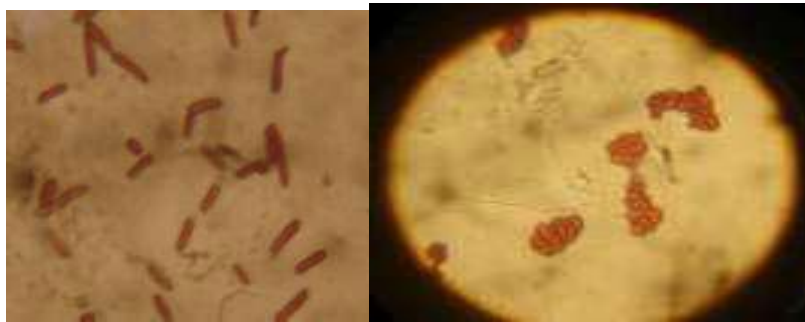


FIG: Isolated rod

shaped *Lactobacillus* species. FIG: Non spore forming *Lactobacillus* sp

SENSITIVITY TO ANTIBIOTIC:

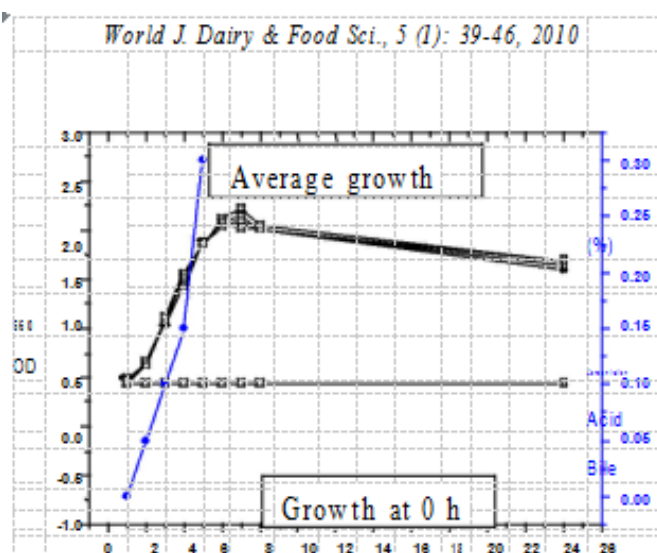
Sensitivity to *Lactobacillus* spp and to different antibiotics was determined in term of minimum inhibitory concentration (MIC).

DISCUSSION:

The isolated *Lactobacillus* were identified as Isolate-1 (1.0mm, white, rough, irregular, round and white colony), Isolate-2 (2.0-2.5mm, white, skinny, smooth and disc like colony).

pH is an important factor which can dramatically effect bacterial growth. In our environmental design we have observed our growth of *Lactobacillus* spp in various pH value ranges from 2.5 to 8.5. The reason for changing this pH value determine weather LAB species can grow in acidic and alkaline conditions and also to predict the pH value for good growth. From the experimental results it was found that the isolated *Lactobacillus* spp from yoghurts is able to survive in extreme pH acidic (pH 2.5 to 3.5) and basic pH (pH 7.5 to 8.5). Maximum growth OD = 2.054 of isolated *Lactobacilli* from Kanchipuram yoghurt maximum growth (OD=1.93) was observed at pH 6.5. From the results we also found a variation of optimum growth pH.[38-41]

FIG: Bile
(Isolate-2)
isolated
yoghurt.



acid tolerance of
Lactobacillus spp

from
Kanchipuram

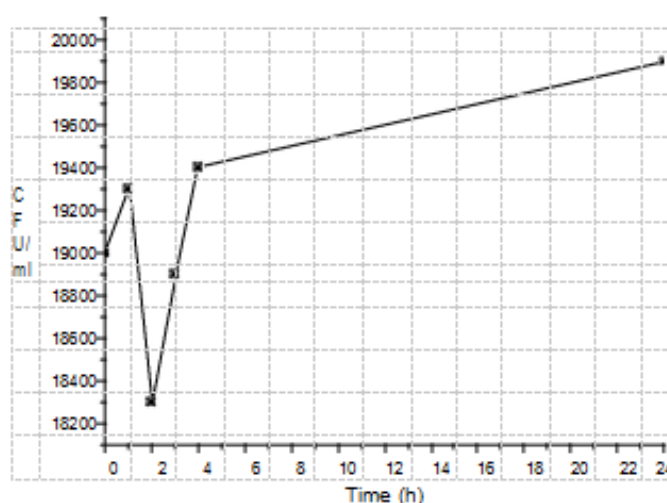


Fig: Survival and partial multiplication abilities of isolated *Lactobacillus* spp. (Isolate-2) in simulated gastric juice at pH 6.6.

NaCl is an inhibitory substance which may inhibit in the growth of certain types of bacteria. The current showed that lactobacillus species isolated from yoghurts were able to tolerate 1-9% of NaCl and good growth was observed at 1% NaCl. Our experimental results have the similarity findings of Elezete and Carlos, incase of lactobacilli isolated from gastrointestinal tract of swine that were tolerable to 4-8% N bacteria (LAB) from fermented products may act as a reservoir of antimicrobial-resistance genes. From the MIC values of 9 tested antibiotics it was found that, *Lactobacillus* spp. Isolated from Kanchipuram yoghurt is sensitive to amoxicillin, moderately sensitive to gentamycin, clindamycin, azithromicin and resistant to kanamycin, nalidixic acid, metronidazol, cefradine and tetracyclin. On the other hand, *Lactobacillus* spp. Isolated from yoghurts of Chennai region of tamilnadu were sensitive to gentamicin, clindamycin, and resistant to amoxicillin, tetracyclin, kanamycin, nalidixic acid, metronidazol, azithromicin and cefradine. This investigation revealed that , some antibiotics such as amoxicillin, gentamycin, clindamycin and azithromicin intake can drastically drop the *Lactobacillus* spp. From intestinal microflora, on the other hand five antibiotics viz. tetracyclin, kanamycin, nalidixin acid, metronidazol and cefradine will not influence the growth of lactobacilli population. From our experiments we did find that *Lactobacillus* spp.isolated from yoghurt of Kanchipuram region have shown broad range of resistances to most of the antibiotics including amoxillin. This was possibly due to wide use of antibiotics in veterinary medicine and agriculture which could be contributing to the dissemination of resistances into yoghurt.[42-45]

CONCLUSION:

In conclusion, the experimental results showed that isolated *Lactobacillus* spp. are able to tolerate inhibitory substances such as 0.005-0.3 bile acid, 0.4% phenol, 1-8% NaCl also able to survive in stimulated gastric (pH 2.2) condition, as well as in alkaline (pH 8.5) condition. The isolated lactobacilli were able to produce organic acid in milk. From the MIC results, we have found that the *Lactobacillus* spp. are

Sensitive to amoxicillin, gentamycin, clindamycin, azithromycin resistant to kanamycin, nalidixic acid, metronidazol, cefradine and tetracyclin which are most frequently used antibiotics in Tamilnadu regarding treatments of human and animals.

REFERENCES

1. Nimal, R.J.G.R., Hussain, J.H., Effect of deep cryogenic treatment on EN24 steel, International Journal of Pure and Applied Mathematics, V-116, I-17 Special Issue, PP-113-116, 2017
2. Parameswari, D., Khanaa, V., Deploying lamport clocks and linked lists, International Journal of Pharmacy and Technology, V-8, I-3, PP-17039-17044, 2016
3. Parameswari, D., Khanaa, V., Case for massive multiplayer online role-playing games, International Journal of Pharmacy and Technology, V-8, I-3, PP-17404-17409, 2016
4. Parameswari, D., Khanaa, V., Deconstructing model checking with hueddot, International Journal of Pharmacy and Technology, V-8, I-3, PP-17370-17375, 2016
5. Parameswari, D., Khanaa, V., The effect of self-learning epistemologies on theory, International Journal of Pharmacy and Technology, V-8, I-3, PP-17314-17320, 2016
6. Pavithra, J., Peter, M., GowthamAashirwad, K., A study on business process in IT and systems through extranet, International Journal of Pure and Applied Mathematics, V-116, I-19 Special Issue, PP-571-576, 2017
7. Pavithra, J., Ramamoorthy, R., Satyapira Das, S., A report on evaluating the effectiveness of working capital management in googolsoft technologies, Chennai, International Journal of Pure and Applied Mathematics, V-116, I-14 Special Issue, PP-129-132, 2017
8. Pavithra, J., Thooyamani, K.P., A cram on consumer behaviour on Mahindra two wheelers in Chennai, International Journal of Pure and Applied Mathematics, V-116, I-18 Special Issue, PP-55-57, 2017
9. Pavithra, J., Thooyamani, K.P., Dkhar, K., A study on the air freight customer satisfaction, International Journal of Pure and Applied Mathematics, V-116, I-14 Special Issue, PP-179-184, 2017
10. Pavithra, J., Thooyamani, K.P., Dkhar, K., A study on the working capital management of TVS credit services limited, International Journal of Pure and Applied Mathematics, V-116, I-14 Special Issue, PP-185-187, 2017
11. Pavithra, J., Thooyamani, K.P., Dkhar, K., A study on the analysis of financial performance with reference to Jeppiaar Cements Pvt Ltd, International Journal of Pure and Applied Mathematics, V-116, I-14 Special Issue, PP-189-194, 2017

12. Peter, M., Dayakar, P., Gupta, C., A study on employee motivation at Banalari World Cars Pvt Ltd Shillong, International Journal of Pure and Applied Mathematics, V-116, I-18 Special Issue, PP-291-294, 2017
13. Peter, M., Kausalya, R., A study on capital budgeting with reference to signware technologies, International Journal of Pure and Applied Mathematics, V-116, I-18 Special Issue, PP-71-74, 2017
14. Peter, M., Kausalya, R., Akash, R., A study on career development with reference to premheerasurgicals, International Journal of Pure and Applied Mathematics, V-116, I-14 Special Issue, PP-415-420, 2017
15. Peter, M., Kausalya, R., Mohanta, S., A study on awareness about the cost reduction and elimination of waste among employees in life line multispeciality hospital, International Journal of Pure and Applied Mathematics, V-116, I-14 Special Issue, PP-287-293, 2017
16. Peter, M., Srinivasan, V., Vignesh, A., A study on working capital management at deccan Finance Pvt Limited Chennai, International Journal of Pure and Applied Mathematics, V-116, I-14 Special Issue, PP-255-260, 2017
17. Peter, M., Thooyamani, K.P., Srinivasan, V., A study on performance of the commodity market based on technicalanalysis, International Journal of Pure and Applied Mathematics, V-116, I-18 Special Issue, PP-99-103, 2017
18. Philomina, S., Karthik, B., Wi-Fi energy meter implementation using embedded linux in ARM 9, Middle - East Journal of Scientific Research, V-20, I-12, PP-2434-2438, 2014
19. Philomina, S., Subbulakshmi, K., Efficient wireless message transfer system, International Journal of Pure and Applied Mathematics, V-116, I-20 Special Issue, PP-289-293, 2017
20. Philomina, S., Subbulakshmi, K., Ignition system for vechiles on the basis of GSM, International Journal of Pure and Applied Mathematics, V-116, I-20 Special Issue, PP-283-286, 2017
21. Philomina, S., Subbulakshmi, K., Avoidance of fire accident by wireless sensor network, International Journal of Pure and Applied Mathematics, V-116, I-20 Special Issue, PP-295-299, 2017
22. Pothumani, S., Anuradha, C., Monitoring android mobiles in an industry, International Journal of Pure and Applied Mathematics, V-116, I-20 Special Issue, PP-537-540, 2017
23. Pothumani, S., Anuradha, C., Decoy method on various environments - A survey, International Journal of Pure and Applied Mathematics, V-116, I-10 Special Issue, PP-197-199, 2017
24. Pothumani, S., Anuradha, C., Priya, N., Study on apple iCloud, International Journal of Pure and Applied Mathematics, V-116, I-8 Special Issue, PP-389-391, 2017
25. Pothumani, S., Hameed Hussain, J., A novel economic framework for cloud and grid computing, International Journal of Pure and Applied Mathematics, V-116, I-13 Special Issue, PP-5-8, 2017
26. Pothumani, S., Hameed Hussain, J., A novel method to manage network requirements, International Journal of Pure and Applied Mathematics, V-116, I-13 Special Issue, PP-9-15, 2017

27. Pradeep, R., Vikram, C.J., Naveenchandra, P., Experimental evaluation and finite element analysis of composite leaf spring for automotive vehicle, Middle - East Journal of Scientific Research, V-12, I-12, PP-1750-1753, 2012
28. Pradeep, R., Vikram, C.J., Naveenchandran, P., Experimental evaluation and finite element analysis of composite leaf spring for automotive vehicle, Middle - East Journal of Scientific Research, V-17, I-12, PP-1760-1763, 2013
29. Prakash, S., Jayalakshmi, V., Power quality improvement using matrix converter, International Journal of Pure and Applied Mathematics, V-116, I-19 Special Issue, PP-95-98, 2017
30. Prakash, S., Jayalakshmi, V., Power quality analysis & power system study in high voltage systems, International Journal of Pure and Applied Mathematics, V-116, I-19 Special Issue, PP-47-52, 2017
31. Prakash, S., Sherine, S., Control of BLDC motor powered electric vehicle using indirect vector control and sliding mode observer, International Journal of Pure and Applied Mathematics, V-116, I-19 Special Issue, PP-295-299, 2017
32. Prakesh, S., Sherine, S., Forecasting methodologies of solar resource and PV power for smart grid energy management, International Journal of Pure and Applied Mathematics, V-116, I-18 Special Issue, PP-313-317, 2017
33. Prasanna, D., Arulselvi, S., Decoupling smalltalk from rpcs in access points, International Journal of Pure and Applied Mathematics, V-116, I-16 Special Issue, PP-1-4, 2017
34. Prasanna, D., Arulselvi, S., Exploring gigabit switches and journaling file systems, International Journal of Pure and Applied Mathematics, V-116, I-16 Special Issue, PP-13-17, 2017
35. Prasanna, D., Arulselvi, S., Collaborative configurations for wireless sensor networks systems, International Journal of Pure and Applied Mathematics, V-116, I-15 Special Issue, PP-577-581, 2017
36. Priya, N., Anuradha, C., Kavitha, R., Li-Fi science transmission of knowledge by way of light, International Journal of Pure and Applied Mathematics, V-116, I-9 Special Issue, PP-285-290, 2017
37. Priya, N., Pothumani, S., Kavitha, R., Merging of e-commerce and e-market-a novel approach, International Journal of Pure and Applied Mathematics, V-116, I-9 Special Issue, PP-313-316, 2017
38. Raj, R.M., Karthik, B., Effective demining based on statistical modeling for detecting thermal infrared, International Journal of Pure and Applied Mathematics, V-116, I-20 Special Issue, PP-273-276, 2017
39. Raj, R.M., Karthik, B., Energy sag mitigation for chopper, International Journal of Pure and Applied Mathematics, V-116, I-20 Special Issue, PP-267-270, 2017
40. Raj, R.M., Karthik, B., Efficient survey in CDMA system on the basis of error revealing, International Journal of Pure and Applied Mathematics, V-116, I-20 Special Issue, PP-279-281, 2017
41. Rajasulochana, P., Krishnamoorthy, P., Ramesh Babu, P., Datta, R., Innovative business modeling towards sustainable E-Health applications, International Journal of Pharmacy and Technology, V-4, I-4, PP-4898-4904, 2012

42. Rama, A., Nalini, C., Shanthi, E., An iris based authentication system by eye localization, International Journal of Pharmacy and Technology, V-8, I-4, PP-23973-23980, 2016
43. Rama, A., Nalini, C., Shanthi, E., Effective collaborative target tracking in wireless sensor networks, International Journal of Pharmacy and Technology, V-8, I-4, PP-23981-23986, 2016
44. Ramamoorthy, R., Kanagasabai, V., Irshad Khan, S., Budget and budgetary control, International Journal of Pure and Applied Mathematics, V-116, I-20 Special Issue, PP-189-191, 2017
45. Ramamoorthy, R., Kanagasabai, V., Jivandan, S., A study on training and development process at Vantec Logistics India Pvt Ltd, International Journal of Pure and Applied Mathematics, V-116, I-14 Special Issue, PP-201-207, 2017

