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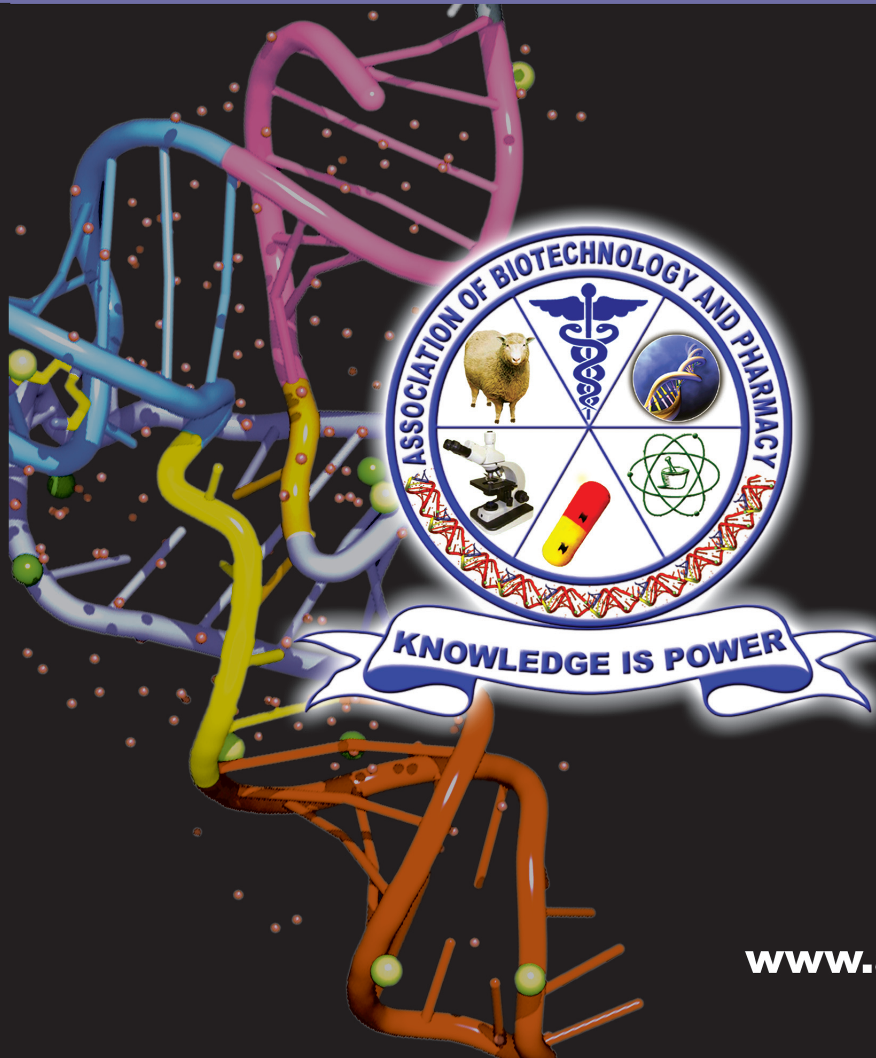

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Information to Authors

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Mahavadi, S., Rao, R.S.S.K. and Murthy, K.S. (2007). Cross-regulation of VAPC2 receptor internalization by m2 receptors via c-Src-mediated phosphorylation of GRK2. Regulatory Peptides, 139: 109-114.

Lehninger, A.L., Nelson, D.L. and Cox, M.M. (2004). Lehninger Principles of Biochemistry, (4th edition), W.H. Freeman & Co., New York, USA, pp. 73-111.

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Multiple Parameter Optimization for Maximization of Exopolysaccharide Production from *Lactobacillus paraplantarum* KM1 by Response Surface Methodology

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Abstract

Statistical experimental design was applied to optimize the medium for exopolysaccharides (EPSs) production from *Lactobacillus paraplantarum* KM1. A design comprising of incubation time, temperature, pH, carbon concentration (lactose) and nitrogen concentration (ammonium sulfate). A quadratic model was found to fit the EPS production. The optimum values of tested variables for EPS production were 25% w/v lactose, 35æ%C, pH 6.5 and 32 h for cultivation time. A great variation in EPS production was observed that ranged from 0 to 34.6 mg/ml. This culture condition enhanced EPS production. An overall increase of 40% was observed by optimizing the conditions through response surface methodology (RSM) in *L. paraplantarum* KM1. There was no significant difference between the predicted value and actual values of response were found and findings reflect the adequacy of RSM for optimization of EPS production by *Lactobacillus paraplantarum* KM1. The adequacy of this model for further analysis was possible since R^2 was greater than 0.70.

Keywords: Exopolysaccharides, *Lactobacillus paraplantarum*, RSM.

Introduction

Exopolysaccharides (EPSs) are long chains of monosaccharide's residues consisting of sugars and sugars derivatives. The various sources of EPSs include plants, fungi, bacteria and algae (1). Among all the EPSs producers, lactic acid bacteria (LAB) have gained special attention. LAB is gram positive bacteria which are generally regarded as safe (GRAS) microorganisms. The LAB producing EPSs have great health potential due to various advantageous effects like anti-ulcer, antitumor, antioxidants, cholesterol lowering effect and enhancement of immune system (1). The valuable applications of EPSs from LAB in the enhancement and improvement of texture, rheology and mouth feel have made it more popular among the health conscious consumers (2,3).

For optimization of EPSs a collection of statistical and mathematical techniques is used which is termed as Response Surface Methodology (RSM). RSM is used to design optimization experiment for reducing the cost of expensive analysis methods (e.g. finite method) and their associated numerical noise. The shape of the response surface is represented graphically, either in the three-dimensional space or as contour plots. A central composite design (CCD) is used to construct a second order model and also to estimate turning parameters of a second order model (4). The general parameter for optimizing different process conditions is by varying one parameter at study while keeping others constant. But the drawback of One Variable at a Time (OVAT) is that it does not study the interactions of different parameters (5). Therefore, RSM is used to optimize the process conditions of EPS from LAB in this study through CCD. In the present study, different parameters i.e. pH, temperature,

nitrogen concentration, carbon concentration and incubation time for maximum production of EPS by lactic acid bacteria is studied.

Materials and Methods

***Bacterial strains and culture conditions*:** EPS producing LAB strain i.e. *Lactobacillus paraplantarum* KM1 was evaluated from human milk. The culture was maintained by biweekly transfers into sterile litmus milk or skim milk medium at 1% level by inoculated at 37°C for 24h and held at -4°C between transfers. *Lactobacillus paraplantarum* KM1 registered under the accession number KX671558. For the optimization by response surface methodology (RSM), it was grown at 37°C in MRS.

***Determination of EPS production*:** MRS media (20ml) was inoculated (10%) with strain pregrown in MRS medium. After 24 h of incubation, trichloroacetic acid 40% (w/v) was added as 1/3 of volume of cultivation broth centrifuged at 10000 rpm at 4°C for 30min and insoluble material removed. 3 volumes of cold 95% ethanol were added to the supernatant. Precipitated were allowed to sediment at 4°C for 24 h. Centrifugation at 10000 rpm for 30 min at room temperature (precipitates collected). Total EPS (expressed as mg/ml) was estimated in sample by phenol-sulfuric acid method (6).

Optimization of process parameters by using response surface methodology (RSM): The MRS medium was used for further optimization by applying RSM of central composite design (CCD).
***Experimental design*:** The levels of five independent variables, i.e., incubation time (A), temperature (B), pH (C), carbon concentration of lactose (D) and nitrogen concentration of ammonium citrate (E), were optimized by RSM. The ranges of different parameters optimized by RSM were mentioned as low to high in Table-1.

The central composite design (CCD) with five factors at five levels was employed to investigate the first- and higher-order main effects of each factor and interactions amongst them. The five coded levels investigated in the current study were (-2), (-1), (0), (+1) and (+2).The full experiment plan as per the experimental design, the minimum and maximum ranges of the variables with the coded and actual values. The statistical software package "Design Expert®" version 6.0 (Stat Ease, Inc, Minneapolis, USA) was used to generate polynomials and the contour plots. All experiments were carried out in triplicates. For a five factor system, the following model equation was generated;

$$Y = \beta 0 + \beta 1 A + \beta 2 B + \beta 3 C + \beta 4 D + \beta 5 E + \beta 11 A2 + \beta 22 B2 + \beta 33 C2 + \beta 44 D2 + \beta 55 E2 + \beta 12 AB + \beta 13 AC + \beta 14 AD + \beta 1\,5 AE + \beta 23 BC + \beta 24 BD + \beta 25 BE + \beta 34 CD + \beta 35 CE + \beta 45 DE$$

Where Y was response variable, β0 was intercept, β1, β2, β3, β4, β5 were linear coefficients, β11, β22, β33, β44 and β55 were quadratic coefficients, β12, β13, β14, β15, β23, β24, β25, β34, β35 and β45 were the second-order interaction coefficients and A,B,C,D,A^2, B^2, C^2, D^2, E2 AB, AC, AD,AE, BC, BD, BE, CD, CE, DE were level of independent variables.

***Analysis of variance (ANOVA)*:** A second-order polynomial equation was established on the basis of analysis of variance. The optimum ratio of the medium components was found using the Design-Expert 6.0 software optimization toolbox. Standard deviation, PRESS, R^2 values were also analyzed.

Model validation

The mathematical model generated during RSM implementation was validated by conducting check point studies. The experimentally obtained data were compared with the predicted one, and the prediction error was calculated.

Results

Response Surface Methodology may be summarized as a collection of experimental strategies, mathematical methods and statistical inference for constructing and exploring an approximate functional relationship between a response variable and a set of design variables (7).

***Regression model of response*:** In this method, prior knowledge of significant culture conditions

and media components was necessary for achieving a more realistic model. Table 2 shows the maximum and minimum levels of variables chosen for trials (Run) in the CCD. For RSM based on Central Composite *Lactobacillus paraplantarum* KM1 design, used for the optimization of independent variables for EPS production, 50 experimental runs with different combinations of five factors were carried out. The independent variables used for the factorial analysis were incubation time (A), temperature (B), pH (C), Carbon concentration (D) and nitrogen concentration (E), while the dependent variable selected was EPS activity (Y). The complete experimental design with coded factors and response values has been given in (Table 2) for *Lactobacillus paraplantarum* KM1, which shows considerable variation in the amount of EPS production depending upon the interaction of various levels of five independent variables in the medium.

By applying multiple regression analysis on the experimental data obtained for *Lactobacillus paraplantarum* KM1. A quadratic model was generated for the response of EPS activity. The significant model terms were evaluated by analysis of variance (ANOVA) in the optimization study (p<0.01) and had been identified as A, B

C, D, E, A^2, B^2, C^2, D^2, E^2, AB, AC, AD, AE, BC, BD, BE, CD, CE, DE, the second-order interaction coefficients and A, B, C, D, A^2, B^2, C^2, D^2, E2 AB, AC, AD, AE, BC, BD, BE, CD, CE, DE were the levels of independent variables.

For five factor system, the following model equation was generated:

Exopolysaccharides production= +34.85 + 0.61 *A+ 0.86 *B + 0.083 *C + 0.79 *D - 0.085 *E -1.80 *A^2 – 5.39 *B^2 – 2.58 *C^2- 1.06 *D^2 + 0.51 *E^2 + 0.40 *A *B - 0.18 *A *C + 0.13 *A *D - 0.076 *A *E+0.45 *B *C+0.15 *B *D -0.12 *B *E + 0.26 *C *D – 6.25 *C *E + 0.15 *D *E

where Y is the EPS activity (mg/ml), A is incubation time, B is temperature, C is pH, D is carbon concentration and E is nitrogen concentration. By optimizing the above parameters by using RSM, maximum EPS activity obtained was 32.81 mg/ml at 35°C temperature and 6.5 pH @ 10% inoculum using 1.50% (w/v) carbon and 2% (w/v) concentration.

According to CCD of RSM, A, D, A^2, B^2, C^2, D^2 were significant model terms. Interactions of other factors were also found equally important for exopolysaccharide production. The response surface curves (Figures 1) were plotted for the variation in exopolysaccharide production, as function of concentration of two variables when all the other factors were kept at their 'O' (central) levels.

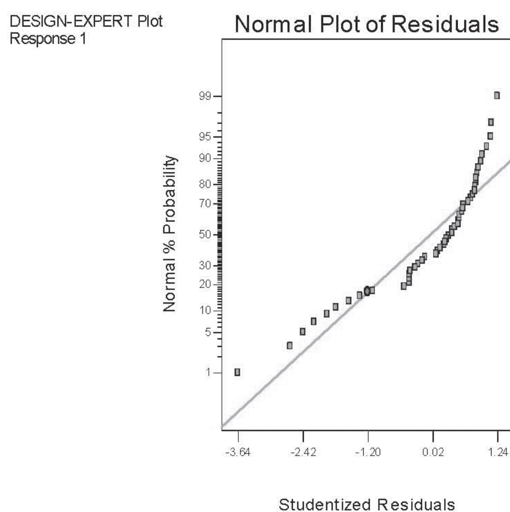

Fig. 1. Parity plot showing distribution of experimental and predicted values of EPS production by Lactobacillus paraplantarum KM1

***Analysis of variance (ANOVA) for response surface of Lactobacillus paraplantarum* KM1:** Analysis of variance provided response (EPS production (mg/ml)) as a function of the initial values of time, temperature, pH, carbon source (lactose), nitrogen source (ammonium citrate), (Table 3). The coefficient of determination (R^2) was calculated as 0.7233 for exopolysaccharide production of *Lactobacillus paraplantarum* KM1 indicating that the statistical model can explain 92.83% of variability in the response. The R^2 value

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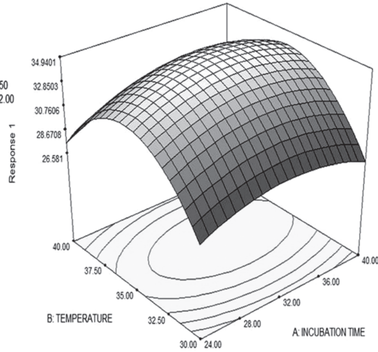

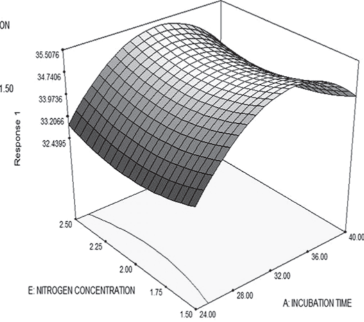

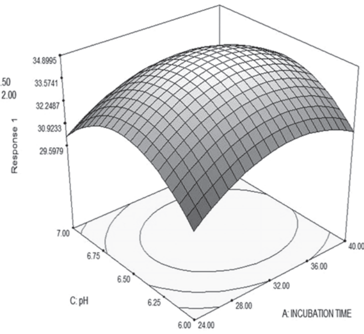

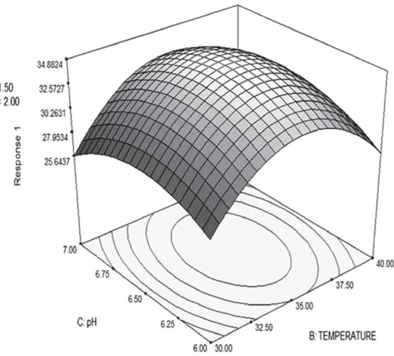

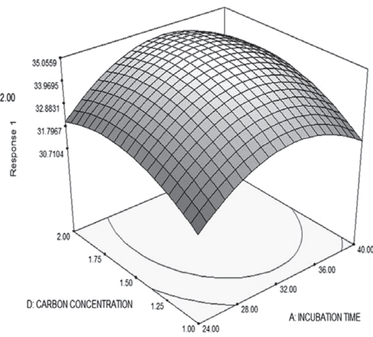

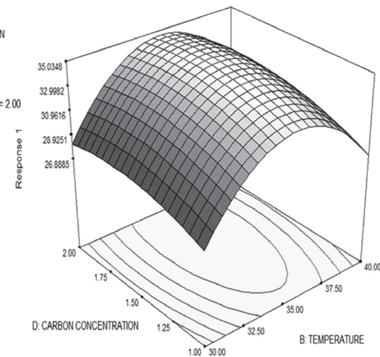

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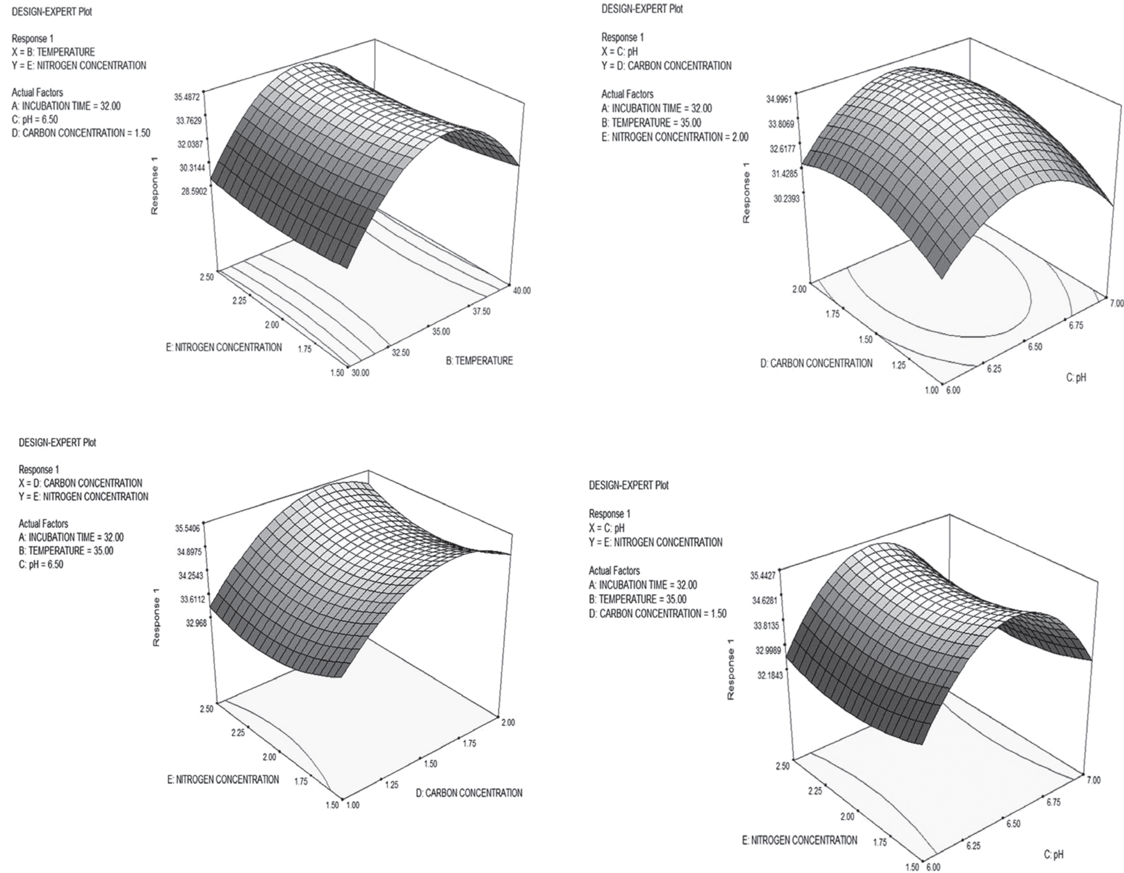

Fig. 2. Three dimensional response surface curves for exopolysaccharide production plotted between incubation period & temperature; incubation period & pH; incubation period & carbon conc.; incubation period & nitrogen conc.; temperature & pH; temperature & carbon conc.; temperature & nitrogen conc.; pH & carbon conc.; pH & nitrogen conc.; carbon conc. & nitrogen conc.

Table 1. Range of values for independent variables used in Central Composite Design (CCD) of RSM

Independent variables	Units	Low	High
Incubation time	Hours (h)	12.97	51.03
Temperature	C	0	46.89
pH		5.31	7.69
Carbon concentration (lactose)	%	0.31	2.69
Nitrogen concentration (ammonium citrate)	%	0.81	3.19

was between 0 and 1 which indicated that the model was significant in predicting the response. The closer the R^2 to 1.0, the stronger the model and better it is predicted (8). The purpose of statistical analysis is to determine the experimental factors, which generate signals that are large in comparison to the noise. The predicted R^2 of 0.7233 was in reasonable agreement with the adjusted R^2 of 0.8944. This indicates that there is a good agreement between the experimental and predicted values for exopolysaccharide production. The adjusted R^2, corrects the R^2 (Cofficient of determination) value for the sample size and for the number of items in the model.

The value of "Lack of Fit F-value" of 6.92 implies the Lack of Fit is significant,idicating that the model equation was adequate for predicting the EPS under any combination of values of the variables. The three dimensional response were generated by origin Software expert 8.0. The result that predicted value and experimental value (mg/ml) was significant which suggetsed that the models gave good fit.

Fig. 2 are three dimensional counter plots with the period incubation period & temperature; incubation period and pH; incubation period and carbon conc.; incubation period and nitrogen conc.; temperature and pH; temperature and carbon conc.; temperature and nitrogen conc.; pH and carbon conc.; pH and nitrogen conc.; carbon conc. and nitrogen conc.; have better effect on the exopolysaccharide production. Interactions of other factors were also found equally important for EPS production of the organisms. These experimental findings are in close agreement with the model predictions.

***Validation of the model*:** The statistically optimal values of variables were obtained when moving along the major and minor axis of the contour, and the response at the centre point yielded maximum EPS production. These observations were also verified from canonical analysis of the response surface. The canonical analysis revealed a minimum region for the model. The stationary point presenting a maximum EPS production for *L. paraplantarum* KM1 had the critical values as 32.81 mg/ml at 37°C temperature and 6.5 pH @ 10% inoculum using 1.50% (w/v) carbon and 2% (w/v) concentration in 32h.

Numerical optimization was carried out to obtain the best combination for maximum EPS production. The desired goals for each variable and response were chosen and different weights were assigned to each goal to adjust the shape of its particular desirability function. The software generated seven optimum conditions of independent variables with the predicted values of responses for *Lactobacillus paraplantarum* KM1.

Response surface methodology (RSM) is a statistical tool used for the modeling and optimization of multiple variables, which determines the optimum process conditions by combining experimental designs with interpolation by first or second order polynomial equations in a sequential testing procedures (9). Central composite experimental design maximizes the amount of information that can be obtained while limiting the number of individual experiments (10). Thus smaller and less time consuming experimental designs could generally suffice for the optimization of many processes in very less time. The major objective of RSM is to determine the optimum region of the factor space in which operating specifications are satisfied.

Discussion

EPS production in modified MRS to study the influence of different conditions on biopolymer and the cell growth. RSM with central composite design was subsequently applied to determine the effect of significant parameters and their interactions and to identify the optimum values. Finally, the optimum conditions were experimentally validated. The study showed that the EPS production by *L. paraplantarum* KM1 could be improved by optimization of medium condition such as carbon cocncentrations, nitrogen concentration, pH, incubation time and temperature. The sugar source is essential for

the growth as well as EPS production by mucoid lactococci as it provides the energy necessary for both processes. Furthermore, a fraction of sugar source is used for the biosynthesis of biomass and EPS precursors. Amino acids are not directly involved in EPS biosynthesis but serve as carbon and nitrogen sources, which are essential for growth. The higher glucose concentration (carbon source) provides the high yield of EPS (11). For *Lactococcus lactis* subsp lactis, a higher EPS production and the better cell growth is observed for growth on glucose compared to fructose, although the transcriptional level of the EPS gene clusters is independent of the carbohydrate source (12). However, there are reports in which higher EPS production was observed under nitrogen-limited conditions than under carbon limited conditions (13,14).

The influence of different substrate limitations on EPS production by closely related organism namely *Lactococccus lactis* subsp. *cremoris.* They observed that reduction of the growth rate from 0.5 to 0.1/ h resulted in an increase of the specific EPS yield, but a further reduction of dilution rate to 0.05h/1 resulted in a decrease of the polymer yield. At all growth rates tested, the efficiency of EPS production EPS production (mg EPS/g glucose consumption) was slightly higher under glucose limitation than in complete chemically defined medium. Biosynthesis of biomass and EPS biosynthesis follow roughly the same metabolic pathways. This results in the same metabolic control for EPS production and for growth. The uncoupling of growth and acid production explains the reduction in efficiency of EPS production in the cultures not glucose limited (15). Production of EPS and synthesis of cell surface polysaccharides both require isoprenoid lipid carriers, sugar nucleotides and energy, and competition between the two processes is possible for any of these factors (16). At higher growth rates, more intermediates per time unit are needed for the biosynthesis of cell surface polysaccharides, and the intermediates are apparently used in favor of the synthesis of these polysaccharides; this may explain the reduction of the EPS yields at higher growth rates.

The EPS quantity reported ranges from 50350 mg/L for *S. thermophilus*, from 60-150 mg/L for *L. delbrueckii* subsp *bulgaricus*, from 50-60 mg/L for *L. casei* (17). However, the amounts of EPS produced by LAB in situ are low and their production is unstable, particularly in milk. Consequently, improvement of the EPS concentration in situ should result in an increased functionality of EPS producing LAB. The effects of temperature, pH and Bacto-casitone concentration for production of EPS by *Lactobacillus delbrueckeii* subsp. *bulgaricus* RR in a semidefined medium using RSM. They observed 300mg/L of EPS production occurred at 38ºC and pH 5.0 respectively with a predicted yield of 295mg/L of EPS (18).

The design expert software recommended solution and was based on maximum desirability. But predicted higher EPS yield was selected for actual performance to find the adequacy of design of experiment. The final trial comprising of all the parameter suggested by the expert (incubation time 32 h, temperature 35°C, pH 6.5, carbon concentration and nitrogen concentration of 1.50% and 2.0% respectively) was carried in triplicate.

Conclusion

An increase in carbon concentration found to improve the EPS recovery and nitrogen concentration also had significant effect. Among the processing variables temperature ($P<0.01$), pH ($P<0.01$) and incubation time ($P<0.01$) have significant effect on medium at quadratic level. Maximum microbial growth was observed, where growth medium was supplemented with 1.5% carbon concentration and 2.0% nitrogen concentration, incubated at 35æ%C and pH was maintained at 6.5. The experimental values (actual values) were compared with that of predicted values (Table 1). There was no significant difference between the predicted values and actual values of responses as the calculated t-values for all the parameters were found to be less than the table values. On the basis of the findings of the present study it is concluded that the selected

Table 2. Optimization of process parameters of exopolysaccharides production by response surface methodology (RSM) from *Lactobacillus paraplantarum* KM1

Run	Incubation time (h)	Temperature (C)	pH	Carbon Conc. (%)	Nitrogen Conc. (%)	Actual value	Predicted value
1	32	35	6.5	1.5	2	34.00	34.80
2	40	30	7	2	1.5	25.50	23.71
3	24	30	6	1	1.5	23.80	22.68
4	24	30	7	1	1.5	23.12	21.86
5	40	40	7	1	2.5	25.84	24.57
6	32	35	6.5	1.5	2	33.83	34.80
7	32	35	5.31	1.5	2	14.45	18.33
8	24	40	7	1	1.5	25.97	23.18
9	40	30	7	2	2.5	25.56	24.69
10	32	35	6.5	0.31	2	19.99	25.98
11	24	30	6	1	2.5	23.80	22.33
12	40	40	7	2	2.5	29.75	27.51
13	32	35	6.5	2.69	2	27.20	30.61
14	40	40	7	2	1.5	29.41	27.36
15	24	30	6	2	1.5	24.48	23.34
16	40	30	6	1	1.5	24.61	22.75
17	24	30	7	1	2.5	23.39	22.46
18	24	40	7	2	2.5	27.50	25.75
19	24	30	6	2	2.5	25.50	23.80
20	24	40	6	1	1.5	24.34	22.62
21	40	30	6	2	2.5	25.16	24.59
22	24	40	7	2	1.5	27.54	25.79
23	32	35	6.5	1.5	2	34.50	34.80
24	40	30	6	2	1.5	25.00	23.96
25	24	40	6	1	2.5	24.34	23.29
26	40	30	7	1	1.5	23.30	22.06
27	51.03	35	6.5	1.5	2	18.85	24.34
28	32	35	**6.5**	1.5	2	34,00	34.80
29	40	40	6	1	2.5	26.35	23.96
30	40	40	6	2	2.5	28.73	26.37
31	12.97	35	6.5	1.5	2	17.85	21.75
32	40	40	6	2	1.5	28.32	26.47
33	24	0	7	2	2.5	24.24	24.02
34	32	35	6.5	1.5	2	33.66	34.80
35	32	35	6.5	1.5	2	34.03	34.80
36	24	30	7	2	1.5	24.48	23.28
37	32	35	6.5	1.5	3.19	33.66	38.14
38	24	40	6	2	1.5	25.56	23.07
39	32	35	6.5	1.5	2	34.22	34.80
40	32	35	7.69	1.5	2	13.60	19.18
41	32	35	6.5	1.5	0.81	33.32	37.89
42	40	40	7	1	1.5	26.69	24.50
43	24	40	7	1	2.5	25.56	24.67
44	32	35	6.5	1.5	2	34.00	34.80
45	40	30	7	1	2.5	26.52	22.85
46	40	40	6	1	1.5	26.28	24.14
47	32	46.89	6.5	1.5	2	0	2.84
48	32	23.11	6.5	1.5	2	0	6.56
49	40	30	6	1	2.5	24.34	22.24
50	24	40	6	2	2.5	25.50	24.40

Table 3. ANOVA for Response Surface of *Lactobacillus paraplantarum* KM1 Analysis of variance table [Partial sum of squares]

Source	Sum of squares	DF	Mean Squares	F value	Prob > F
Model	2089.36	20	104.47	21.75	< 0.0001 significant
A	16.04	1	16.04	3.34	0.0779
B	32.40	1	32.40	6.75	0.0146
C	0.30	1	0.30	0.061	0.8059
D	27.12	1	27.12	5.65	0.0243
E	0.31	1	0.31	0.065	0.8001
A^2	180.41	1	180.41	37.56	<0.0001
B^2	1617.29	1	1617.29	336.74	<0.0001
C^2	369.79	1	369.79	76.99	<0.0001
D^2	62.99	1	62.99	13.12	0.0011
E^2	14.33	1	14.33	2.98	0.0948
E^2	14.33	1	14.33	2.98	0.0948
AB	5.15	1	5.15	1.07	0.3089
AC	1.03	1	11.03	0.21	0.646
AD	0.52	1	0.52	0.11	0.7456
AE	0.19	1	0.19	0.039	0.8453
BC	6.46	1	6.46	1.35	0.2555
BD	0.76	1	O.76	0.16	0.6932
BE	0.45	1	0.45	0.094	0.7614
CD	2.12	1	2.12	0.44	0.5115
CE	1.25	1	1.25	2.60	0.9987
DE	0.68	1	0.68	0.14	0.7097
Residual	139.28	29	4.80		
Lack of Fit	133.16	22	6.056.92	0.0069	significant
Pure Error	6.13	7	0.88		
Corrected Total	2228.65	49			
R^2 =0.7233, Adj R^2 = 0.8944					

combination is the best one in terms of optimization of responses delineated at the beginning of the study. The model can be adequately used for optimizing the parameters for higher EPS production.

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Comparative Microbiological and Physico-Chemical Properties of Commercially available Baker's Yeast and Fruit Juice Isolate (FJ1)

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Abstract

Seven commercially available Baker's yeasts formulations were procured from local market and stored under the conditions mentioned by the manufacturer. Microbiological analysis of the product was done using five different selective media viz. Rose Bengal chloramphenicol agar for moulds, Baired Staph agar for *Staphylococcus aureus*, McKonkey agar for faecal coliforms, *B. cereus* selective agar for *Bacillus cereus* and *Salmonella-Shigella* agar for *Salmonella*. The result revealed that all the commercial products differ w.r.t. to the presence of contaminants. The level of contaminants in some of the samples was above the safe limit. These contaminants may cause a substantial loss economically in the bakery industry and may also cause health problems due to the production of cytotoxic substances. Further these products were used for isolation of different yeast strains present in them using normal microbiological procedures and the isolates were compared w.r.t morphological (texture, colour, surface, elevation and margin), physiological (growth, pseudohyphae formation, budding and the presence of pellicle) and biochemical (sugar fermentation ability in both non-sugar dough as well as in high- sugar dough, invertase activity, carbon utilization as well as assimilation, nitrogen assimilation, carbohydrate and trehalose content) characteristics with a laboratory isolate (FJ1). Freeze tolerance of all these yeast isolate was studied upto 90 days.

Key words: Baker's yeast, microbial contaminants, invertase activity, trehalose, fermentation

Introduction

Commercial yeasts have many practical uses in baking, distilling, wine and brewing industries and are also being used for the production of enzymes, amino acids, vitamins, and substances for therapeutic purposes such as hormones, antibiotics and vaccines. However, the most widely recognized role of yeasts is their leavening and fermenting ability in bread and other fermented products (1). As industrialization increased the manufacture of fermented products, the demand of yeast grew exponentially (2). Baker's yeast (*Saccharomyces cerevisiae*) is the common name for the strains of yeast generally used as a leavening agent in baking. It is still one of the most important fermentation products based on the volume of sales and its use for bread-making which is a staple food for a large section of world's population (3). In addition to producing high-quality yeast for baking, it is an important objective for yeast manufacturers to increase product shelf life. New products with better and sophisticated properties make the search for new and improved industrial strains a promising talk. Despite the rich yeast flora of fruit juices, there is little or no information on the dough leavening ability of these yeast isolates (4). Keeping it in mind an attempt is hereby made to isolate yeast from fruit juices and a potent isolate named as

FJ1 was compared with the commercial yeast strains.

The shelf life of commercially manufactured yeast is often taken for granted because of the intrinsic factors such as lowering the pH during manufacturing lowers the chances of contaminants. However, the microbial spoilage ecology of commercially manufactured yeast subsequent to initial contamination is often influenced by a number of factors. Commercially manufactured yeast is nutrient-rich and is comprised of water (70%), proteins (15%), carbohydrates (10.5%), minerals (3%) and fats (1.5%), and is thus actually highly susceptible to contamination and growth of a variety of spoilage microorganisms (5). To meet customer demands, commercial yeast manufacturers must consistently produce a high-quality product with guaranteed storage life. Although there has been research executed on the bacterial populations associated with commercially manufactured compressed yeast blocks (6), limited work has been carried out on the bacterial populations associated with commercially manufactured cream and dry yeast products (7). In fact, the bacterial populations responsible for the spoilage of these three different commercially manufactured yeast products are largely unknown (8).

Therefore this study was conducted to highlight the major pathogenic micro-organisms present in the different baker's yeast formulations including FJ1 and to compare these yeast preparations w.r.t morphological, physico-chemical and cultural characteristics. Furthermore, their sugar fermentation ability on both non-sugar dough and high- sugar dough, growth on maltose and sucrose respectively, invertase activity, carbohydrate and trehalose content were also compared.

Materials and Methods

Isolation and maintenance of yeast

The yeast strains used in this study were isolated from seven different commercial baker's yeast formulations and a laboratory isolate FJ1 was obtained from fermenting orange juice by standard microbiological procedures and were maintained by periodic sub-culturing on Glucose Yeast Extract (GYE) agar.

Microbiological analysis of Commercial Baker's yeast : The comparative qualitative microbiological analysis was done for the presence of Molds, *Bacillus, Staphylococcus*, Faecal coliforms and *Salmonella* in all the yeast preparations by the methods given by (3). Different selective media were used for checking the presence and absence of various pathogenic micro-organisms.

Comparative cultural characteristics of different yeasts : Growth in liquid media was examined to know the cellular morphology of different yeast isolates using Malt extract (ME) broth by the method (9). Similarly, Malt extract agar was used for the observation of morphological/ cultural properties in solid media. Cultural characteristics include colony texture, color, surface, elevation, and margin.

Physiological and biochemical characteristics of different yeasts

Fermentation and assimilation of carbon compounds : The fermentation basal medium of (10) was used and fermentation of different carbon compounds (galactose, xylose, fructose, arabinose, sucrose, maltose, mannitol, dextrose, cellobiose, starch and lactose) by different commercial yeasts and the FJ1 isolate was studied. Similarly, carbon assimilation was checked using the basal medium of (9) supplemented with different carbon sources (starch, citric acid, sucrose, arabinose, melibiose, rhamnose, lactose, glucose, raffinose, maltose and mannitol). Comparative growth was observed as a measure of Optical Density at 540 nm after 24 hrs of incubation.

Assimilation of N compounds

The assimilation of $NaNO_2$ and KNO_3 was studied by auxanographic method of (9).

Pseudohyphae formation

Ability to form pseudohyphae was checked by Dalmau plate method (10) using rice agar. Results were noted microscopically wherein the

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coverslip was examined under a microscope for the presence/absence of pseudohyphae.

Sugar fermentation ability in non-dough and high-sugar dough : The ability of all the strains to ferment maltose and sucrose as non- sugar and high-sugar dough were tested with Bromo Cresol Purple or BCP method, whereby, growth was reported as the intensity of yellow color developed against the color of the control substrate (purple) and were expressed as plus/ minus (yellow/purple).

Total carbohydrate and trehalose determination : Total carbohydrate and trehalose contents of yeast were determined by the Anthrone reagent method (11). Standard curve for trehalose and carbohydrate estimation was made by using trehalose and glucose as standard solutions upto 100 µg/ ml.

Invertase activity : Invertase activity was measured spectrophotometrically at 525 nm by the methods of (12). Results obtained in terms of Optical Density were defined in terms of glucose released, where one unit of invertase is defined as µg of glucose released at 30°C per minute per mg of yeast (dry basis) under the experimental conditions.

Freeze tolerance : Freeze tolerance ability of different yeast strains was compared by the method of (13) wherein methylene blue solution (0.01% in distilled water) was used to identify the non-viable cells in the haemocytometer chamber.

Results and Discussion

Comparison of the microbiological quality of commercial yeast strains and laboratory isolate FJ1 is presented in Table 1. Various contaminating micro-organisms including *Bacillus cereus, Staphylococcus and Molds* were prevalent in most of the commercial preparations. *Salmonella* and *E.coli* was not found in any of the commercial yeasts preparations. Faecal coliforms were present only in Prestige and Kothari preparations. The presence of *Staphylo coccus* in almost all the commercial yeasts and in FJ1 possibly signifies the aerial mode of contamination. Earlier also, a study conducted by (1) aimed at verifying the quality of different commercial yeasts used in breadmaking showed that the liquid baker's yeast was characterized by the lowest microbial contamination and by the highest leavening activity. Thus dry and compressed forms were contaminated by different microorganisms, and the extent of contamination depended on the type of baker's yeast formulation. They reported that, one of the major contaminating bacteria found prevalent in commercial yeast preparations was *Bacillus* which is the main cause of ropiness in the bread. In the present study, *Staphylococcus* was found to be the most prevalent bacterium followed by *Bacillus*.

O'Brien *et al* determined the effects of various storage temperatures on the shelf life and bacterial populations associated with commercially manufactured cream, compressed and dry yeast. Results showed that Cream and compressed yeast samples became bacteriologically and visually spoiled over time when stored at elevated temperatures (10, 25 and 37°C). This also revealed the populations of Enterococcaceae including *Enterococci,* predominant in the finished dry yeast product, while *Lactobacillus* sp. is the dominant bacterial population associated with cream and compressed yeast products.

Various morphological/cultural properties presented in Table 2 showed marked difference in the shape, color, surface, elevation and margin whereas no such difference was present in the texture of the yeast isolates. The shape varied from oval to coccus. Color varied from cream to white. Platinum yeast showed striated surface, rest was smooth. Elevation showed marked difference wherein Allinson, Kothari and Falora showed raised elevation, FJ1 showed pulvinate and Kipps, Red star and Platinum showed convex elevation. Margin varied from entire to smooth. Among Physiological properties Kothari, Kipps and FJ1 isolate showed a significant presence of pseudo hyphae (Table 2). Pellicle formation was not seen in any of the yeast preparation instead bottom sediments were seen. Budding was

Table 1. Comparative microbiological analysis of various commercial yeasts and lab isolate

SAMPLE	*B. Cereus*	*Staphylococcus*	*Moulds*	*Salmonella*	*E.coli*	*Faecal Coliforms*
Prestige	+	+	+	-	-	+
Kipps	+	+	+	-	-	-
Kothari	+	+	+	-	-	+
Platinum	-	+	+	-	-	-
Red Star	-	+	+	-	-	-
Falora	+	+	+	-	-	-
Allinson	-	-	+	-	-	-
FJ1	-	+	-	-	-	-

Table 2. Morphological and physiological properties of different commercial yeasts and lab isolate

Sample	Morphological/cultural properties					Physiological Properties			
	Shape	Texture	Color	Surface	Elevation	Margin	Pellicle	Budding	Pseudo-hyphae
Prestige	Oval	Butyrous	White	Smooth	Convex	Entire	-	+	-
Kipps	Oval	Butyrous	White	Smooth	Convex	Entire	-	+	+
Kothari	Oval	Butyrous	White	Smooth	Raised	Entire	-	-	+
Platinum	Coccus	Butyrous	White	Striated	Convex	Smooth	-	-	-
Red Star	Coccus	Butyrous	Cream	Smooth	Convex	Smooth	-	+	-
Falora	Coccus	Butyrous	White	Smooth	Raised	Smooth	-	-	-
Allinson	Oval	Butyrous	Cream	Smooth	Raised	Smooth	-	-	-
FJ1	Oval	Butyrous	White	Smooth	Pulvinate	Entire	-	+	+

Table 3. Comparative assimilation of inorganic nitrogen source

Sample	KNO_3 (Nitrate)	$NaNO_2$ (Nitrite)
Prestige	-	-
Kipps	-	-
Kothari	-	-
Platinum	-	-
Red Star	-	-
Falora	+	+
Allinson	+	-
FJ1	+	-

observed in Prestige, Kipps, Red Star and FJ1 isolate.

Assimilation of inorganic source of nitrogen was checked in nitrate and nitrite as shown (Table 3). The bleak ability of assimilation was shown by the yeast preparations. FJ1, Falora and Allinson were found to assimilate nitrate and Falora alone was found to assimilate Nitrite. Ability to ferment dough was seen in both the non-sugar and high-sugar dough. All the yeasts were able to ferment Maltose (non-sugar). The data indicated that only Kothari, Kipps and Platinum were able to ferment Sucrose in the high-sugar dough.

Table 4. Dough fermentation ability in non-sugar and high-sugar dough

Sample	Low Sugar (Maltose)	High Sugar (Sucrose)
Prestige	+	-
Kipps	+	+
Kothari	+	++
Platinum	+	+
Red Star	+	-
Falora	+	-
Allinson	+	-
FJ1	+	-

Table 5. Comparative ability of commercial yeasts and lab isolate to ferment different carbon sources

SampleSugar	Prestige	Kipps	Kothari	Platinum	Red Star	Falora	Allinson	FJ1
Galactose	++	++	+	++	++	-	+	+
Xylose	++	++	+	++	++	+	+	+
Fructose	++	++	+	++	++	-	+	++
Arabinose	++	++	+	++	+	-	-	++
Sucrose	++	++	+	++	++	+	+	++
Maltose	++	++	+	++	++	-	+	++
Mannitol	++	++	+	++	++	+	+	+
Dextrose	++	++	+	+	++	+	+	+
Cellobiose	++	++	+	+	++	+	+	+
Lactose	++	++	+	++	++	-	+	+

* All values are mean of three replicates
* Incubation temperature- 25±2°C
* Incubation period- 72 hrs.

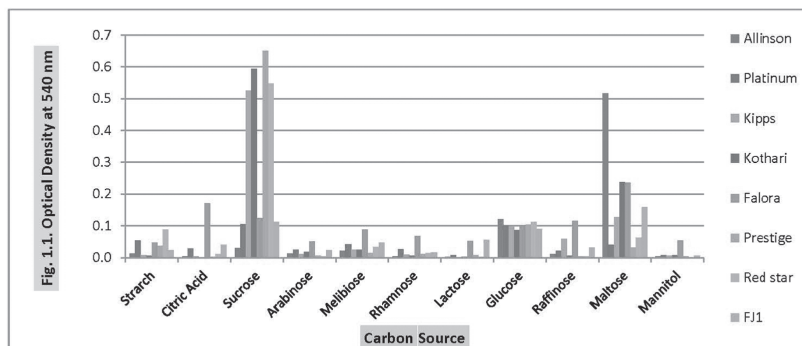

Fig. 1. Carbon assimilation by commercial yeasts and FJ1 isolate

The carbon assimilation was analysed in terms of increase in optical density at 540 nm after 72 hr of incubation at 28°C. Sucrose was the most preferred carbon source with Prestige yeast showing maximum assimilation followed by maltose and glucose. Allinson showed maximum assimilation for maltose and glucose while others did not show any significant difference in their assimilation values. Other sources of carbon showed bleak assimilation.

Relatively higher carbohydrate content was observed in Allinson and FJ1 isolate. It ranged between 28-34% among the different yeast isolate studied. No significant difference in the carbohydrate content of different yeast strains was observed. The carbohydrate content of FJ1 was at par with the other commercial isolates. The results were in agreement with the study done by (14) which suggested a carbohydrate content ranging between 30-32% with commercial baker's yeast strains for high sugar bread dough containing relatively high carbohydrate content in the range 34-39%.

Trehalose has been reported by many investigators to have certain roles in heat and desiccation resistance and cryoresistance in frozen bread dough method (15, 16). Resistance to dehydration of *S. cerevisiae* containing high trehalose content was also increased if a high level of intracellular trehalose accumulated in stationary-phase cells or cells incubated in the absence of nitrogen source (17). Trehalose content (Table 9) was found to be maximum in FJ1 isolates (5.00%), thereby possibly depicting its cryotolerance property followed by Falora (3.38%), Platinum (3.27%), Kipps (2.79%), Allinson (2.73%), Kothari (2.06%), Prestige (1.81%) and Red Star (1.51%). A significant difference was observed in the trehalose content of various commercial yeasts and lab isolate. Among all commercial yeast strains, Falora contained the highest trehalose content (3.381 %).

Since baker's yeast is highly sensitive to high osmotic pressure created by sugar and/or salt in bread dough, yeast with low invertase helps to prevent them adverse effect of high osmotic pressure (18). Thus, lower invertase activity of these new strains was considered beneficial for leavening ability in low- and high sugar bread doughs. There was a marked difference in the invertase activity among the different isolates. It varied from 205.04 unit/mg in Allinson to 30.32 unit/mg in Falora. FJ1 isolate showed relatively higher (165.14 unit/mg) invertase activity than Red Star, Kothari, Kipps, Prestige and Falora.

Table 6. Comparative carbohydrate, trehalose and invertase activity of different commercial yeasts and lab isolate.

Commercial yeasts/ FJ1	Carbohydrate content % (g/100g DW)	Trehalose Content % (g/100g DW)	Invertase Activity (unit/mg)
Prestige	29.66	1.81	75.45
Kipps	31.78	2.79	130.97
Kothari	29.67	2.06	44.13
Platinum	28.76	3.27	186.49
Red Star	30.99	1.51	62.64
Falora	32.22	3.38	30.32
Allinson	33.62	2.73	205.0
FJ1	33.89	5.00	165.14
CD (5%)	2.99	0.29	12.30

Table 7. Freeze tolerant ability of different commercial yeasts and lab isolate.

Commercial yeasts/FJ1	10 days	20 days	30 days	40 days	50 days	60 days	90 days
Allinson	100	100	98.0	97.0	93.0	90.0	72.0
Prestige	100	96.0	94.0	90.0	88.0	83.0	64.0
Kipps	100	100	95.0	90.0	84.5	70.5	58.0
Kothari	100	97.0	92.0	88.5	81.0	76.5	62.5
Platinum	100	97.0	93.0	90.0	82.0	73.5	59.5
Falora	100	98.0	94.0	90.5	83.0	73.0	54.0
Red Star	100	100	97.0	87.5	81.2	72.5	61.0
FJ1	100	100	97.0	93.0	90.0	87.0	71.0

*All values are mean of three replicates **CD (5%)** = 5.05 (after 90 days of incubation)

Freeze tolerance ability of the yeast depicts its ability to survive under refrigeration conditions. The freeze tolerance ability of the isolates was noted for 90 days which showed asignificant difference in Kipps and Falora while no significant difference was observed in all other yeasts. FJ1 isolate in comparison to Allinson yeast showed lesser viability after 90 days.

Conclusion

Yeast is the foremost constituent in the baking industry. Prevalence of contaminating microorganisms was underestimated because of the inherent low pH of the substrate (molasses) being used for its multiplication. But this study concluded the presence of various food-borne pathogens in the commercial yeast preparations. Contaminants like *B. cereus*, *Staphylococcus* and Molds have been found to be prevalent. Faecal coliforms were present only in two commercial brands. Assimilation of inorganic nitrogen in the form of nitrate was observed in FJ1, Falora and Allinson yeast, whereas nitrite was assimilated by Falora yeast. All the isolates were able to ferment lactose (non-sugar dough) whereas only Kothari, kipps and platinum were able to ferment sucrose (high-sugar dough). Sucrose was the most preferred carbon source (with Prestige yeast showing maximum assimilation) followed by maltose and glucose. Allinson yeast showed maximum assimilation of maltose which is the main sugar available for fermentation in flour. Allinson and FJ1 have significantly higher carbohydrate content (33.62 and 33.89%), while for all other 6 brands it was at par. Trehalose content was highest for FJ 1 (5.0 g/100 g DW) followed by Falora, Platinum and Allinson, whereas invertase activity was highest in Allinson.

FJ1 isolate was selected on the basis of its highest trehalose content whereas among the commercial strains Allinson was selected as reference strain on the basis of its preferred carbohydrate assimilation, high carbohydrate content, moderate trehalose content and high freeze tolerance. Both these strains are presently being tested for their dough rising ability and fermentation efficiency for use in bread making in our laboratory.

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Sonic Stimulation can affect Production of Quorum Sensing Regulated Pigment in *Serratia marcescens* and *Pseudomonas aeruginosa*

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Abstract

Effect of nine different mono-frequency sound stimuli on two gram-negative bacteria (*Pseudomonas aeruginosa* and *Serratia marcescens*) was investigated. Frequency of the test sound ranged from 100 Hz – 2,000 Hz. Both the test bacteria responded differently to sonic stimulation. Sound corresponding to 600 HZ caused a notable reduction in quorum sensing (QS) regulated production of the pigment pyoverdine by *P. aeruginosa*. 400 Hz sound affected prodigiosin production by *S. marcescens* the most. 500 Hz sound could enhance prodigiosin production without affecting growth of the producing bacterium, suggesting the effect purely to be QS modulatory. This study has demonstrated the capacity of the sound waves of affecting bacterial growth and quorum sensing regulated metabolite production.

Key words: Sound, Quorum sensing, Sonic stimulation, Prodigiosin, Pyoverdine

Introduction

Sound is one of the most widely distributed environmental factors. One or another kind of sound can be found in almost all corners of the natural world. Effect of a variety of environmental factors such as temperature, light, pH, oxygen concentration, etc. on microorganisms has been studied well. However despite sound being a universally present factor, its effect on the microbial life forms has not received enough attention from the community of microbiologists. Though microorganisms do not possess any auditory cell component, there are reports (1, 2, 3) indicating them to be affected notably by sonic stimulation. The precise mechanisms explaining how microbes sense and respond to external sound stimuli are not yet understood. It is required to conduct the investigations regarding influence of sound on many different bacteria, algae, protozoa, and fungi, to find out whether there is any common pattern underlying microbial response to sound. Ultrasound in general is known to be deleterious to microbial cells (1), and is widely used in cell lysis protocols. However there is no large body of literature to throw light on interaction of sonic range (20 Hz - 20 KHz) of sound with microbes.

Our previous studies (2, 4) indicated *Serratia marcescens* responding differently to sound stimulation as compared to other test organisms. In the present study, we subjected *S. marcescens* and *Pseudomonas aeruginosa* to sonic stimulation at different sound frequencies, and observed the effect on their growth and quorum sensing (QS) regulated pigment production. In our previously published studies, we used multi-frequency sound in form of music, whereas in the present study mono-frequency sound was employed to investigate whether these two bacteria respond to sonic stimulation in a frequency dependent fashion.

Materials and Methods

***Bacterial culture*:** *Serratia marcescens* (MTCC 97) was procured from the Microbial Type Culture Collection (MTCC), Chandigarh. This organism

was grown in nutrient broth (HiMedia, Mumbai) supplemented with 1% v/v glycerol (HiMedia,). Incubation was made at 28º C for 48 h. *Pseudomonas aeruginosa* was procured from Microbiology Department of M.G. Science Institute, Ahmedabad. The organism was grown in *Pseudomonas* broth (2% peptone; 1% potassium sulphate; 0.14% $MgCl_2$; pH 7±0.2). Incubation was made at 35°C for 24 h.

Sound generation: Sound beep (s) of required frequency was generated using NCH® tone generator. The sound file played during the experiment was prepared using WavePad Sound Editor Masters Edition v.5.5 in such a way that there is a time gap of one second between two consecutive beep sounds.

Sound stimulation: Inoculum of the test bacterium was prepared from its activated culture, in sterile normal saline. Optical density of this inoculum was adjusted to 0.08-0.10 at 625 nm (Agilent Technologies Cary 60 UV-vis spectrophotometer). The test tubes (Borosil, 25x100 mm; 38 mL) containing inoculated growth medium (6 mL including 5%v/v inoculum) were put into a glass chamber (Actira, L: 250 x W: 250 x H: 150 mm). A speaker (Minix sound bar, Maxtone Electronics Pvt. Ltd., Thane) was put in this glass chamber at the distance of 15 cm from the inoculated test tubes. Sound delivery from the speaker was provided throughout the period of incubation. This glass chamber was covered with a glass lid, and one layer of loose-fill shock absorber polystyrene, in such a way that the polystyrene layer gets placed below the glass lid. Silicone grease was applied on the periphery of the glass chamber coming in contact with the polystyrene material. This type of packaging was done to minimize any possible leakage of sound from inside of the chamber, and also to avoid any interference from external sound. Similar chamber was used to house the 'control' (i.e. not subjected to sound stimulation) group test tubes. One non-playing speaker was also placed in the glass chamber used for the control tubes at a distance of 15 cm from tubes, where no electricity was supplied and no sound was generated (5). Intensity of sound, measured with a sound level meter (acd machine control Ltd.) at a distance of 15 cm from the speaker, varied with the frequency, in the range of 75-99 dB (Table 1). Sound level in control chamber was found to be below the detection level of the sound level meter. Schematic of the whole experimental set-up is shown in Figure 1.

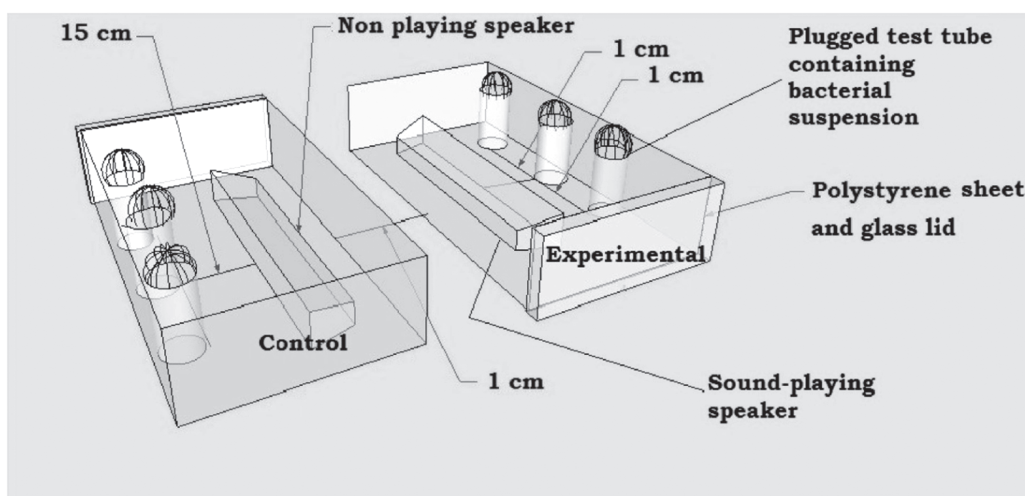

Figure 1. Schematic of the experimental set-up

Table 1: Level of sound intensity for different frequencies

Sr. No	Frequency of the test sound (Hz)	#Intensity as measured by the sound level meter (dB)
1	100	80
2	200	85
3	300	90
4	400	86
5	500	84
6	600	75
7	700	82
8	1000	86
9	2000	99

#Volume regulating knob of the speaker was set at a constant level of 9 for all frequencies

Intermittent mixing of the contents of the test tubes to minimize heterogeneity was achieved by vortexing the tubes at an interval of every 3 h using a vortex mixer. Whenever the tubes were taken out for vortexing, each time positions of tubes of a single chamber were inter-changed, and their direction with respect to speaker was changed by rotating them 180º. This was done to achieve a high probability of almost equal sound exposure to all the tubes, and their content.

***Growth and pigment estimation*:** At the end of incubation, after quantifying the cell density at 764 nm (6), the culture tubes were subjected to extraction of the respective pigment, as described below.

Prodigiosin extraction *(7):* One mL of the culture broth was centrifuged at 10,000 rpm (10,600 g) for 10 min. Centrifugation was carried out at 4°C, as prodigiosin is a temperature-sensitive

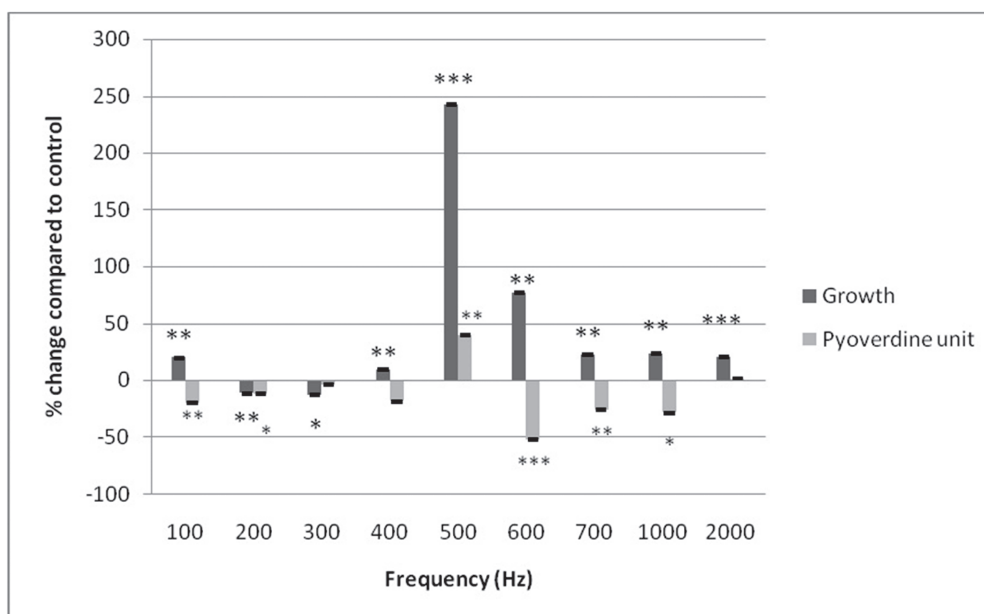

Fig. 2. Effect of different sonic frequencies on growth and QS-regulated pyoverdine production in *P. aeruginosa*

Bacterial growth was measured as OD_{764}; OD of pyoverdine was measured at 405 nm, and pyoverdine unit was calculated as the ratio OD_{405}/OD_{764} (an indication of pyoverdine production per unit of growth); *p<0.05, **p<0.01, ***p<0.001

compound. The resulting supernatant was discarded. Remaining cell pellet was resuspended in 1 mL of acidified methanol (4 mL of HCl into 96 mL of methanol; Merck), followed by incubation in dark at room temperature for 30 min. This was followed by centrifugation at 10,000 rpm for 10 min at 4°C. Prodigiosin was obtained in the resulting supernatant; OD was taken at 535 nm. Prodigiosin unit was calculated as OD_{535}/OD_{764}. This parameter was calculated to nullify the effect of change in cell density on pigment production.

Pyoverdine extraction *(8) :* Two mL of the culture broth was mixed with chloroform (Merck, Mumbai) in 2:1 proportion followed by centrifugation at 12,000 rpm (15,300 g) for 10 min. This resulted in formation of two immiscible layers. OD of the upper water-soluble phase containing yellow-green fluorescent pigment pyoverdine was measured at 405 nm. Pyoverdine unit was calculated as OD_{405}/OD_{764}. This parameter was calculated to nullify the effect of change in cell density on pigment production.

Results and Discussion

Pseudomonas aeruginosa: Effect of nine different sonic frequencies was investigated on growth and pigment (pyoverdine) production of *P. aeruginosa.* All the nine sonic treatments were able to alter the growth of *P. aeruginosa* significantly, whereas pigment production was altered significantly at six different sonic treatments (Figure 2). Sound corresponding to 300 Hz, 400 Hz, and 2000 Hz was able to alter final cell density only, but not the pigment production. As production of the pigment pyoverdine in *P. aeruginosa* is regulated by quorum sensing (QS) (9), all the frequencies capable of altering pyoverdine production may be said to have QS-modulatory effect. The maximum (52.13%) effect on QS-regulated pigment production was observed at 600 Hz.

Serratia marcescens: Out of the nine sonic treatments, seven (except 500 Hz and 600 Hz) were able to alter the growth of *S. marcescens* significantly (Figure 3). Production of the pigment (prodigiosin) was affected by eight different sonic

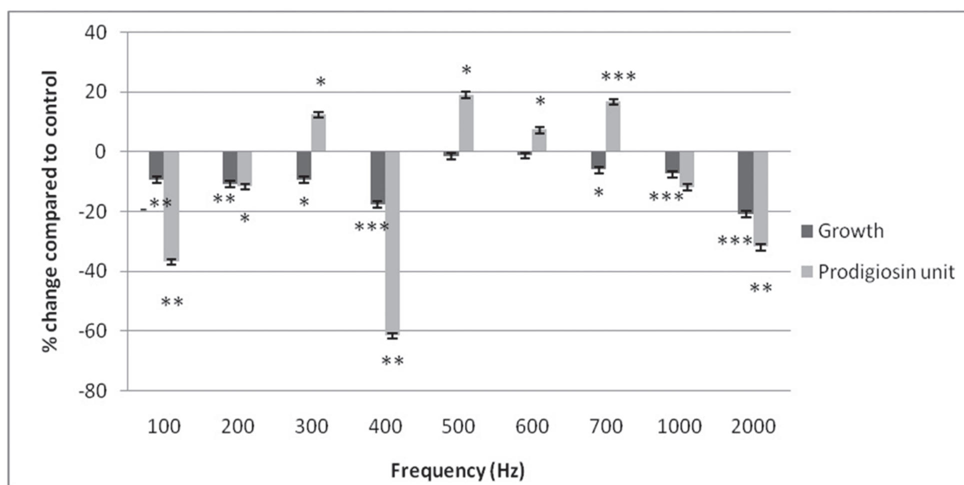

Fig. 3. Effect of different sonic frequencies on growth and QS-regulated prodigiosin production in *S. marcescens*

Bacterial growth was measured as OD_{764} ; OD of prodigiosin was measured at 535 nm, and prodigiosin unit was calculated as the ratio OD_{535}/OD_{764} (an indication of prodigiosin production per unit of growth); *p<0.05, **p<0.01, ***p<0.001

frequencies; only 1000 Hz sound did not exert effect on this parameter. Thus, all the test frequencies were able to alter either one or both the test parameters (e.g. growth and pigment production).

All the growth affecting sonic treatments caused *S. marcescens* to achieve a lower final cell density than control. In our previous studies (10, 2, 3) too, sonic stimulation was found to have an inhibitory effect on *S. marcescens* growth. 400 Hz sound affected prodigiosin production by *S. marcescens* the most; here visible prodigiosin production also started earlier in the control tube than the experimental tube. Production of the pigment prodigiosin in *S. marcescens* is known to be under regulation of QS (11). As evident from Figure 3, except 1000 Hz, all other test frequencies had significant effect on QS-regulated prodigiosin production, and thus they can be said to possess QS modulatory effect.

Sound stimuli of 500 Hz and 600 Hz could enhance prodigiosin production (by 18.93% and 7.19%, respectively) without affecting growth, suggesting the effect purely to be QS modulatory. Identifying the specific sonic frequencies and the differential gene expression induced by them associated with enhanced prodigiosin production can be very useful, as prodigiosin and its derivatives are of potential commercial and therapeutic value. They can find application as food colourant, proapoptotic agents for cancer treatment, potential sunscreen, etc. (12). Prodigiosin has been reported to possess a variety of biological properties such as antimicrobial, antimalarial, anticancer, immunosuppressive, and quorum sensing inhibitory (13) activities. Prodigiosin or its analogues have been considered effective biological control agents against harmful algae, have been considered cell growth regulators, and can be used as a natural dye (14).

Results of this study indicate that same frequency of sound may be responded differently by different species of bacteria. Both the bacteria used in this study are gram-negative bacteria, and hence do have some similarities with respect to their cell envelope structure, and the QS circuit possessed by them. In general gram-negative bacteria have a *luxI* / *luxR* type of AHL (acyl homoserine lactone) based QS system (15). Despite these similarities, there were differences in response given by these two bacteria to same type of sonic stimulation. QS-regulated pigment production was not affected in case of *P. aeruginosa* at 300 Hz, 400 Hz, and 2,000 Hz; whereas all these three sonic frequencies were able to alter QS-regulated production of the pigment prodigiosin in *S. marcescens*. Similarly the 500 Hz and 600 Hz sound, which had only QS-modulatory effect on *S. marcescens*, affected both growth as well as pigment production in *P. aeruginosa.* In part, this difference in bacterial response perhaps may be attributed to the fact that *P. aeruginosa* has multiple QS systems (16), and an alternative pathway may become more active in case of other pathway(s) being interfered. We studied effect of sonic stimulation only on one QS-regulated trait, however a large part of bacterial genome and a multitude of their activities are believed to be regulated by QS (17), and hence many other pathways are likely to be affected simultaneously when a sonic frequency capable of modulating bacterial QS is employed.

It is not illogical to think that traits not directly associated with QS can also get affected by sound waves. Sound waves can be thought of exerting a pan-genome effect on the microbial cells exposed to them. Differential gene expression in sound-stimulated culture of the bacterium *Chromobacterium violaceum* has recently been reported by us (18). Effect of sonic vibration on yeast metabolome was studied by Aggio et al. (19). Intracellular macromolecular synthesis and growth of *Escherichia coli* was shown by Gu et al. (20) to be affected owing to sound exposure. Effect of sonic stimulation on germination of *Bacillus atrophaeus* endospores was studied by Liu et al. (21). They found the sonic stimulation to promote the germination speed of the endospores. These authors postulated that the acoustic energy absorbed by the spores might change membrane permeability and enhance enzyme activities,

resulting in faster germination. They indicated the possibility of dormant endospores undergoing germination owing to a rapid release of unknown chemical mediators for quorum sensing.

One of the possible mechanisms by which sound waves might exert their effect on bacterial cells, can be through oscillation of mechanosensitive channels. All cells, including those of bacteria have the ability to detect and respond to mechanical forces. When the membrane is under pressure, channel proteins can be directly stretched open. External vibration/ pressure can alter the ion flux through mechanosensitive channel proteins. The mechanosensitive channel in yeast is also sensitive to membrane stretch. It is possible that proteins located in the lipid bilayer can respond to the changes (originating from the vibrations created by sound waves) in the mechanical environment provided by the lipid bilayer (22). Vibrations generated owing to travelling of sound waves in the nutrient medium, may lead the cells to undergo repeated expansion and contraction, giving rise to a different sound; which may further be sensed by nearby cells and induce some response in them. These mechanisms can involve the conversion of ATP and membrane potentials to movement. Intracellular conversion of the sound received by bacterial cells into electromagnetic waves is also theoretically conceivable (23).

Conclusion

External sound stimulation can be considered as a type of stress for the microorganisms receiving it. Our present and previous studies and few other researchers have already indicated that microbial growth, metabolism, and their population behavior can get altered owing to sonic stimulation. Study of the sound stimulated microbial cultures with respect to their altered gene expression can be very interesting and useful with respect to bioengineering of strains to overproduce the desired metabolite(s). For example, research into how the biology of pigment production in *S. marcescens* gets affected at the molecular level (i.e. at the level of transcriptome/ proteome) by sound stimulation can pave the way for bioengineering of prodigiosin overproducing strains. Understanding how the sonic stimulation makes bacterial culture achieve a lesser or higher cell density can enrich our knowledge regarding stress response behaviour of bacterial populations. If we can reach a stage of knowing that how a particular microbial species inside their human host will respond to a given sonic stimulation, in long run sonic waves can have therapeutic implications too. May not be in near future, but modulation of the relative abundance of different microbial populations within human microbiome, perhaps can be one of the outcomes of research in the area of microbial interaction with sonic range of sound waves.

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Development of NEUROAchFET circuit for Patients Having Neurological Disorder

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Abstract

Many people across the world suffer from Parkinson's, Alzheimer's and other neurological disease. The patients having neurological disorder cannot transmit signals due to inability of detecting Acetylcholine (a neurotransmitter enzyme). An acetylcholine field effect transistor (AchFET) has been fabricated and used for detecting neuronal signals in this paper. AchFET is actually an enzyme modified field effect transistor (ENFET) which uses the concept of ion sensitive field effect transistor (ISFET).The circuit used here is simple and compatible to biological environment. The AchFET when used in the electronic circuit reproduces action potential same as obtained by experimental data published earlier. Although there are various models developed, but none of them takes into account the synaptic transmission. This model can be helpful for synaptic transmission for patients having problem in acetylcholine detection and the circuit has been named as the NEUROAchFET circuit.
Keywords:NEUROAchFET, Acetylcholine, ENFET,synaptic transmission

Conflicts of Interest: There is no conflicts of interest.

Introduction

Various neuron models were developed since Lapicque model to replicate neuron signals. Roy, Harmon and Lewis, Johnson and Hanna FitzHugh-Nagumo, Fitghugh, developed electronic models which can be used to simulate action potential and its related signals (1-5). The electronic model developed in this paper simulates neuron signals exactly like the experimental signals obtained by Hodgkin-Huxley model (6-19).

The nerve signals are transferred from one neuron to another and then to the brain. The action potential is the signal which carries information to brain and vice versa. This action potential is generated when a disturbance is exerted in the cell. If the external energy is more than the threshold point , action potential occurs. The neuron follows an all or none response. If the external energy is less than the threshold voltage , the action potential cannot be observed. The membrane of the neuron consists of three types of ions: sodium, potassium and leakage ions which mostly consists of chloride ions. Neuron cell possess a resting state where the sodium ions remain outside of the cell. The potassium ions inside the cell and leakage ions mostly chlorine ions moves to and fro in the cell. When disturbance is felt, the sodium ions goes inside the cell and the potassium ions comes outside the cell so as to maintain its charge neutrality. This produces a hump in the signal causing repolarization and then depolarization occurs when the ions goes in the resting state.

Hodgkin and Huxley had described the neuronal signals mathematically (1). The ionic current(I_i) is divided into sodium (I_{Na}), potassium(I_K) and leakage ions(I_l) mostly chloride ions. There is a capacitive current C_M also which occurs due to the charge possessed by the

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neuron. The movement of ions is time dependent. Each ionic current is represented by the product of its conductance and its potential difference. The sodium, potassium and leakage conductance is represented by g_{Na}, g_K and g_l. As mentioned, the ions flow to and fro in the membrane when certain gates are open .When all the gates are open, the current is maximum. The probability of opening of gates is represented by m, n and h. m and h control the flow of sodium ions. n is the probability of opening of gates of potassium. The equations formulated (1) are responsible for the neuron signals generated.

Hodgkin-Huxley model is a reliable model where they have performed voltage clamp experiments to study the biophysical nature of neuron. The circuit designed in this work can simulate action potential, individual current etc similar to the literature available. The device used here is Acetylcholine field effect transistor which can sense acetylcholine(a neurotransmitter) and thus will help to transmit neuro-signals(6)-(7). Detailed fabrication and simulation results are described in the sections below.

NEUROAchFET

A junctionless dual gated AchFET have been fabricated in a glass substrate of 5mm* 2mm*50mm (6)-(8). Indium Tin Oxide (ITO) is deposited on the substrate taking it as bottom gate. A thin layer of SnO is layered to act as an insulator to avoid leakage current. On top of it, PEI (polyethylenimine) doped CNT (carbon nanotube) is deposited which acts as n-type source for drain(D) and source(S). $Hf0_2$ is used as gate insulator which will increase capacity and reduces direct tunneling leakage current. The gate, drain and source(Fig.1) is made with aluminium for contact purpose and the device is covered with polydimethylsilaxane(PDMS). Acetylcholine is immobilized in the sensing membrane on the surface before the device is packed with PDMS. Acetylcholine when reacts with water breaks down into choline and acetic acid releasing hydrogen ions. The schematic for the mechanism of AchFET is shown in Fig.2.These ions then change the potential difference between gate and source, changing the drain current eventually. The drain current is recorded by varying drain source voltage(V_{DS}) and keeping the reference electrode(top gate) and bottom gate constant. The schematic diagram of AchFET is shown in Fig. 1.The characteristic curve of the AchFET for different concentration of Acetylcholine is similar to a MOSFET as shown in Fig.3(6).The AchFET works best in the temperature range of 30-35° C. The maximum response is obtained at 0.6 V at drain voltage of 0.4V.The sensitivity of the device is 1.25 V/dec and for drain current 500μA/mM/mm^2.

NEUROAchFET circuit

The NEUROAchFET circuit developed is shown in Fig. 4.The sodium(g_{Na}) and potassium(g_k) conductance is represented by each AchFET used. It has been observed that the characteristic

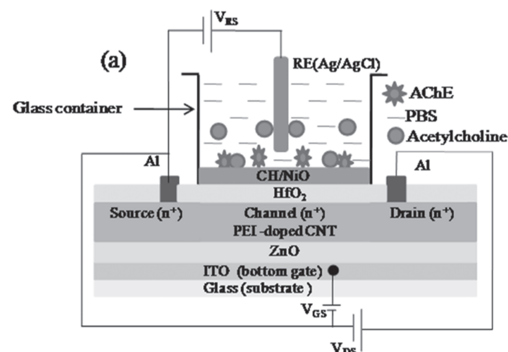

Fig.1. Schematic diagram of AchFET

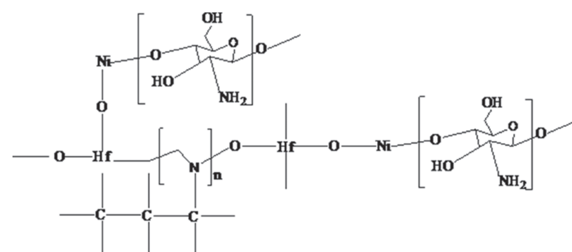

Fig.2. Schematic of the mechanism of AchFET

curve of the AchFET is similar to the axon membrane and with the help of feedback and RC circuit , proper neuron signals can be generated. The drain source voltage (V_{DS}) is taken as the membrane voltage and the drain current(I_D) as the ionic current of sodium and potassium ions. A low voltage is maintained to get the resting potential of the membrane by biasing the AchFET. V_{DS} is feedback via a circuit to the gate and the conductance of AchFET will changes when V_{DS} is applied. Amplification of the signal is done by OPAMP while the time dependent conductance of sodium and potassium is maintained by the RC circuit. To obtain a delayed rise in potassium, diode is introduced with proper biasing. When negative voltage is applied, capacitor becomes less negative and diode starts conducting resulting in delayed rise in gate voltage. When the two circuits of potassium and sodium conductance is connected with leakage conductance and membrane capacitance, an action potential is obtained.

Results and Discussion

The simulated action potential from NEUROAchFET model is shown in Fig.5. The simulated graph is similar to Hodgkin Huxley's experimental results and Roy's model shown in Fig.6 and Fig.7(1)-(5).Fig. 5 shows the action potential rising from resting potential. The action potential rises to a peak showing depolarization where sodium ions enters the membrane and again regains resting potential by letting out sodium ions(repolarization). The sodium and potassium conductance variance with voltage (V_{DS})

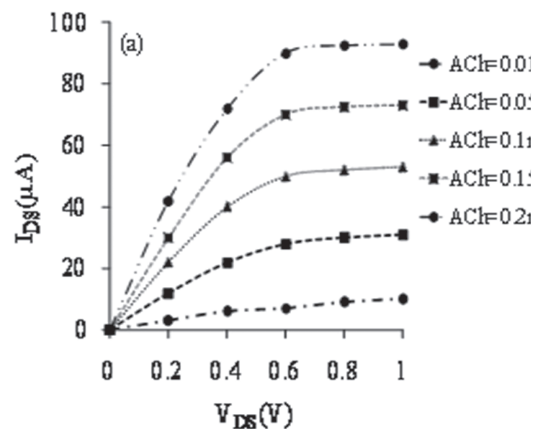

Fig. 3. Characteristic curve of AchFET

of the circuit is also shown in Fig.8 and Fig.9 and compared with Roy's model(5) and Hodgkin-Huxley model in Fig.10 and Fig.11.Sodium conductance increases with depolarization rapidly and decreases. While potassium conductance is prolong and rises slowly. All signals obtained from the circuit is similar to the literature available till date.(10)-(21).A comparison of Hodgkin Huxley data and NeuroAchFET model in tabular form is established and shown in Table 1. It has been

Table 1. Conductance variance of sodium and potassium with respect to voltage of NeuroAchFET circuit and Hodgkin-Huxley model.

NeuroAchFET	Voltage Clamped experimental data of HH (1952)				
Voltage (mV)	Sodium Conductance (mmho)	Potassium Conductance (mmho)	Voltage (mV)	Sodium Conductance (mmho	Potassium Conductance (mmho
0	0	0	0	16	0
20	10	8	20	20	10
40	15	10	40	28	11.1
60	25	12	60	34	12
80	30	12	80	33	12
100	40	12	100	40	13

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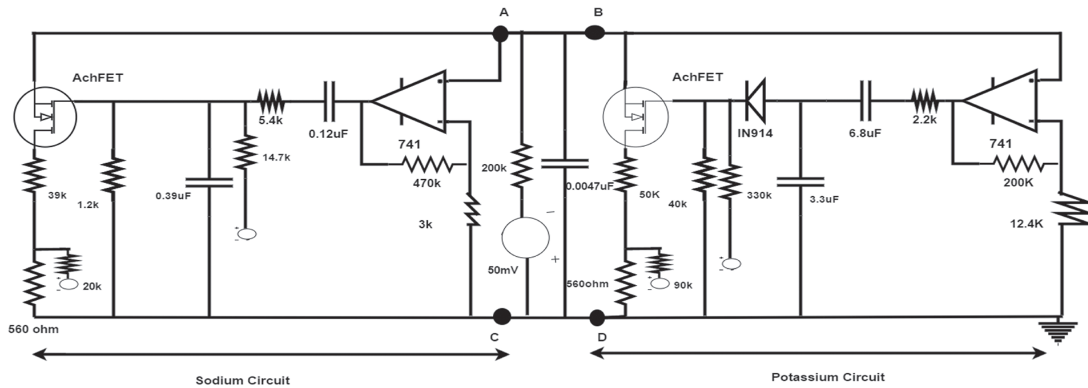

Fig.4: NeuroAchFET model

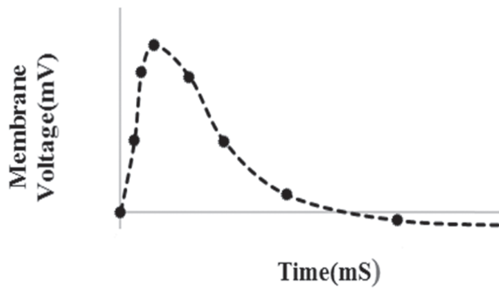

Fig.5. Action potential obtained from NEUROAchFET

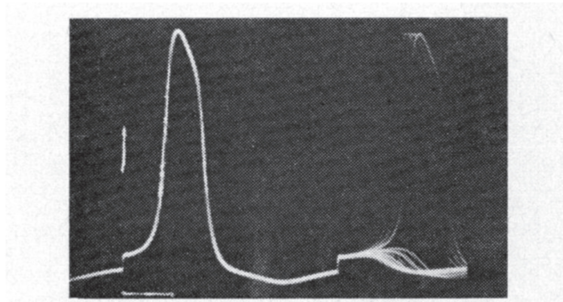

Fig.7: Action potential obtained by Roy model(5)

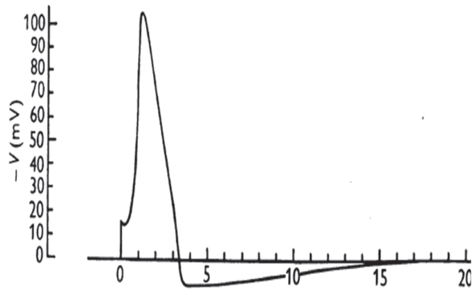

Fig.6: Action potential obtained by Hodgkin-Huxley

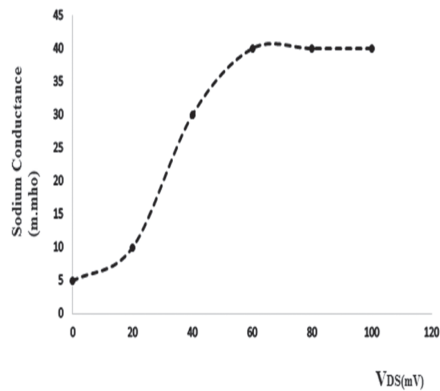

Fig.8. Sodium conductance at V_{DS} equal to 20 mV, 40 mv, 60 mV, 80 mV and 100 mV.

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observed that the results obtained is similar to the experimental data of Hodgkin-Huxley model. Therefore, the circuit can be used in neurological field for reproducing neuron signals and as a teaching tool.

Conclusion

NeuroAchFET circuit is designed for application in neurology. It can reproduce action potential, sodium and potassium currents satisfactorily. Furthermore, since the circuit can detect acetylcholine, it can be used for

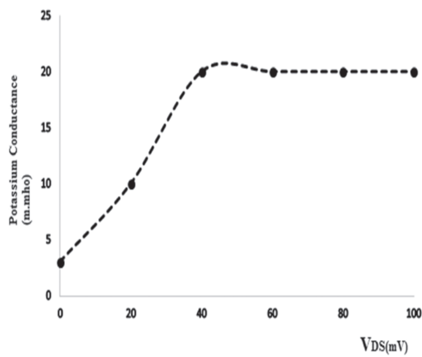

Fig.9. Potassium conductance at V_{DS} equal to 20 mV, 40 mv, 60 mV, 80 mV and 100 mV.

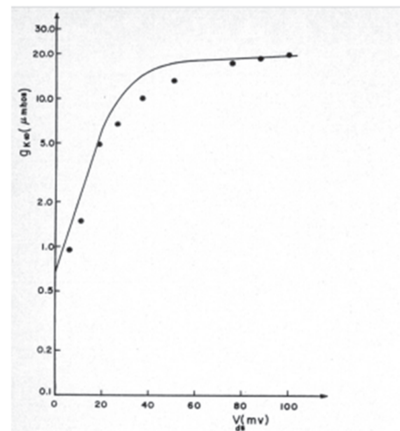

Fig.11: Potassium conductance variance with drain source voltage. Dots are the points taken from Hodgkin-Huxley.

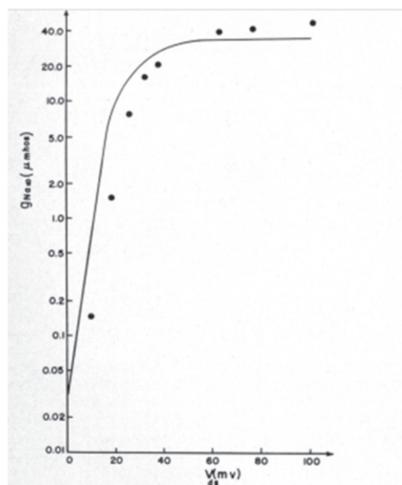

Fig.10: Sodium conductance variance with drain source voltage. Dots are the points taken from Hodgkin-Huxley

neurological patients. Also it has the capability of generation of spontaneous activity, pacemaker, changing frequency of pacemaker which can save millions of patients.

Acknowledgment

The authors like to extend their gratitude to the Tezpur University for providing facilities in the Laboratory where the simulations were performed.

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Screening, optimization and production of a novel β- cyclodextrinase by *Bacillus flexus* MSBC 2

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Abstract

CGTase (Cyclodextrin Glycosyl Transferase), is an important starch modifying enzyme catalyzing conversion of starch and related polysaccharide into cyclodextrins (CDs), which have important applications in numerous industries. In the present study, a potent CGTase producing bacteria was isolated from corn field soil. The bacteria was identified by microscopic and biochemical tests along with 16s rRNA to be as *Bacillus flexus* MSBC 2. From plate assay it was seen that only β-CD was produced. Submerged fermentation parameters for CGTase production was optimized and the best process parameters after optimization was found to be incubation period 5 days, pH 10.0, temperature 37 °C, peptone with yeast extract as best nitrogen source and 2% corn starch as best carbon source . A 3.0 fold increase in enzyme production was observed after optimizing the cultural conditions. The bacterium is specifically producing β- CGTase which is of high commercial value in industries as generally bacterial CGTases produce mixture of cyclodextrins.

Keywords: CGTase, *Bacillus flexus* MSBC 2, β- cyclodextrin.

Introduction

Modified starches like cyclodextrins, maltodextrin derivatives have important commercial applications. Thus, starch modifying enzymes are of great importance, of which Cyclodextrin GlycosylTransferace (CGTase) is one such enzyme. CGTase (2.4.1.19) belongs to α- amylase, family of Glycosyl hydrolase 13 (GH 13) (3). It produces cyclodextrins (CDs) via cyclization reaction an intramolecular transglycosylation reaction. CGTases generally produce mixture of CDs namely; α -, β- and γ- CD depending on the number of glucose units i.e. 6, 7 and 8. Thus, CGTase producing one type of CD are of commercial importance (1).

CDs are α-1,4 linked glucopyrunose units, non reducing, torus shaped, it possesses hydrophobic interior and hydrophilic exterior,(4) which can form inclusion complexes with organic and inorganic guest molecules altering their physical and chemical properties. As a result of this CDs have numerous applications in pharmaceutics, textile, cosmetics, agriculture, food, supramolecular chemistry, environmental protection, membrane and analytical chemistry (5).

Due to its wide applications, CGTase producing micro-organisms are been screened for enzyme of desirable traits suitable for industrial applications (1). Most common producers of CGTase are members of *Bacillus* sp. (6, 7 & 8). CGTase is inducible in nature (2) and thus, its production is affected by the

nature of substrate used in the fermentation medium and its properties depend on the source of micro-organism (9). Also, the enhancement of enzyme production is found to be influenced by physical and nutritional parameters, thus evaluating these parameters are important for enhancing metabolic production and microbial growth (10 & 11). Numerous strategies like computational techniques and genetic engineering are also used to enhance the production of CGTase (6, 8 & 11). This study presents the screening of *Bacillus flexus* MSBC 2, which is a potent producer of CGTase and the optimization of cultural conditions for maximum enzyme production.

Materials and Methods

Isolation screening and identification of β-CGTase producing bacteria: Soil sample was collected from sugarcane and corn fields, Maddur, Karnataka. 1.0 g of the soil sample was suspended in 10 mL of distilled water, serially diluted sample was screened according to rapid screening method described by Park *et al*. (3), where 100µL of serially diluted suspension was plated on to Horokoshi media –II (HM-II), pH was adjusted to 10.0 in order to isolate alkaophilic bacteria producing CGTase and incubated at 37 °C for 24 - 36 h. The media composition was as follows 1.5% soluble starch, 0.5% peptone, 0.5% yeast extract, 0.1% K_2HPO_4, 0.01% $MgSO_4$, 0.02% phenolphthalein, 0.01% methyl orange, 1.0% Na_2CO_3 (autoclave separately). Bacteria showing maximum zone of clearance was selected for further studies.

The selected strain was characterized as per Bergey's Manual of Determinative Bacteriology and further confirmed with 16s rRNA technology.

Qualitative analysis for the type of CD produced: The bacterial culture was inoculated onto the Petri plates containing HM II media with dyes 0.035 mM congo red, 3 mM phenolphthalein with 0.035 mM methyl orange and 0.035 mM bromocresol green respectively for 24 - 36h at 37 °C, to check the production of α-, β-, and γ- CD (12).

Preparation of inoculum: The selected strain was sub cultured on to Horikoshi media II (pH 10.0), in the slants excluding indicators at 37°C for 24h, a loop full of culture was then transferred in to HM-II broth excluding indicators, the culture showing cell density of 0.18 OD at 600nm was used as standard inoculum.

Determination of CGTase activity and protein content: CGTase assay was carried out as per slightly modified phenolphthalein method (13), 4% soluble starch in 0.1M phosphate buffer, pH 7.0 was prepared by heating it in boiling water bath for 3 min. To 650 µL of substrate, 250 µL of 0.1 M phosphate buffer pH 7.0 was added along with 100 µL of enzyme. This reaction mixture was incubated at 60 °C for 15 min. 4 mL of 0.04 mM phenolphthalein prepared in 125 mM sodium carbonate, was added immediately after incubation period. Blank contained 0.1 mL of distilled water excluding enzyme. The decrease in absorbance was read at 540 nm. One unit (U) of enzyme activity is, the amount of enzyme required to liberate 1µmol of β - CD per minute under standard assay conditions. Standard curve was plotted from known β- CD concentrations of 0-500 µg/mL. Protein content was determined according to the method described by Lowry *et al*. (14).

Optimization of cultural parameters: Various factors influencing CGTase production was optimized by standardizing one factor, keeping all other variables constant, optimized parameter was then incorporated into the experiment for optimization of next factor. Post incubation, supernatant obtained by centrifuging broth at 8,500 rpm (4 °C) for 10 min served as crude enzyme. All the experiments were carried out in triplicates. CGTase assay of optimized process parameters was carried out as per slightly modified

phenolphthalein method as described above (13).

· Time course of CGTase production:

2% inoculum was added into the HM-II broth(pH 10.0), exclusive of indicators and incubated at 37 °C, 2 mL of cultural filtrate was withdrawn every 24h for a period of seven days. The filtrate obtained was centrifuged at 10,000 rpm for 10 min at 4 °C, supernatant obtained served as crude enzyme source and CGTase activity was assayed as mentioned earlier.

· Effect of incubation temperature on *Bacillus flexus* MSBC 2 for CGTase production:

Culture medium (pH 10.0) were inoculated with 2% inoculum and incubated at different temperatures i.e., 20 °C, room temperature - 28±2 °C, 37 °C, 45 °C and 55 °C. CGTase activity was checked on the optimum day of enzyme production.

· Effect of initial pH of the medium on enzyme production:

Effect of pH was evaluated by adjusting pH to 7.0, 8.0, 9.0, 10.0, 11.0 and 12.0 with a digital LI 120 pH meter (ELICO) before sterilization. pH 10.0 served as control. After sterilization at 121 °C for 15 min, media was inoculated with 2% inoculum and incubated at 37 °C for 5 days and CGTase activity was assayed according to slightly modified phenolphthalein method (13).

· Influence of different nitrogen sources on CGTase production by *Bacillus flexus* MSBC 2:

Different nitrogen sources (1%, w/v): peptone, yeast extract, casein, urea, ammonium nitrate, ammonium chloride, beef extract and ammonium sulphate were used. Peptone along with yeast extract served as control. Media was inoculated with 2% inoculums, pH 10.0 and incubated at 37°C. The best nitrogen source was optimized by evaluating the enzyme activity on 5th day.

· Influence of different carbon sources and carbon source concentration on CGTase production by *Bacillus flexus* MSBC 2:

Various carbon sources such as corn starch, potato starch, amylopectin, wheat starch and starch hydrolysate were tested, by replacing it with soluble starch which served as control. Media was inoculated with 2% inoculum and incubated at 37 °C, peptone along with yeast extract as nitrogen source, pH 10.0. Enzyme production was assessed on 5th day.

Varying concentrations of corn starch i.e. 0.5%, 1.0%, 1.5%, 2.0%, 2.5% and 3.0% was taken and enzyme activity was checked on the 5th day.

Statistical Analysis

Experiments were carried out in triplicates and one way ANOVA was performed for statistical analysis using GraphPad Prism v6.

Results and Discussion

***Isolation, screening and identification of β-CGTase producing bacteria*:** Serially diluted soil samples from corn and sugarcane fields were subjected to screening according to rapid screening method (3).Numerous colonies showing distinct yellow hallo was observed on 3rd day.The colonies were then individually plated onto the media and the culture showing maximum zone of clearance was selected for further studies. CGTase producers can be isolated form soil regions of corn root, oat cultures, sugarcane and hyper saline soda lake (15,16). The isolate was identified as *Bacillus* sp. from microscopic examination and the strain was further confirmed as *Bacillus flexus* MSBC 2 through 16s rRNA sequencing.

***Plate assay for the type of CD produced*:** Isolated stain *Bacillus flexus* MSBC 2 was plated on to the medium containing respective indicators namely methyl orange,

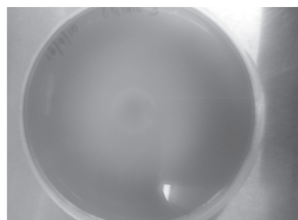

Fig. 1. Screening and isolation of CGTase producing bacteria. Bacteria screened and isolated through rapid screening method showing highest zone of clearance.

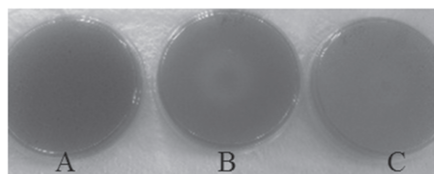

Fig. 2. Plate assay for the type of CD produced. (A) Bromocresol green for γ- CD (B) Phenolphthalein for β-CD (C) methyl orange α- CD.

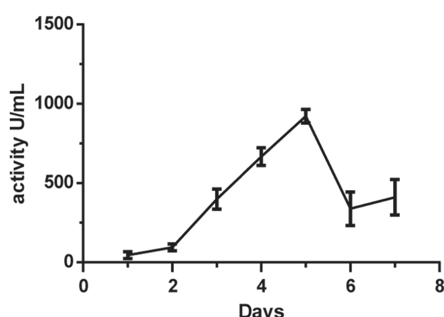

Fig. 3. Time course for optimum enzyme production by *Bacillus flexus* MSBC 2. . CGTase production on different days grown in HMII media with pH 10.0 and inoculated 2% inoculums and incubated at 37 °C. 5th day displayed optimum activity. Results obtained were statistically significant at $p < 0.05$.

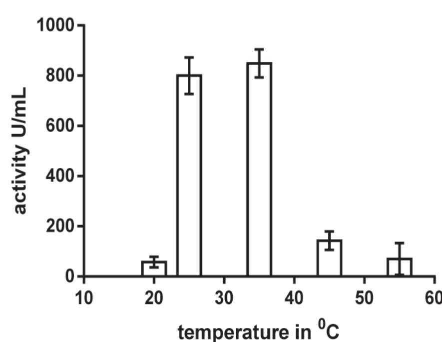

Fig. 4. Effect of incubation temperature on production of CGTase. HM II (pH 10.0) media was inoculated with 2% inoculum and incubated at respective temperatures. 37 °C displayed optimum enzyme activity and as temperature increased enzyme production declined. The values represented differ significantly from each other at $p < 0.05$.

phenolphthalein and bromocresol green for α- β- and γ- CD. Zone of clearance was observed on the plate containing phenolphthalein indicating that only β- CD was produced. Fig. 2. Plate assay for the type of CD produced. (A) Bromocresol green for γ- CD (B) Phenolphthalein for β-CD (C) methyl orange α- CD.

Optimization of cultural parameters: Time course: CGTase activity was assayed for every 24 h after incubation to identify the optimum incubation period for maximum enzyme production. The enzyme production was observed on the 2nd day and optimum production was observed on 5th day. Depending

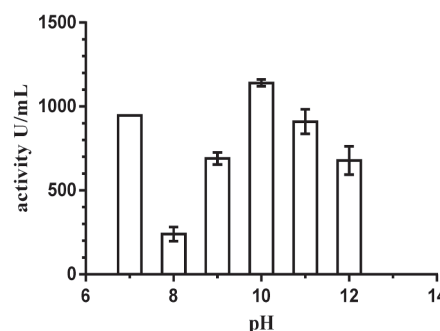

Fig. 5. Effect of initial pH on CGTase production. HM II media was set to respective pH, inoculated with 2% inoculum and incubated at 37 °C for 5 days. pH 10.0 served as control. The values represented differ significantly from each other at $p < 0.05$.

on the source and environmental conditions from which microorganism is obtained time course for enzyme production may vary, *Bacillus firmus* obtained from soil oat culture displayed optimum activity on 5th day (1) whereas, *Bacillus* TS1-1 displayed highest activity at 24 h (6).

Effect of temperature on CGTase production: Incubation temperature plays an important role in enzyme production as it affects the growth rates of micro-organisms. Influence of incubation temperature on CGTase production was studied. Maximum enzyme production was observed at RT and 37 °C. Optimum production was displayed at 37 °C, which was statistically significant ($p < 0.05$). There was a decline in enzyme production when temperature increased beyond 37 °C. Studies conducted by Sivakumar *et al*.,(20) on *Bacillus megaterium* showed similar results where 27 and 37 °C were optimum for enzyme production. The values represented differ significantly from each other at $p < 0.05$.

Effect of pH of the medium on alkaline protease production: Initial pH of medium is an important factor in deciding growth of microorganism either directly or indirectly (17) and enzyme activity, as any change in pH may result in change in shape of substrate and/or enzyme (18).The initial pH of the medium had significantly ($p< 0.05$) influenced the enzyme production. There was an increase in the production of enzyme as pH increased, also the growth of the organism and enzyme production was observed at neutral pH, this may be due to the ability of micro-organism to grow at neutral pH conditions. However, enzyme production was maximum at pH 10.0. There was a significant decrease observed when the pH was further increased to 11.0 and 12.0. pH 6.0 was found to be optimum for *Bacillus* sp. G1(19) for high enzyme production.

Effect of nitrogen sources on CGTase production: Influence of different nitrogen

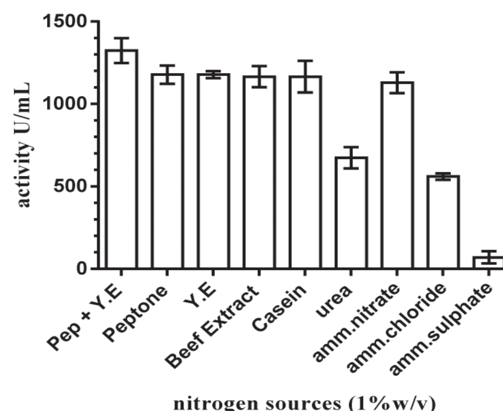

Fig. 6. Effect of nitrogen sources (1.0% w/v) on CGTase production by *Bacillus flexus* MSBC 2. The fermentation media at intial pH of 10.0 was inoculated with 2% inoculums incubated at 37 °C for 5 days. Peptone with yeast extract served as control. The values represented differ significantly from each other $p < 0.05$.

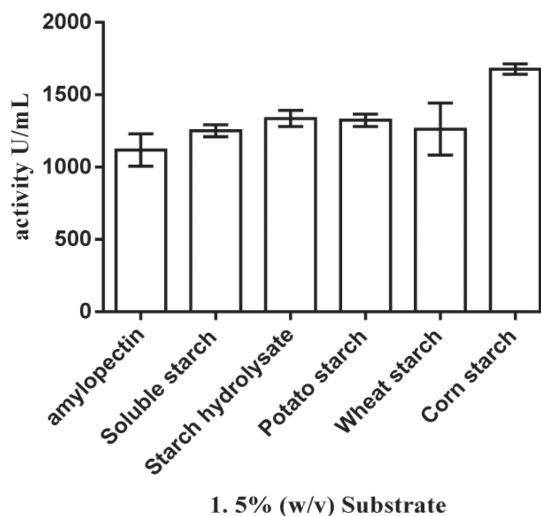

Fig. 7. Influence of various carbon sources (1.5% w/v) on enzyme production by *Bacillus flexus* MSBC 2. Organism was able to utilize various carbon sources and optimum activity was observed in the medium containing corn starch. Values obtained were statistically significant from each other at $P <0.05$.

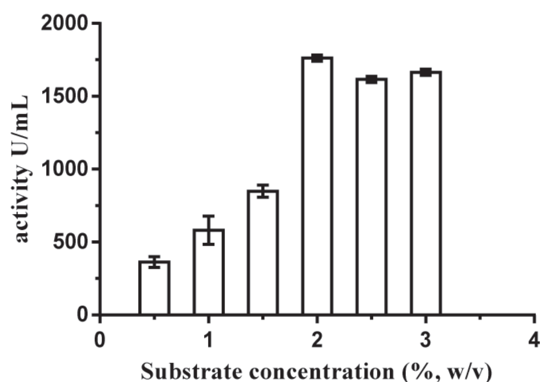

Fig. 8. Substrate of 1.5% served as control and 2% corn starch was found to be the optimum. The results analysed through graph pad prism v6 were statistically significant from each other at $p < 0.05$.

sources on enzyme production were tested, wherein the bacteria was able to use all the nitrogen sources except for ammonium sulphate and ammonium chloride. Significant activity was obtained in the media supplemented with peptone and yeast extract ($p < 0.05$). Generally studies show that yeast extract is found to be the best nitrogen source for CGTase production.

***Effect of substrate and carbon source CGTase production*:** The type of CD produced depends on the substrate and source of enzyme, thus influence of various carbon sources (1.5% w/v) were tested for maximum enzyme production. Corn starch displayed optimum CGTase activity and results obtained were statistically significant at $P_{0.05}$. However, sufficient enzyme activity was observed in the media containing other carbon sources indicating that micro-organism can utilize a wide range of substrates for CGTase production. Corn starch in hydrolysed form was found to best substrate in study conducted by Sivakumar and Shakila(20) where as it was a poor substrate for *Klebsiella pneumonia* As-22 (20,21). This may be due to inducible nature of enzyme production, thus choice of appropriate substrate is necessary for inducing enzyme production by micro-organism.

The effect of altering substrate concentration on CGTase production has been tested and depicted in the **Fig. 8**. The optimum enzyme production was observed at 2.0% concentration which was statistically significant at $P_{0.05}$. Similar results was obtained with *Bacillus firmus* strain No.37 (22), where 2% corn starch resulted in optimum yield of the enzyme.

Fig. 8. Different substrate concentration were analysed for highest CGTase production.

Conclusion

The isolated bacterium, *Bacillus flexus* MSBC 2 was able to use wide range of carbon sources and could grow in alkaline environment which is best suited for industrial processes. Plate assay showed production of only â- CD, which can be used for the commercial production. Further the optimization of fermentation parameters have resulted in a 3.0 fold increase in enzyme production.

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Purification Strategies for Microbial Pectinases

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Abstract

Microbial pectinases currently occupy a place of prominence among biocatalysts owing to their capability to catalyze a diversity of reactions in aqueous and non-aqueous media. Specific action of these enzymes has initiated remarkable attention among industrialists and researchers. Pectinases from a large number of fungal and a few bacterial sources have been purified to homogeneity. This has assisted in successful sequence and three dimensional structure determination leading to a better understanding of their unique structural function relationships during various hydrolytic and synthetic reactions. Since protein purification is normally done in a series of sequential steps involving a mixture of different techniques. This article presents a critical review of different approaches which have been employed for the purification of fungal and bacterial pectinases. This will be immense help for researchers while planning microbial pectinase purification.

Keywords: Bacterial, fungal, pectinases, purification

Introduction

Enzymes are well known delicate biocatalysts that accomplish a multitude of chemical reactions in the metabolism of almost all organisms' *viz.,* plants, animals, fungi, bacteria and viruses. From an industrial perspective, only a constrained number of enzymes are commercially exploited in the detergent, food, leather processing, pharmaceutical, diagnostics, and fine chemical industries (1-3). Pectinases constitute a diverse group of enzymes which catalyze the hydrolysis of pectin polymers (4). Pectin polymers are structural polysaccharides which forms an important component of middle lamella and primary cell wall of higher plants (Fig-1) (4).

Primarily, these are made up of á (1-4) linked D-galacturonic acid residues (5). Depending on their mode of catalysis, the enzymes hydrolyzing pectin are broadly known as pectinases (Table-1 and Fig-2), which include endo-polygalacturonase (E.C. 3.2.1.15), exo-polygalacturonase (E.C. 3.2.1.6.7), pectin lyase (E.C. 4.2.2.10) and pectin esterase (E.C. 3.1.1.11) (6-7).

Pectinases are alone account for about one quarter of the world's food enzyme production (8). It has been reported that microbial pectinases account for 25% of the global food enzymes sales (9). Pectinases are of great significance (Table-2), extensively used in the clarification of fruit juices, extraction of vegetable oil, treatment of pectin waste waters, degumming of plant fibers, pulp and papermaking, and for coffee and tea fermentation (10-16).

Most of the commercial production of pectinases is limited to some species of microorganisms *viz., Bacillus licheniformis* (17), *Bacillus cereus* (17), *Bacillus subtilis* (18), *Bacillus polymyxa* NCIM 2534 (19), *Lactobacillus plantarum sp.* (20), *Lactobacillus pentosus* SJ65 (20), *Bacillus sp.* (21-27), *Staphylococcus aureus* (17),

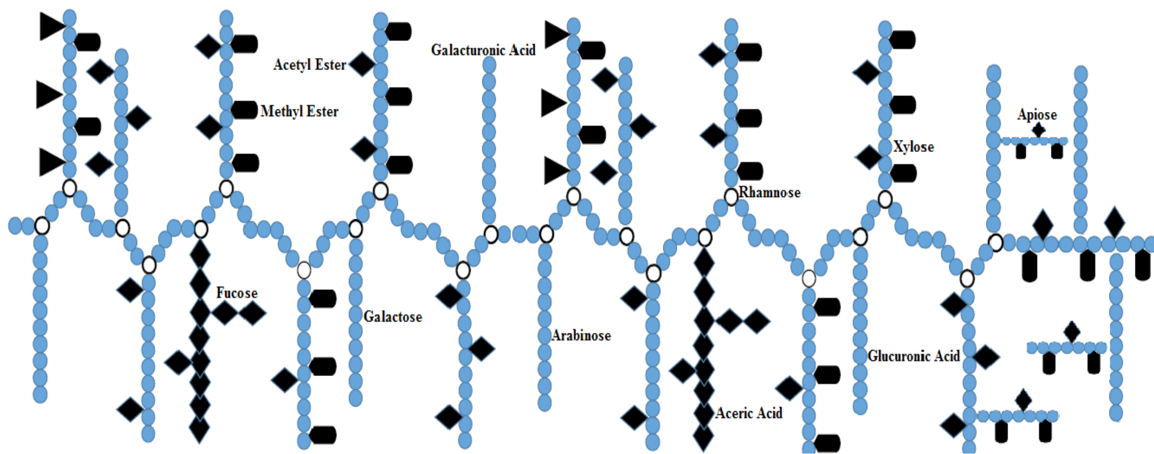

Fig. 1. Basic Structure of Pectin

Table-1. Pectolytic enzymes classification based to the mode of action

No	Enzyme	E.C. Number	Main Substrate	Mode of Action	Product
1	**Esterases** Pectin methyl esterase Pectin acetyl esterase	3.1.1.11 3.1.1.6	Pectin Pectin	Hydrolysis Hydrolysis	Pectic acid + methanol Pectic acid + methanol
2	**Depolymerases** *Hydrolases*Proto pectinases Endo poly- Galacturonase Exopolygalacturonase	 3.2.1.1.5 3.2.1.6.7	Proto pectin Pectic acid Pectic acid	Hydrolysis Hydrolysis Hydrolysis	Pectin OligoGalacturonates MonoGalacturonates
3	**Lyases** Endo pectate lyase Exo pectate lyase Endo pectinlyase	4.2.2.2 4.2.2.9 4.2.2.10	Pectic acid Pectic acid Pectin	Trans elimination Trans elimination Trans elimination	Unsaturated Oligo Galacturonates Unsaturated Oligo Galacturonates Unsaturated methyl Oligo Galacturonates

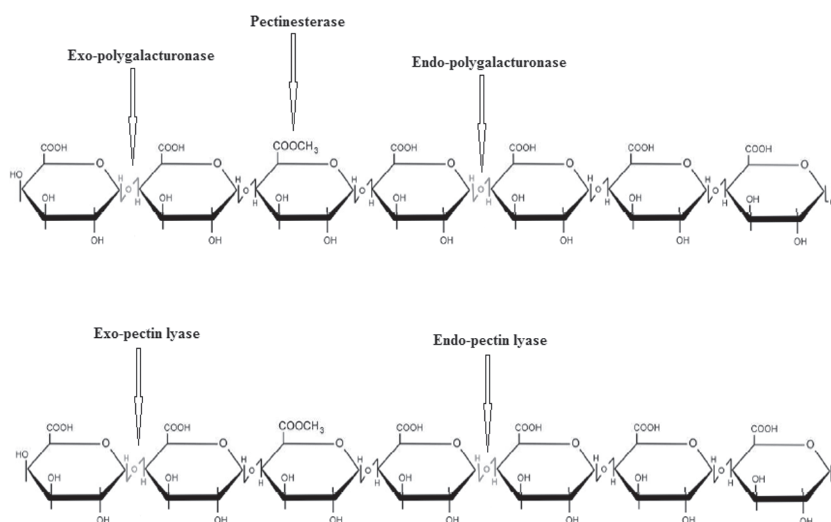

Fig. 2. Pectinases Mode of Catalysis

Erwinia sp. (27), *Leuconostoc lactis* VJ52 (20), *Pediococcus pentosaceous* (20), *Streptomyces sp.* (28), fungi *viz., Aspergillus niger* (27-44), *Aspergillus terreus* (45), *Aspergillus versicolor* (42), *Aspergillus flavus* (30, 31, 42), *Aspergillus awamori* (46), *Aspergillus ocharceous* (31), *Aspergillus japonicas* (30), *Aspergillus foetidus* (47), *Aspergillus aculeatus* (47), *Aspergillus candidus* (48), *Aspergillus carbonarius* (49-51), *Fusarium oxysporum* (27, 42), *Fusarium moniliforme* (52), *Gliocladium viride* (48), *Kluyveromyces* (27, 53), *Mucor racemous* (42), *Mucor hiemalis* (42), *Penicillium canescens* (27, 48), *Penicillium citrinum* (42), *Penicillium griseoroseum* (54), *Penicillium jenseni* (42), *Penicillium dierckxii* (55), *Penicillium occitanis* (56), *Penicillium frequentans* (57, 58), *Penicillum oxalicum* (59, 60), *Penicillium viridicatum* (61), *Rhizopus stolonifer* (27, 42, 48, 62), *Botrytis cinerea* (63), *Cladosporium cladosporioides* (64), *Chaetomium globosum* (30), *Lentinus edodes* (65), *Polyporus squamosus* (66), *Rhodotorulla sp.* (67), *Trichoderma viride* (42), *Thermoascus auriantacus* (68) and yeast *viz., Saccharomyces cerevisae* (16, 27).

Pectinases contain glycine rich consensus sequence (69). Knowledge of the three-dimensional structure of pectinase plays a significant role in designing and engineering pectinase for specific purposes. Most commercial applications do not require homogeneous pectinase preparations; a certain degree of purity, however, enables efficient and successful usage. In addition, purification of enzymes allows successful determination of their primary amino acid sequence and three-dimensional structure. The X-ray studies of pure pectinase enable the establishment of the structure-function relationships and contribute for a better understanding of the kinetic mechanisms of pectinase action on hydrolysis and synthesis (69). Further, purified pectinase preparations are needed in industries employing the enzymes for the bio catalytic production of fine chemicals and pharmaceuticals.

Purification of pectinolytic enzymes : The main constraints in traditional purification strategies include low yields and long time periods. Alternative new technologies such as membrane processes, and aqueous two-phase systems are

gradually coming to the forefront in the purification of pectinases (44, 45, 53, 60, 70). Industries today look for purification strategies that are inexpensive, rapid, high yielding and agreeable to large-scale operations. Purification protocols available in literature are important for consultation when attempting to purify any new preparation. Thus, a review article summarizing up-to-date literature on purification of pectinases serves as a ready reference for researchers engaged in the area of protein purification. The earlier research in this field highlight clearly the importance of designing optimal purification schemes for various microbial pectinases (25, 28, 63).

The present review enlists the various purification procedures applied by different workers. The extent of purification differs with the order of purification steps and this aspect is assessed through the different purification protocols pursued by various investigators and the attempts made in our laboratory for designing purification protocol for bacterial and fungal pectinases have also been discussed. On analyzing 112 articles on pectinases published from 1990 to 2015, some conclusions have been drawn about the percent of different methods used in the purification of these enzymes.

Pre-purification steps : The analysis of enzyme activity in the crude extract does not indicate either an isolated action or the presence of a multi enzyme system working in synergy on the substrate degradation. The characterization of purified enzymes is an important research line since it provides discrimination between the enzyme complex components about substrate degradation mechanism, optimum activity conditions and enzyme synthesis regulation. Most of the microbial pectinases are extracellular and the fermentation process is usually followed by the removal of cells from the culture broth, either by centrifugation or by filtration. The cell-free culture broth is then concentrated by ultrafiltration, ammonium sulphate precipitation or extraction with organic solvents. About 80% of the purification schemes attempted thus far have used a precipitation step, with 60% of these using ammonium sulphate and acetone followed by a combination of several chromatographic methods such as gel filtration and affinity chromatography.

Table-2. Application of Pectinases

S.No	Area	Application
1	Juice industry	Juice clarification
2	Textile industry	They are capable of depolymerizing the pectin breaking it into low molecular water soluble oligomers improving absorbency and whiteness of textile material and avoiding fiber damage
3	Pulp and paper industry	Effective in bio bleaching of mixed hard wood and bamboo Kraft pulp
4	Wine industry	Improve wine characteristics of colour and turbidity, improvement of chromaticity and stability of red wines
5	Biological applications	In protoplast fusion technology and plant pathology
6	Coffee and tea fermentation	Fermentation by breaking pectins present in tea Leaves
7	Oil extraction	By avoiding emulsification formation

Precipitation is usually used as a fairly crude separation step, often during the early stages of a purification procedure, and is followed by chromatographic separation. Increase in pectinases activity depends on the concentration of ammonium sulfate solution used (10, 71). Large quantities of material can be handled, and this step is less affected by interfering non-protein materials than chromatographic methods. In comparison to other techniques, which give lower yields (60-70%), precipitation methods often have high average yield (80%) (72). Salting out (ammonium sulfate precipitation) is useful for concentrating dilute solutions of proteins. It is also useful for fractionating a mixture of proteins, since large proteins tend to precipitate first while smaller ones will stay in solution. Rosenberg *et al* obtained 4.6 fold purification with 89.6% of polygalacturonase recovery when they used 70% ammonium sulfate saturation (73).

Hara *et al* obtained around 1.5 fold purification levels with 85% of enzyme recovery when they used 90% ammonium sulfate saturation to precipitate polygalacturonase from *Aspergillus niger* (74). Whereas 3 fold of purification and 68.3% of yield were attained at 70% ammonium sulfate saturation as reported by Darrieumerlou *et al* (75). Charlotte *et al* purified two isozymes of polygalacturonase from *Botrytis cinerea* by addition of 90% ammonium sulfate (63). Kashyap *et al* partially purified pectinase from *Bacillus sp.* DT7 by addition of 40-100% solid ammonium sulphate to the cell-free supernatant (26). Gyan *et al* purified pectinase from *Bacillus subtilis* by addition of 60% ammonium sulphate saturation (25). Das *et al* purified pectinase from *Streptomyces sp.* GHBA10 by addition of 80% ammonium sulphate precipitation (28). Mehraj *et al* purified polygalacturonase produced by *Aspergillus foetidus* MTCC 10367 by addition of 80% ammonium sulphate fractionation (76). Essam *et al* purified pectinase from *Thermomyces lanuginosus* by addition of 60% ammonium sulphate (77).

Chromatographic steps (Table-3)

In order to characterize and study the properties of microbial pectinases the enzymes must be purified. Important purification methods for the isolation of different pectinases are briefly summarized in this section. Most of the time, a single chromatographic step is not sufficient to get the required level of purity. Hence, a combination of chromatographic steps is required. Ion exchange chromatography is the most common chromatographic method; used in 67% of the purification schemes analyzed and in 29% of these procedures, it is used more than once (77).

The most frequently employed ion-exchangers are the diethylaminoethyl (DEAE) group in anion exchange and the carboxymethyl (CM) in cation exchange. Strong ion exchangers based on triethylaminoethyl groups and Sephacryl are becoming more popular in pectinase purification (74-84). Gel filtration is the second most frequently employed purification method, used in 60% of the purification schemes and more than once in 22% of them (81).

Pectinases from various sources of microorganisms have been purified to homogeneity. An exo-PG has been separated from mycelial extracts of *Aspergillus niger* by eluting from DEAE cellulose with 0.2 M sodium acetate buffer at pH 4.6. purification was efficient with 209 fold increase in specific activity with a recovery of 8.6% and the enzyme displayed its full activity only in the presence of Hg^{2-} ions (85). A second PG was isolated with 205 fold increase in specific activity with a recovery of 1%. These two PGs are differentiated by their optimum pH and PGII was not inhibited by chelating agents and did not require Hg^{2-} for activity (86). Benkova *et al* developed a purification statergy for the isolation of extracellular PG and PE (87). The enzyme was salted out with ammonium sulphate and precipitated with ethanol after gel filtration through Sephadex G-25. Repeated chromatography on DEAE-cellulose column

Table-3. Source, Substrate and Methods for Pectinase Production and Purification

S.N.	Name of the Organism	Type of Fermentation and Substrate Used	M.W in kDa	Purification Process	Ref.
1	*Aspergillus sp.*	Submerged and Solid State Fermentation Pectin, Glucose, Wheat bran extract, Corn meal, Sucrose, Soy bran, Sugar cane bagasse and Grape pomace	36-79	Amonium sulphate saturation, Gel filtration and Ion exchange chromatography	4, 54, 55, 58, 60, 80, 100
2	*Amycolata sp.*	Submerged Fermentation	30	Ultrafiltration, Ion exchange and Hydrophobic interaction chromatography.	105
3	*Bacillus sp.*	Submerged Fermentation and Solid State Fermentation Vegetable Waste	106	Amonium sulphate saturation, Gel filtration and Ion exchange chromatography	8, 17
4	*Botrytis cinerea*	Submerged Fermentation Vegetable Waste	52	Amonium sulphate saturation, Gel filtration and Ion exchange chromatography	63
5	*Erwinia carotovora*	Submerged Fermentation Pectin	42	Amonium sulphate saturation	64
6	*Kluyveromyces marxianus*	Submerged Fermentation Pectin	75	Amonium sulphate saturation, Gel filtration and Ion exchange chromatography	83
7	*Macrophomina phaseolina*	Submerged Fermentation Pectin	56	Amonium sulphate saturation, Gel filtration and Ion exchange chromatography	84
8	*Mucor sp.*	Submerged Fermentation and Solid State Fermentation Sugar beet pulp and Citrus pectin	66	Amonium sulphate saturation and Gel filtration	81, 82
9	*Penicillium sp.*	Submerged Fermentation and Solid State Fermentation Citrus pectin, Sugar beet pectin, Pectin, Orange, Sugar cane bagasse, Wheat bran bagasse and Wheat bran	31-45	Amonium Sulphate saturation, Gel filtration, Ion exchange and Affinity chromatography	54, 55, 56, 58, 60, 80, 103
10	*Rhizopus oryzae*	Submerged Fermentation Pectin	31	Gel filtration and Ion exchange chromatography	55
11	*Sclerotium sp.*	Submerged Fermentation Pectin	38	Gel filtration, Ammonium sulfate precipitation and Ion exchange chromatography	62
12	*Streptomyces sp.*	Submerged Fermentation Pectin	32	Amonium sulphate saturation, Gel filtration and Ion exchange chromatography	6
13	*Thermoascus aurantiacus* CBMAI-756	Solid State Fermentation Wheat bran and Orange bagasse	29.3	Gel filtration and Ion-exchange chromatography	104
14	*Trichoderma sp.*	Solid State Fermentation and Submerged Fermentation Sugar beet pulp	30-72	Amonium sulphate saturation, Gel filtration and Ion exchange chromatography	82

yielded a homogeneous preparation of enzyme. Exo-PG, Endo-PG and pectinesterase have been seperated from the culture filtrate of *Trichoderma reesei* by Sephadex chromatography (88).

Polygalacturonase from *Rhizopus stolonifer* has been purified up to 10 fold by ethanol precipitation followed by CM-Sepharose 6B ion exchange chromatography and gel filtration by Sephadex G-100 (89). PG and PL (pectinlyase) from *Aureobasidium pullulans* LV10 have been seperated by CMSepharose 6B followed by column chromatography (DEAE-cellulose column) and gel filtration on sephadex G-100 (89). PG and PL (pectinylase) have been seperated into PG I and PG II and PL I and PL II, respectively. Pectatelyase (PGL) was synthesized by *Amycolata* species and the extracellular crude enzyme has been purified to homogeneity by both cation and anion exchange columns and hydrophobic interaction chromatography (89). It has been observed that purification resulted in a 4 fold increase in specific activity with 37% recovery. Pectinases from *Clostridium acetobutylicum* ID 9136 a UV mutant has been purified by cation exchange chromatography on a Sepharose column by eluting with NaCl (90). Endo pectate lyase synthesized by *Bacillus macerans* has been purified by ammonium sulphate precipitation followed by DEAE Sephadex A-50 chromatography and CM Cellulofine chromatography (91). Similarly endo pectate lyase I/IV have been isolated from the culture filtrate of *Erwinia carotovora* by CM Sepharose CL 6B chromatography, Sephadex S-200 gel filtration and isoelectric focusing (92).

Kobayashi *et al* purified the first bacterial exo-PG from *Bacillus sp.* strain KSM-P443 to homogeneity (93). This enzyme releases exclusively mono-galacturonic acid from polygalacturonic acid (PGA), Di, Tri, Tetra and Penta galacturonic acids. They also determined the N-terminal sequence and concluded that no sequence matched with other pectinases reported to date. An extracellular endo PG produced by *Aspergillus awamori* IFO 4033 was purified homogeneity using cation exchange and size exclusion chromatographic columns (94). Sakamoto et al isolated propectinase N (PPN) and propectinase R (PPR) from the culture filtrate of *Bacillus subtilia* IFO3134 (95). These enzymes have been purified by hydrophobic interaction chromatography on butyl-toyopearl 650 M, cation exchange chromatography on CMtoyopearl 650 M and gel filtration on sepharose 12HR. These enzymes have been found to be stable over a wide range of pH and temperature. Endo pectate lyase produced by *Erwinia caratovara* FERM P-7576 has been selectively co-sedimented with an extracellularly produced lipopolysaccharide lipid complex (96). The cell free broth was precipitated and the enzyme separated by gel chromatography with a specific activity of 710 U/mg of protein. Co-sedimentation has been affected by pH and Ionic strength. Denis *et al* studied the effect of shear stress on purification of five isoenzymes of pectate lyase produced by *Erwinia chrysanthemi* 3937 in ultrafiltration equipment (97). Activity was not affected during 7 h of pumping and 36% activity was lost after 25000 passes.

New affinity matrices have been developed for the purification of pectinases, which possess better mechanical and chemical stability than those cross linked one with pectic acid (98). The culture filtrate was desalted on Sephadex G-25 column. The supports used were salinized controlled pore glass, silica gel salinized with 5-aminopropyl triethoxylsilane. All supports were activated with 3(3-dimethyl amino propyl) carbodiimide and best results were obtained with salinized controlled pore glass. Gupta et al developed an affinity precipitation technique for separation of selective proteins using heterobifunctional ligands (99). They used a soluble form of the ligand for affinity binding and then precipitation was induced for separating the protein complex. Alginate was used as successful ligand for pectinases. Wu *et al* studied the partitioning behavior of endo-PG and total protein from *Kluyveromyces marxianus* culture broth in polyethylene glycol (PEG) and PEG-potassium phosphate aqueous two phase system (ATPS)

(100). Both enzyme and total protein partitioned into the bottom phase in broth systems. Since, the enzyme protein content in the total protein of the fermentation broth was higher, they proposed that separating endo-PG was concentration rather than separation.

An efficient concentration was achieved using ATPS with polymer recycling and dialysis. Immobilized metal ion affinity polysulphone hollow fiber membranes with a high capacity for protein adsorption were tested for commercial pectic enzyme fractionation (101). The flow through (unbound) fraction has higher activity for pectinlyase and PE was retained on the column. Similarly Savary *et al* developed a rapid and simple method to separate PE from PG and other pectinases in potato enzyme preparations using perfusion chromatography (102). PE was eluted at low salt concentrations (80 mM NaCl) and PG at high salt concentrations (300 mM NaCl). The development of such columns looks promising for economical purification strategies for pectinases. A literature survey showed that extensive work has been carried out on the purification of PG, PE and PGL and less attention has been focused on PMG propectinase and oligogalacturonases. Charlotte *et al* purified crude extracts of polygalacturonase from *Botrytis cinerea* to homogeneity by applying Biologic duo flow system (Bio-Rad) liquid chromatography (63). Desalting of the crude protein was performed by gel filtration on HI Prep desalting column (Pharmacia) equilibrated with 50 mM Tris-HCl buffer and the desalted protein extract was loaded on an anion exchange column (Econo Pac High Q) (PG-I 6% and PG-II 3.7% recovery and eluted with 50 mM Tris-HCl buffer with a step gradient from 0 to 1 M NaCl. These fractions were applied to Size exclusion chromatography on SEC 250 column after this PG-I recovery 0.07 and PG-II 0.06% recovery.

Kashyap *et al* purified pectinase from *Bacillus sp.* DT7 by anion exchanger, DEAE-Sephacel equilibrated with 20 mM Tris HCl (pH 7.5) buffer (26). The proteins were eluted with a NaCl gradient (0.2 M). Using DEAE-Sephacel chromatography, 67.2 fold purification of the enzyme was achieved and its specific activity was found to be 730.3 U/mg of protein. Fractions showing pectinase activity were pooled, concentrated using a Centricon P-10 unit, and loaded on Sephadex G150 column. Protein was eluted with Tris-HCl buffer (pH 7.5). As pectinase activity was detected in fractions 21 ± 30, these fractions were pooled, concentrated and dialysed against 0.01 M Tris-HCl buffer (pH 8.0). This phase of purification yielded a 131.8 fold increase in the purification of pectinase and its specific activity was 1433 U/mg protein. Gyan *et al* purified pectinase from *Bacillus subtilis* by agarose ion-exchange column elution was carried out by linear gradient of NaCl 0.1 M-0.6 M (25). After this the specific activity was 4.5 U/mg and enzyme was 1.5 fold purified. Maximum yield observed about 80%.

Das *et al* purified pectinase from *Streptomyces sp.* GHBA10 by gel filtration chromatography (Sephadex G-200 column) equilibrated with 0.2 M phosphate buffer 7.5 pH (28). The activity of the crude and purified pectinase was evaluated as 250 U/l and 658 U/l, respectively. The specific activity of the crude and purified pectinase was recorded as 744 U/mg and 2610 U/mg, respectively. After column chromatography, 3.5-fold increase in the specific activity was noted. The crude Polygalacturonase produced by *Aspergillus foetidus* MTCC 10367 was subjected to gel filtration chromatography on a Sephadex G-100 column. Polygalacturonase activity for purified enzyme was found to be 40.1 U/ml, protein concentration was 0.5 mg/ml and specific activity was determined as 80.2 U/mg. A purification fold of 119.59 and % recovery of 71.55 was achieved (76). Pectinase enzyme was partially purified by Sephadex G-100 column chromatography. It showed an increased specific activity of *P. chrysogenum* pectinase to 145.84 IU/mg protein and enzyme recovery of 48.12%. Preparations of the enzyme obtained from culture filtrates of *P. italicum* were subjected to ion exchanger DEAE-cellulose and carboxymethyl cellulose chromatography at pH 8 and 6, respectively. The

preparations yielded the elution pH value of 8.6 and the elution volume of 15.1 ml and about three-fold higher specific activity (103).

The polygalacturonase from *Mucor circinelloides* ITCC 6025 was purified about 13.3 fold with a specific activity of 31.74 IU/mg giving a yield of 3.4% after Sephacryl S-100 gel-permeation chromatography which resulted in almost a single peak when absorbance was recorded at 280 nm (81). The crude enzyme solution obtained by *Thermoascus aurantiacus* CBMAI-756 culture on solid-state fermentation applied on Sephadex G-75 gel column showed only one peak of enzyme activity, which was detected between 160.0 ml and 256.2 ml. This step resulted in an increasing in the specific activity from 60.0 U/mg to 331.6 U/mg protein, in 5.2 fold enzyme purification and 58.8% yield. In the second step, 50 ml of enzymatic extract was applied on ion-exchange chromatography, using 20 mM acetate-NaOH buffer, at pH 4.0. Two protein peaks were observed from the elution volumes of 42.0 ml and 88.2 ml before the start of the salt gradient and three between 0.15 M and 0.7 M NaCl. Polygalacturonase was eluted at 0.9 M salt concentration. The specific activity increased from 331.6 U/mg to 5351.5 U/mg protein, with 89.2 fold enzyme purification and 14.2% yield (104). The crude pectate lyase from an *Amycolata sp.* was applied to a DEAE Sepharose column. Most of the pectate lyase did not bind to the anion-exchange resin and eluted from the column with the starting buffer. A small fraction of pectate lyase bound weakly to the resin and was eluted at a very low NaCl concentration (105).

Essam *et al* obtained *Thermomyces lanuginosus* pectinase specific activity up to (5.812 Umg^{-1}) by Sephadex G-200 Gel Filtration column chromatography (77). Saflastirilmasi *et al* purified pectinase from *Trichoderma viridi* by ion exchange column chromatography (sephadex G100), the enzyme purity increased 5.59 fold, with a specific activity of 97.2 U/mg (106). Mohsen *et al* purified polygalacturonase from *Asperigillus niger* U-86 by Sephadex G-75 column and obtained 9.5 fold of purification and 60.2 %. Recovery (107). Contreas *et al* purified 470 fold the PGI from a culture extract of *Aspergillus kawakii* with a recovery of 8.6 % of the initial activity in three steps: acetone precipitation, Sepharose Q and Sephacryl S-100 column chromatographies (108). The PG from *Thermoascus aurantiacus* was isolated with 21 fold increase in specific activity with a recovery of 24.6 % by Sephadex G-75 gel filtration followed by SP-Sepharose ion exchange chromatography (109).

Celestin et al purified 9.37 fold one pectinase produced by *Acrophialophora nainiana* which has exopolygalacturonase and pectin lyase activity, 60.6% of the enzyme was recovered after three steps: Sephacryl S-100 gel filtration, DEAE-Sepharose ion exchange and another gel filtration on Sephadex G-50 (110). Kashyap *et al* developed a purification strategy for the isolation of the pectin lyase from *Bacillus* sp. DT7 (26). The enzyme was precipitated with ammonium sulphate followed by DEAE-Sephacel and Sephadex G-150 column chromatographies. The pectin lyase produced by *Aspergillus flavus* was purified 58 fold with a recovery of 10.3% of the initial activity in three steps: ammonium sulphate fraction, DEAE-Cellulose ion exchange and Sephadex G-100 gel filtration (110). Semenova *et al* isolated five pectinases produced by *Aspergillus japonicus*, PGI, PGII, PEI, PEII and PL, by hydrophobic and ion exchange column chromatographies (111). The polygalacturonase from *Streptomyces lydicus* was purified with 57.1 fold increase in the specific activity and a yield of 54.9% after ultrafiltration followed by CM-Cellulose and Sephadex G-100 column chromatographies (112).

Conclusions

Most purification patterns for pectinases are based on multistep strategies. However, in recent years, new techniques have been developed that may be skillfully used, usually as a first step in purification processes leading to high recovery. A mixture of traditional chromatographic processes can indeed be expected to yield rapid and high recovery. As the tertiary structures of several pectinases are known today, one can also imagine

that the design of novel, highly specialized purification procedures based upon molecular recognition will be developed, allowing even easier and more efficient separation and recovery. An overview of various pectinase purification procedures shows that no conclusions can be drawn regarding an optimal sequence of chromatographic methods that maximizes recovery yields and purification fold. Based on the nature of the pectinase produced by the organism, one has to design the protocol for purification involving precipitation and chromatographic steps. However, it will also be guided by the purity of the enzyme required for its usage, which is important from the economic viewpoint. Very few examples of enzyme substrate affinity purification for pectinase exist. Affinity for cofactors and substrate analogues are other possibilities that have not been fully exploited in the purification of pectinases.

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Algorithm to Simulate a Chemically Induced DNA Logic Gate and Boolean Circuit

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Abstract

A simple proof-of-principle type of simulation of chemically implemented logic gate is proposed in this paper using the structural switching of single stranded DNA strand from i-motif to hairpin and vice versa which is triggered by regulating H^+ and copper (II) ions. The advantage of such chemically induced gates lies in its fast response time, controllability and reusability feature. Later on this simulation process is expressed in the form of algorithm to evaluate AND-OR Boolean circuit.

Key words: DNA, AND gate, OR gate, Boolean Circuit.

Introduction

Watson and Crick claimed novel prize in 1953 for the discovery of the double helical structure of DNA. For ages DNA is known as primary genetic material responsible for transmitting genetic information from one generation to another until Adleman(1), for the first time recognized the information processing property of DNA and demonstrated the first ever DNA synthetic computer by solving an instance of HPP. With time, enormous numbers of research are reported to employ DNA computing approach for solve several diverse fields of problems. DNA Boolean circuit simulation draws most of the interest and efforts. Ogihara and Ray (2,3) proposed the first DNA system that can simulate bounded Boolean circuit with complexity proportional to size of circuit. Since then several theoretical and experimental models and algorithms have been proposed. Analogous to silicon logic gate, molecular logic gate is expected to act as fundamental for biological or molecular computers (4)-(14).However the efficiency of most of such model is limited by involving too many error prone and time consuming biochemical processes. Recently secondary structure of DNA such as G-quadruplex and i-motif structure finds their way into several gate simulation models due to their unique properties such as highly specific binding properties, polymorphic versatility, and self assembly (15-19). There are several models reported where i-Motif structure coexisted with G-quadruplex but such models experienced the set back in terms of its complex maintenance and high cost (20,21). In few models G-quadruplex structure is solely used (22) whereas in some other models i-motif structure is solely used in construction of gate designing (23,24). Yunhua et al.(24) demonstrated DNA logic gates using only i-motif structural induction in response to the presence of H^+, Ag^+, and I^- as inputs. This simple technology has potential to simulate the functionality of OR and INHIBIT gate.

Henry Albert Day et al. (25) published the switchability of pH induced i-motif structure to hairpin and vice-versa. During the experiment they initially induced i-motif structure in human telomeric sequence at pH 5.5(folding time of 100 milli seconds) which was altered to hairpin structure by adding Cu^{2+} at room temperature without changing the pH . The folding time of i-motif structure to hairpin on adding Cu^{2+} was estimated to be 44 $\pm$ 2 seconds. Further they

successfully reverse the hairpin structure to i-motif structure by adding EDTA. The total time span of a complete cycle of folding or unfolding is 100 seconds only.

In this application based paper, it is proposed that AND-OR gate can be chemically induced by using the switching property proposed by H.A Day and his group. The author proposed an algorithm to simulate logic gates and evaluation of Boolean circuit using secondary structural switching of C-rich chain of DNA. Instead of encoding the gate strand and the input strands in the form of DNA sequence, in this paper the ions and pH are programmed and controlled. It is expected that this model have several advantages such as cost effectiveness, fast response time, reusability and easy implementation.

Preliminaries

DNA i-motif : For decades it's been believed that DNA remain in only B-form double helical structure as proposed by Watson and Crick but after extensive studies in following years it is well established that DNA can be found to be in several different secondary structures such as in A and Z forms, triplexes, three and four way junctions and quadruplexes. i-motif or i-tetraplex structure is one of such secondary structures of DNA which recently gains lot of interests. It is a four stranded structure formed by intercalating two pair duplexes of anti parallel orientation which are held together via hydrogen bond between cytosine$^+$–cytosine base pairs. There are several ways to enhance the formation of i-motif such as acidic pH of the solution or at neutral pH with the presence of certain ions like Ag^+ (27) etc. In human genome i-motif sequence present as complementary sequence of G-quadruplex forming sequence such as human telomeric i-motif sequence (hTeloC)(26). Several research works are reported expecting to use i-motif structures for anticancer drug development and gene regulation (28,29) and several other applications such as construction of biosensors(pH sensors), logic circuits, nanomachines and functional materials like proton-fuelled i-motif nanomotor, DNA ''bipedal walkers''.

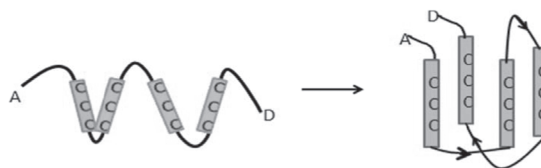

Fig. 1: Switching of C-rich sequence to i-motif structure

DNA hairpin : DNA hairpin structure is obtained when two self complementary segment in a single stranded DNA fold itself to self-hybridize. The resulting structure consists of two parts: stem part and the loop part. Stem part are the two self complementary sequences and the loop portion is the sequence which doesn't take part in the hybridization. The stem-loop structure owes its stability to the length, involvement of G-C pairs, and number of mismatches in the stem. Hairpin structure finds its application in several biomedical and nano-technology field.

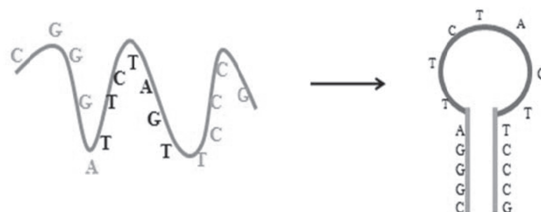

Fig. 2. Single stranded to hairpin structure.

Logic Gate and Boolean circuit simulation: In a Boolean circuit, computational units which are responsible for carrying out information processing are called gates, connected to each other by a network of inputs and outputs. The output processing of gates are based on the present inputs available at that moment and don't have any memory about the past. The size and the depth of the circuit are the standards to measure the complexity of any circuit. Boolean circuit can be categorized in three types depending on the inputs and outputs associated:

- Unbounded fan-in Boolean circuit: No limitation to the numbers of inputs to both AND-OR gates.

- Semi-unbounded fan-in Boolean circuit: AND gate is limited to maximum of two inputs and no limitation to the numbers of inputs to OR gate.
- Bounded fan-in Boolean circuit: Both AND and OR gates can have maximum of two inputs. In this paper a Bounded fan-in Boolean circuit is proposed.

i. AND gate simulation : AND gate evaluates 1 if all of its inputs are true otherwise it gives output as 0. The functionality of AND gate can be considered as serial connection. In this paper theoretic realization of AND gate is demonstrated at molecular level by regulating H^+ and Cu^{2+} ($CuCl_2$) content. The entire process can be represented in the form of an algorithm (illustrated in Fig 3):

AND_operator (main_strand, T_1)
{
T_1•! T_1 U main_strand;
if (X_1 = =0)
{
no titration of H^+ to T_1; //no change in the structure of main_strand
if (X_2==0 || X_2==1)
No Cu^{2+} is added; // no change in the structure of main_strand
Output =0;
}
else
if (X_1==1)
{
Titrate H^+ to T_1; // main_strand change to i-motif structure
if (X_2= =1)
add Cu^{2+} to T_1; // i-motif structure change to hairpin structure;
Output =1;
else
don't add Cu^{2+} to T_1;// No change in the i-motif structure;
Output =0;
}
}

Fig. 3. Algorithm for AND gate

In the above algorithm the main_strand is the DNA strand(5'-TAA-CCC-TAA-CCC-TAA-CCC-TAA-CCC-3') upon which all structural changes are observed, depending on the inputs (either H^+ or Cu^{2+} or both). T_1 is the test tube where the reactions are carried out. The output is read as 1 when the structure of main_strand attain the hairpin shape which happens only when both the inputs are present and in any other case the structure of main_strand remains in either linear strand or in i-motif structure which is read as output 0 (illustrated in Fig 4.).

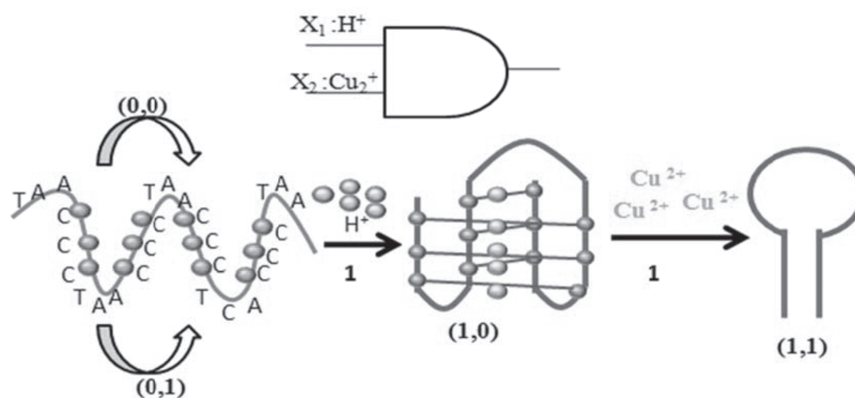

Fig. 4. Demonstration of AND gate.

Table 1: Two input AND gate

X_1	H^+	0	0	1	1
X_2	Cu^{2+}	0	1	0	1
Output		0	0	0	1

OR gate simulation : OR gate evaluates to 1 if any of its inputs are true otherwise as 0. The functionality of OR gate is like parallel connection. Unlike AND gate where H^+ and Cu^{2+} acts as inputs, OR gate simulation is realized regulating only H^+ in the test tube. The entire process can be represented in the form of an algorithm (illustrated in Fig 5):

ORgate_operation (main_strand, T_1)
{
T_1•! T_1 U main_strand;
if (X_1==1 || X_2==1|| X_1==1&& X_2==1)
Titrate H^+ to T_1;//main_strand change to i-motif structure
Output=1;
Else
No H^+ is titrated to T_1;// no change to main_strand
Output=0;
}

Fig 5. Algorithm for OR gate

Main_strand is a C-rich strand having potential to attain i-motif structure (5'-TAA-CCC-TAA-CCC-TAA-CCC-TAA-CCC-3'). When H^+ is added to a test tube containing main_strand the pH of the solution changed to slight acidic and hence i-motif structure is obtained. The output is read as 1 in case of this molecular OR gate when the structure of main_strand attains the i-motif structure, which happens when either of the input is present (illustrated in Fig 6.).

Table 2: Two input OR gate

X_1	H^+	0	0	1	1
X_2	H^+	0	1	0	1
Output		0	1	1	1

iii. Boolean Circuit Evaluation : Fig 7. illustrated a diagram of a three leveled Boolean circuit with level 0 consists of inputs, level 1 consists of intermediate gates (AND and OR) and level 2 has AND gate. Any Boolean circuit can be visualized as a directed acyclic graph with all the gates as nodes connected to each other in such a way that the output of one level serves as input to the higher level. The algorithm to emulate a Boolean circuit is shown in Fig 8.

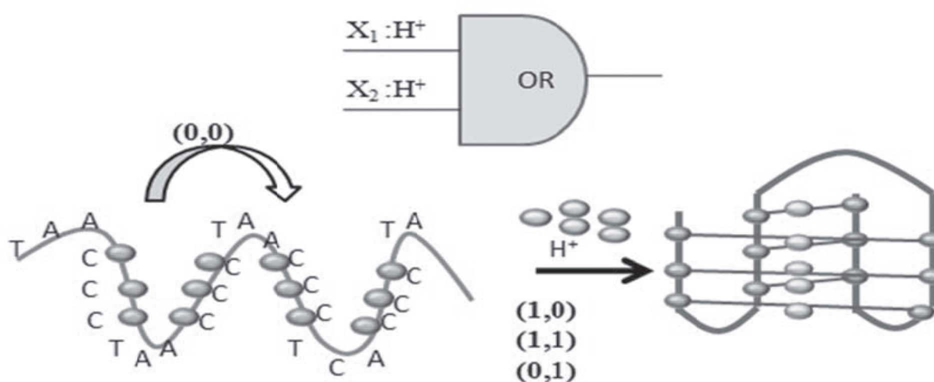

Fig 6. Demonstration of OR gate.

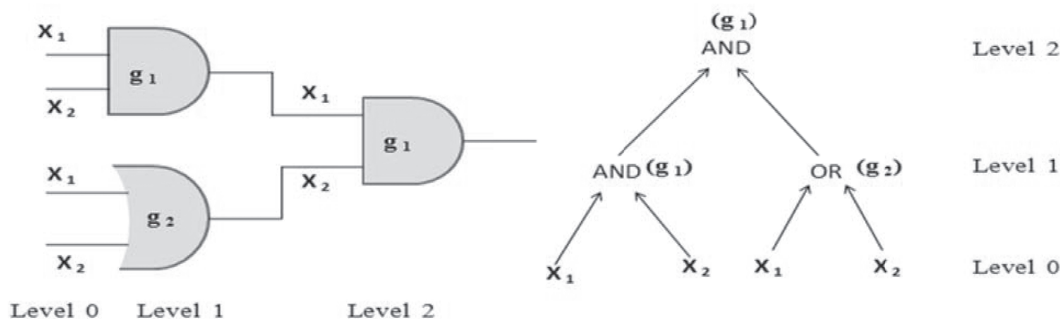

Fig 7. Instance of a AND-OR Boolean circuit.

```
Boolean_operator (main_strand,T1)
        for j=1 to j=level_max
             for k=1 to kÂ=gate_max
               if (gk==AND)
                 AND_operator(main_strand,T1);
                    if(gk==OR)
                ORgate_operation(main_strand,T1);
             END for
        END for
```

Fig 8. Algorithm for AND-OR Boolean circuit

As the time incurred for each cycle of switching is only a few 100 seconds [25], it can be concluded that the proposed logic gate has fast response time. Similarly the model is reusable as several cycles of switching are possible for same strand of DNA as the linear strand can be restored every time by controlling the amount and concentration of pH, Cu^{2+} or EDTA in the solution.

The model exploits the potential of three different structural outputs (linear, i-motif, hairpin) on the basis of pH condition and cationic concentration in the solution which provide a new insight to design DNA logic gate and Boolean circuits.

Conclusion

In this paper a new gate design strategy is proposed to simulate AND and OR gate individually as well as to evaluate any AND-OR Boolean circuit by controlling H^+ and/or Cu^{2+}. The conformational structural change of C-rich DNA strand to i-motif structure and hairpin structure is utilized throughout the operations. The advantage of such chemically induced gates lies in its fast response time, controllability and reusability feature. Also the model offers a great potential in designing several nano machines as three structural outputs are possible using same strand of DNA. However this model lacks the feature of full automation and parallelism.

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Statistical Optimization for Tannase Production by *Mucor circinelloides* Isolate F6-3-12 under Submerged and Solid State Fermentation

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Abstract

Mucor circinelloides isolate F6-3-12 was selected out of three fungal isolates that can utilize tannic acid as substrate. The fungus was identified by phenotypic analysis and 18S rRNA gene sequencing. Extracellular tannase was produced by solid-state fermentation (SSF) and submerged fermentation (SmF) from plants known for their high tannin content to be used as sole carbon source and supporting solid substrate namely pomegranate rind, sumac leaves and green tea leaves powder , pomegranate rind was selected for maximum tannase production under SSF. Optimization using sequential statistical strategy for some vital factors; substrate concentration, temperature, substrate: moisture ratio, incubation period, inoculum age and KH_2PO_4 were adjusted by the sequential two design of response surface methodology. *Mucor circinelloides* isolate F6-3-12 produced the highest tannase level (2.07 IU) during the first 72 h of fermentaton using pomegranate rind concentration of 1 g/flask in SSF then decline after 96 h. Tannase production was enhanced from 2.07 IU to 5.83 U/gds using Plackett-Burman design and improvement up to 12.24 U/gds upon applying the Box–Behken design that considered 2.82 and 2.1 fold enhancement.

Key Words: *Mucor circinelloides,* tannase, 18S rRNA gene sequencing, sequential statistical strategy, solid-state fermentation.

Introduction

Tannase referred as tannin acyl hydrolase (E.C. 3.1.1.20), is hydrolytic enzyme that is an inducible enzyme which hydrolyze ester bonds present in ellagitannins, gallotannins, complex tannins (1). They are significant enzymes distributed throughout the animal, plant and microbial kingdoms of wide application in food, pharmaceutical and chemical industries. Tannins are natural water-soluble polyphenols found in plants as secondary metabolites (2, 3). Industrially it is produced by microbial means using SmF, where the activity is expressed intracellular that imply additional costs in its production (4). Commercially, tannase were used for preparing instant tea, manufacture of acorn liquor also in gallic acid production, which is used as precursor in the synthesis of antibacterial agent trimethoprim, and also propylgallate as food preservative. Gallic acid also exhibits biological activities as antioxidant (5 ,6) and clarifying agent in juices and coffee-flavored drinks (1, 7) and printing inks. SSF has advantages over SmF techniques for tannase production as using available raw materials as substrates, low energy used, less expensive downstream processing, non-aseptic conditions, less water usage, little fermentation space, higher concentration of the products and high reproducibility (1, 8, 9, 10). Fungi are the most adapted microorganisms for SSF as they are known to tolerate low water and high osmotic pressure conditions and can utilize

agro wastes (11). Changing one variable of the fermentation conditions at a time keeping the others at fixed levels is time-consuming and never guarantees determination of optimal conditions. Designing suitable fermentation conditions is of significant importance because it reduces time, expense and increase product yield and productivity (12).

Materials and Methods

Chemicals: Tannic acid, Quinine HCL, Potato dextrose agar was obtained from Sisco India. Commercial green tea, pomegranate rind and sumac leaves were obtained from local market. Other chemicals were of analytical grade.

Microorganism and inoculums: Fresh fungal spores inoculated on potato dextrose agar were used as inoculum, 2 ml spore suspension (contain-ing around 10^6 spores/ml) was added to 100 ml of the production medium in 250 ml Erlenmeyer flasks and incubated at 30°C for 7days on a reciprocal shaker (200 rpm). The fungal strain was routinely grown on potato-dextrose agar (PDA) medium at 30°C and preserved at - 80ºC in 50%(v/v) glycerol.

Phenotypic and genotypic Characterization: Genetic identification using molecular taxonomy was performed to identify and study the phylogeny. Molecular identification of the selected fungal isolate was performed based on its internal transcribed spacer ribosomal DNA (ITS-rDNA) sequences. Mycelia were collected by centrifugation (at 4 °C and 5000 rpm for 20min) and DNA was extracted by using protocol of Gene Jet Plant genomic DNA purification Kit (Thermo#K0791). PCR was performed by using Maxima Hot Start PCR Master Mix (Thermo# K0221), and PCR clean up to the PCR product made by using GeneJET™ PCR Purification Kit (Thermo#K0701) in Sigma Company of Scientific Services, Egypt (www.sigma-co-eg.com). Finally, sequencing of the PCR product was performed a t GATC Company (German) by using ABI3730xlDNA sequencer, using forward and reverse primers, and by combining the traditional Sanger technology with the new 454 technology. Purified DNA was subjected to PCR amplification using a pair of ITS1(52 -TCCGTA GGTGAA CCTGCGG-32) and ITS4(52 -TCCTCCGCT TATTGATAT GC-32) primers for ITS-rDNA amplification (13). Sequence data was analyzed in the Gene Bank data base by using the BLAST program available on the National Center for Bio-technology Information website (www.ncbi.nlm.nih.gov). The unknown sequence was compared to all of the sequences in the database to assess the DNA similarities (14). Multiple sequence alignment and molecular phylogeny were performed using BioEditsoftware. The phylogenetic tree was displayed using the TREEVIEW program (15, 16).

Culture conditions: Two culture systems; submerged fermentation (SmF) and solid state fermentation (SSF) were used for tannase production. Composition of production medium (Modified Dox medium) was (g/L): $NaNO_3$; 6, KCL; 0.52, $MgSO_4.7H_2O$;0.52, KH_2PO_4; 0.52, $ZnSO_4$; 0.01, $FeSO_4$;0.01 (17) and 3gm green tea leaves powder per flask (In case of SSF 1gm of green tea leaves per flask) at 30°C, initial pH 5.5 and varying incubation time (200 rpm agitation for SmF). Two ml spore suspension were used as inoculum of the production medium, the growth kinetics of the fungal culture in SmF and SSF were periodically monitored. Crude enzyme extracts from SmF were obtained by filtering, while the extracts from SSF were obtained by centrifugation at 5000 rpm, all experiments were conducted in triplicate.

Three solid substrates which are pomegranate rind, sumac leaves and green tea leaves powder were examined for enzyme production. Each substrate was dried at 70 °C and finely powdered in a grinder mixer and used in SSF. These substrates were used as solid media for tannase production without any pretreatment. One gram powder of each substrate was added to 250 ml Erlenmeyer conical flasks, moistened with 3 ml of modified Dox medium of pH 5.5. The contents were autoclaved at 121.5°C for 20 min, cooled to room temperature and inoculated with 2ml of fungal spore inoculum. The

contents were mixed thoroughly and incubated at 30°C for different incubation periods.

Extraction of tannase enzyme: For the submerged fermentation, after incubation for the desired period, the fungal mycelia were removed by filtration through Whatman No.1 filter paper and the supernatant was treated as crude enzyme for assaying tannase activity. For solid state fermentation, after incubation, the fermented substrates were mixed properly by adding 50 ml of 0.2M acetate buffer (pH 5.5) to the fermented medium. Then, the flasks were kept on the rotary shaker for 1hr and centrifuged at 5000 rpm for 10 min to remove all fungal debris and residue of substrate (18) the clarified extract that represented the crude enzymes was used for assaying tannase activity.

Tannase assay: Tannase assay was estimated by the modified colorimetric method (19). Based on violet colour produced when $FeSO_4$ in presence of Rochelle salt (color reagent) that react with gallic acid. The reaction mixture contained 0.5ml of tannic acid (2.0 % w/v in 0.2M sodium acetate buffer, pH 5.5) was added to 0.5ml of crude enzyme. This reaction mixture was incubated at 40°C for 30 min. The enzymatic reaction was terminated by adding 3.0 ml of precipitant solution (100 ml Quinine HCL (1.0%) mixed with 50 ml of 10.0 % NaCl). A control was prepared side by side using heat denatured enzyme. Then 1ml of the mixture was transferred in open Dwarf tube and centrifuged at 9000 rpm in ultra micro-centrifuge for 15 min. 300µl of the supernatant was transferred to a dry clean test tube and mixed well with 0.5ml of the color reagent. The volume was completed to 5 ml by adding distilled water. The developed color (reddish violet) was measured at 555 nm against boiled enzyme as control, using Spectro UV-VIS labomed.Inc.USA. One unit of enzyme (international units) was defined as the amount of enzyme able to release 1 ìmol gallic acid per minute of culture filtered under the standard assay conditions.

Determination of hydrolysable tannins: One gram of each of green tea or sumac leaves or pomegranate rind was mixed with 10 ml water, autoclaved, then substrates were mixed properly by adding 50 ml of 0.2M acetate buffer (pH 5.5) to each substrate. Then, the flasks were kept on the rotary shaker for 1hr and centrifuged at 5000 rpm for 10 min to remove all residue of substrate; one ml of the clarified extract was transferred to a clean test tube. The tannin compounds are precipitated by adding 2 ml of precipitant solution. One milliliter of the mixture is transferred to Open Dwarf tube and centrifuged at 9000 rpm for10 min. Five hundred micro liter of the supernatant was transferred to a clean and dry test tube and mixed with 0.5 ml of the color reagent. The volume was completed to 5 ml by adding distilled water. The developed color was measured at 555nm. The reading of OD ×6.0 is equivalent to hydrolysable tannins (19).

Protein content: The protein content was determined according to Lowry method (20).

Culture conditions

Effect of incubation time: The fungal culture was inoculated in the autoclaved SSF flasks then incubated at 30°C for different time periods ranging from 72h to 216 h. The enzyme was extracted as discussed above and the crude enzyme was preserved at 4°C for further analysis.

Effect of moistening agents: Different types of moistening agents; modified Dox medium, tap water and distilled water were examined for their role in enzyme production. The solid substrates were moistened by one of these moistening agents and mixed properly, autoclaved, inoculated and incubated at 30°C for the proper time. Then, the enzyme was extracted as above and assayed for tannase activity.

Effect of moisture level: The solid substrate was moistened using modified Dox medium with different ratios (w/v) ranging from 1:4, 1:5and 1: 6 to determine the best ratio of substrate: moistening agent for enzyme production under SSF. The enzyme was extracted and assayed for tannase activity.

Statistical analysis

Plackett-Burman design:The Plackett-Burman experimental design is a fractional factorial design

recommended when more than five factors are under investigation (21,22). This design is practical, especially when the investigator is faced with a large number of factors and is unsure which settings are likely to produce optimal or near optimal responses (23). In this study, the design was used to reflect the relative importance and concentrations of some medium components and fermentation conditions on tannase activity. Seven independent variables were screened in nine combinations, organized according to the Plackett Burman design matrix described in the results section. For each variable, a high level (+) and low level (-) was tested. All trials were performed in duplicates and the averages of products percentage were treated as the responses. The main effect of each variable was determined by the following equation:

$$Exi = (©Mi^+ - ©Mi^-) / N$$

Where Exi is the variable main effect, Mi^+ and Mi^- are tannase activity in trials where the independent variable (xi) was present in high and in low levels, respectively, and N is the number of trials divided by 2.

Box-Behnken Design: In the second phase of medium formulation for optimum tannase activity, the Box-Behnken experimental design was applied (24). In this model, the most significant independent variables, named (X_1), (X_2) and (X_3) were included and each factor was examined at three different levels, low (-), high (+) and central or basal (0). These factors included incubation time (X_1), concentration of $NaNO_3$ (X_2) and moisture content (X_3), all were treated as independent variables. Thirteen combinations and their observations (shown in the results section) were fitted to the following second order polynomial mode:

$$Y=b_o+b_1X_1+b_2X_2+b_3X_3+b_{12}X_1X_2+b_{13}X_1X_3+b_{23}X_2X_3+b_{11}X_1^2+b_{22}X_2^2+b_{33}X_3^2$$

Where, Y is the dependent variable (tannase activity); X_1, X_2 and X_3 are the independent variables; b_o is the regression coefficient at center point; b_1 , b_2 and b_3 are linear coefficients; b_{12} , b_{13} and b_{23} are second-order interaction coefficients; and b_{11}, b_{22} and b_{33} are quadratic coefficients. The values of the coefficients were calculated and the optimum concentrations were predicted using JMP software. The quality of the fit of the polynomial model equation was expressed by R2 (regression coefficient). If the proposed model is adequate, as revealed by the diagnostic tests provided by an analysis of variance (ANOVA), contour plots can be usefully employed to study the response surface and locate the optimum operational conditions (25). The 3D graphs were generated to understand the effect of selected variables individually and in combination to determine their optimum level for maximal production of tannase. The F-test was calculated to determine factors having a significant effect.

Results and Discussion

Biosynthesis of tannase enzyme under submerged fermentation: The present part of the study aims to the examination of three fungi for the biosynthesis of tannase by submerged culture using green tea as substrate. Fermentation was undertaken by shaking culture technique at 30°C using production medium contain 3% green tea in Erlenmeyer flask (250 ml) contain 50 ml modified Dox medium. Fermentation lasted for 2, 3, and 4 and 5 days at 30°C, the fermentation broth was centrifuged and assayed for tannase activity, final pH and protein content.

The results given in table-1 indicated that no relationship between the final pH and the synthesis of tannase, in all cases, the pH values of the culture filtrate of the investigated cultures varied from 4.26-7.83. The results also indicated that the synthesis of tannase depends on the fungus and on the time of incubation. Isolate 1 exhibited the highest tannase activity (1.78 U/ml after 3 days). The other two fungal species showed lower tannase activities; 0.22U/ml after 48 h for isolate 2 and 0 .15 U/ml for isolate 3 after 48 h. Of all the three investigated fungi,isolate 1 was the most promising tannase producer, therefore it was selected for further investigation.

Identification of the fungus: Sequence analysis of 18S rDNA of the selected fungus (isolate 1)

with available NCBI Gen Bank database showed that the tested fungus revealed high identity (99%) to *Mucor circinelloides* (Gene Bank accession number KX349462). The phylogenetic tree was displayed using the TREEVIEW Program that showed the sequences of close relatives obtained from Gen Bank to resolve the phylogenetic relations with ancestor (Fig. 1). *Mucor* is commonly used in industry to produce important organic acids and other industrial enzymes.

Biosynthesis of tannase enzyme by Mucor circinelloides isolate F6-3-12 ***using solid state fermentation (SSF):*** In this experiment three agriculture residues namely pomegranate rind, sumac leaves and green tea powder were used as substrates. This was carried out by inoculating the selected fungus ***Mucor circinelloides*** isolate F6-3-12 in 250 ml Erlenmeyer flasks each containing 3gm of each substrate and 10 ml modified dox medium. At the end of the fermentation period, the fermented substrates were treated as mentioned above. Data recorded in table (2) showed that by using pomegranate rind powder the tannase activity reached 3.82 U/gds (U/g substrate) with high protein content (354 mg/g substrate) this may be due to the highest tannin content of pomegranate rind powder among the other considered substrates (hydrolysable tannin 8.9%).

It's clear that pomegranate rind powder was the best inducer for tannase biosynthesis (3.82 U/gds) after 3 days with specific activity 0.01 and decrease to 2.62U/gds after 4 days, the enzyme production decreased due to the depletion of the substrates. Therefore pomegranate rind powder was used as a sole solid substrate a in the next experiments instead of green tea. Utilization of agricultural wastes into valuable products helped in solving waste disposal problems.

Results obtained from this comparative study of tannase production using both SmF and SSF systems showed that ***Mucor circinelloides*** isolate F6-3-12 had a higher tannase activity using SSF (more than 1.85 times) as compared to SmF, and this observation is similar to the results previously reported (7). It is important to note that for tannase production, the used model (substrate- support) resulted in enhanced enzyme induction and also indicates that ***Mucor circinelloides*** isolate F6-3-12 strain can be adapted for SSF system and utilizes the nutrients in a better form than when it is grown in other culture systems, like SmF. The other two substrates namely sumac leaves and green tea

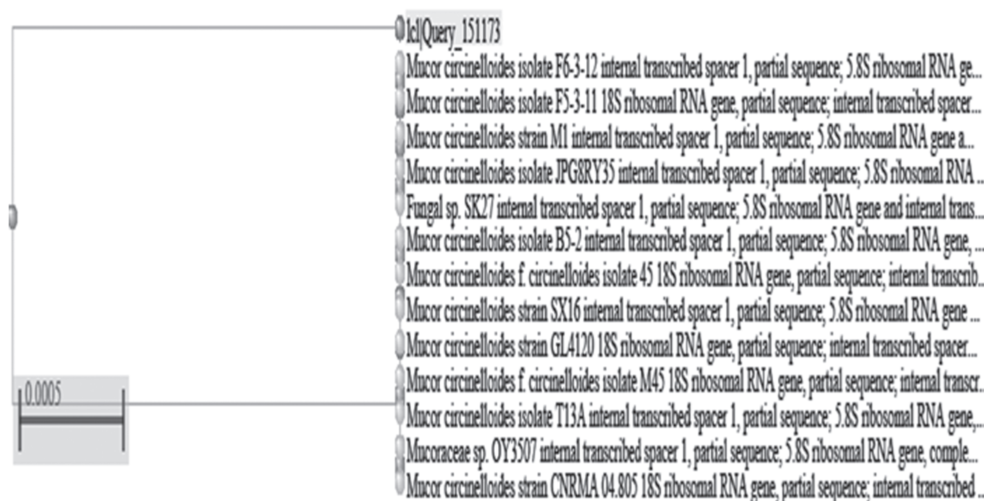

Fig. 1. The phylogenetic tree of *Mucor circinelloides* isolate F6-3-12

leaves produce 2.07 U tannase /gds respectively at 72h. After 120 h, there was a reduction in the amount of tannase due to the depletion of the substrates and the accumulation of gallic acid as reported by Kar *et a*l., (26). Also Bradoo *et al.* (27); and Aguilar and Gutiérrez-Sánchez, ((1) mentioned that tannase is produced during the phase of growth and thereafter declines, also gallic acid showed end product repression (26,27,28). Maximum tannase production was obtained using pomegranate rind as substrate, so it was selected for our further studies on tannase enzyme production.

Influence of incubation time on tannase production: The effect of the incubation period (3, 5, 7 and 9 days) on the tannase productivity was investigated at 30°C. The results (table 3) indicated that an incubation period of 3 days was the most appropriate for the production of tannase (3.82U/gds) with specific activity 0.01U/mg protein, after 5 days the activity decrease to 2.62U/gds with specific activity 0.007U/mg protein and at 7 days decrease to 0.76 U and no activity was detected after 9 days. From the mentioned data, incubation periods of 3 and 5 days were chosen for extra investigation.

Effect of moisturizing agents on tannase biosynthesis:In this experiment the effect of different types of moisturizing agent (dox medium, tap water and distilled water) was examined for tannase biosynthesis. The pomegranate rind was moistened with one of these moistening agents, mixed, autoclaved and incubated at 30 ^{o}C for 3 and 5 days. The results in table (4) indicated that dox medium was the most favorable moisturizing agent with activity 3.82 U/gds and specific activity 0.011U/mg protein after 3 days then decreased to 2.62 after 5 days, followed by tap water and distilled water gave 3.17 U/gds after 3 days, and after 5 days the activity decreased to 2.61 and 2.13 respectively. . Our results agree with that reported by Beniwal *et al*. (29). They found that Czapek Dox medium was the best moisturizing agent for enzyme (2.3U/g) production followed by tap water and distilled water. Majit *et al*. and others (30,31,32) found that tap water was the best moisturizing agent for enzyme production. Ahmed *et al*. (33) also reported distilled water-mediated extraction from the fermented masses.

Extraction of the enzyme from solid substrate at the end of the fermentation process is critical step as it determines the cost of enzyme production due to bounding of the enzyme with the fungal mycelia by means of different noncovalent bonds.

The modified Dox medium was used in different ratios (w/v): 1:4, 1:5 and 1:6 to moist the substrate, a ratio of 1:6 was found to be the best for tannase yield due to the low oxygen supply as the moisture level increases hence a reduction in both enzyme production and biomass. Our results agree with Jana *et al*. (34) using 1:6 solid to solvent ratio.

Optimization of Mucor circinelloides isolate F6-3-12 tannase biosynthesis by multi-factorial design: Fermentation conditions required for maximum rate of tannase biosynthesis were then explored by carrying out a two- phase multi-factorial optimization approach. The first approach involved the application of an incomplete factorial experiment to screen for the optimum culture conditions and medium composition that affect the tannase biosynthesis. The second part was to optimize the most significant factors using a response surface methodology.

Elucidation of fermentation factors affecting tannase biosynthesis: The Plackett- Burman design (21) was used to determine the different culture and medium conditions involved on tannase biosynthesis process. Seven variables were used at two levels, high concentration (+) and low concentration (-) as showed in table (5). The factors tested were shown in Table (6); incubation time, sodium nitrate, KHP_2O_4, moisture level, substrate concentration, temperature and inoculums age. Each row represents a trial run and each column represents an independent variable. All experiments were performed in triplicates and the results (average of the observations) of tannase activity and protein content were calculated. The difference between

Table 1. Survey of some fungal strains for the biosynthesis of tannase in submerged culture using green tea as carbon source

Strain	Final pH Activity U/ml	Tannase mg/ml	Total proteins	Specific activity (U/mg protein)
isolate 1				
48 hrs	4.26	1.08	29.5	0.037
72 hrs	4.80	**1.78**	29.5	0.06
96 hrs	5.27	0.86	43.0	0.02
120 hrs	5.02	-	45.6	-
Isolate				
248 hrs	7.08	0.22	23.3	0.009
72 hrs	6.55	0.073	25.0	0.003
96 hrs	6.52	0.073	30.1	0.002
120 hrs	6.84	0.044	33.0	0.001
Isolate 3				
48 hrs	7.83	0.15	19.5	0.007
72 hrs	7.7	0.073	21.0	0.003
96 hrs	7.39	-	23.0	-
120 hrs	7.22	-	23.0	-

Table 2. Effect of different substrates (containing tannins) as carbon source for biosynthesis of *Mucorcircinelloides*isolate F6-3-12 tannase enzyme by using solid state fermentation (SSF) technique

Substrate	Tannase (U/g ds)	Total proteins (mg/ml)	Specific activity (U/mg protein)
Pomegranate rind72 hrs120 hrs	3.822.62	354354	0.010.007
Sumac leaves72 hrs120 hrs	2.07-	768503.9	0.003
Green tea leave72 hrs120 hrs	2.07-	336372	0.006-

Amount of initial gallic acid in natural substrates (mg/g) :

Pomegranate rinds: 89.520; Sumac Leaves : 57.524; Grean tea leaves : 70.952

both averages of measurements made at high level (+) and the low level (-) of the factor was estimated as the main effect of each variable upon tannase biosynthesis (Table-7).

Table-6 showed a wide variation tannase activity from 0.12 to 5.83 U/gds, this reveal that medium optimization is necessary for high enzyme production. The main effects of the examined factors on the enzyme activity were calculated and ranked by analyzing the Plackett-Burman design data. The tannase biosynthesis is positively affected by incubation time, moisture level and $NaNO_3$ concentration, and temperature in the medium. On the other hand other variables had the negative effect.

A formula composed of (g/ l) is predicted to be near optimum: pomegranate rind, 7g; K_2HPO_4, 2.0; sodium nitrate, 7.0; inoculum age, 9 days

Table 3. Effect of incubation period on *Mucorcircinelloides*isolate F6-3-12tannase biosynthesis under Solid State Fermentation

Time	Tannase (U/g ds)	Total proteins (mg)	Specific activity (U/mg protein)
3 days	3.82	354	0.011
5 days	2.62	354	0.007
7 days	0.76	516	0.001
9 days	-	546	-

Table 4. Effect of moisturizing agents on *Mucorcircinelloides*isolate F6-3-12tannase biosynthesis

Moisturizing agents	Tannase (U/g ds)	Protein (mg/g ds)	Specific activity (U/mg protein)
Modified Dox media			
72 hrs	3.82	258	0.015
120 hrs	2.62	192	0.014
Tape water			
72 hrs	3.17	300	0.011
120 hrs	2.61	270	0.010
Distilled water			
72 hrs	3.17	192	0.016
120 hrs	2.13	108	0.019

Table 5. Factors examined as independent variables affecting Tannase biosynthesis by *Mucorcircinelloides*isolate F6-3-12 and their levels in the Plackett-Burman experiment.

Factor	Symbol	-	0	+
Time	T	2	3	4
Sodium nitrate% $NaN0_3$	NN	5	6	7
KH_2P0_4%	K	1	1.5	2
Moisture level	M	1:4	1:5	1:6
Substrate concentration	S	3	5	7
Temperature	Temp	27	30	33
Inoculum age	in	5	7	9

Table 6. Optimization of media fermentation for the biosynthesis of Mucorcircinelloidesisolate F6-3-12 tannase with Plackett-Burmandesign

Trial No.	T	NN	K	M	S	Temp	IN	Tannase (U/g ds)	Protein (mg/g ds)	Specific activity (U/mg protein)
1	-	-	-	+	+	+	-	1.62	215.6	0.007
2	+	-	-	-	-	+	+	0.42	213.9	0.001
3	-	+	-	-	+	-	+	0.63	217.1	0.002
4	+	+	-	+	-	-	-	3.79	269.1	0.014
5	-	-	+	+	-	-	+	0.12	120.7	0.001
6	+	-	+	-	+	-	-	0.52	274.8	0.001
7	-	+	+	-	-	+	-	0.31	182.4	0.001
8	+	+	+	+	+	+	+	5.83	316.1	0.018
9	0	0	0	0	0	0	0	3.82	223.8	0.017

Table (7): Main effects of independent variables on tannase biosynthesis produced by *Mucorcircinelloides*isolate F6-3-12according tothe results of Plackett-Burman design

Independent variables	Main effect
Time	2.06
Na NO_3	1.88
KH_2 PO_4	0.03
Moisture	2.7
Substrate	0.8
Temperature	0.97
Inoculum age	0.24

Table 8. Examined concentration and the results of the Box-Behnkendesign experiment

Trial	Independent variable			Tannase activity (U/g ds)	Total protein (mg/g ds)	Specific activity (U/mg protein)
	X1 Incubation period	X2 Moisture level	X3 $NaNO_3$			
1	-	-	0	0.42	316.7	0.001
2	+	-	0	3.49	428.7	0.008
3	-	+	0	3.14	214.3	0.01
4	+	+	0	0.4 4	287.4	0.001
5	-	0	-	6.22	347.1	0.017
6	+	0	-	0.87	187.2	0.004
7	-	0	+	12.24	136	0.09
8	+	0	+	11.99	318.3	0.037
9	0	-	-	0.32	315.1	0.001
10	0	+	-	0.41	333.4	0.001
11	0	-	+	1.11	284.7	0.003
12	0	+	+	9.89	496.9	0.02
13	0	0	0	8.14	365.4	0.022

Table 9. Results of ANOVA analysis for optimization of *Mucorcircinelloides*isolate F6-3-12Tannase enzyme production by the Box-Behnken experiment.

Term	Coefficient estimate	*df*	SE	Sum of Squares	t-value	*F*-value	*P*-Value
Corrected Model	-	9	-	262.58678	-	6.9408	0.0230*
Intercept	8.14	1	1.183722	-	6.88	-	0.0010*
X_1	-0.66625	1	0.724879	3.55111	-0.92	0.8448	0.4002
X_2	1.055	1	0.724879	8.90420	1.46	2.1182	0.2053
X_3	3.42625	1	0.724879	93.91351	4.73	22.3412	0.0052*
$X_1 * X_2$	-1.5675	1	1.025134	9.82823	-1.53	2.3381	0.1868
$X_1 * X_3$	1.275	1	1.025134	6.50250	1.24	1.5469	0.2687
$X_2 * X_3$	2.1725	1	1.025134	18.87903	2.12	4.4912	0.0876
X_1^2	-0.7475	1	1.066993	0.52650	-0.70	0.1253	0.5148
X_2^2	-5.645	1	1.066993	119.77498	-5.29	28.4935	0.0032*
X_3^2	0.4375	1	1.066993	0.70673	0.41	0.1681	0.6988

R^2 =R Squared = 0.92589(Adjusted R Squared = 0.792492)

with initial pH 7.0 at 33 °C for 4 days incubation. The remaining variables with less significant main effect were used in all next trials at their zero level; this optimized medium was used for further investigations.

A verification experiment was carried out in triplicate for determining the accuracy of the Plackett- Burman design, the applied near optimum condition resulted in 5.83 U/gds tannase biosynthesis. These results represent about 1.53 fold increases in tannase biosynthesis in comparison to the results showed using the basal condition (3.82U/g).

Culture conditions optimization by Box – Behnken design: In order to search for the optimum concentration of the most significant variables (incubation time, sodium nitrate and moisture level), experiments were performed according to Box - Behnken experimental design. The coded and levels of the three independent variables investigated at three different levels (-1, 0, +1) were listed in Table (8) with 13 trials.

The regression equation coefficients were calculated, and the data were fitted to a second-order polynomial equation.

$$Y_{activity} = 8.14 - 0.66625X_1 + 1.055\,X_2 + 3.42625X_3 - 1.5675\,X_1X_2 + 1.275\,X_1X_3 + 2.1725X_2X_3 - 0.7475\,X_1^2 - 5.645\,X_2^2 - 0.4375\,X_3^2$$

Y activity is the response (tannase yield) and X_1, X_2 and X_3 are the coded values of the test (incubation period, moisture level and sodium nitrate), respectively. The two dimensional contour plots as the graphical representations of the regression equation (Fig.2) determine the interactions of the three factors and showed the optimum levels of each and their interactions using SAS JMP 8 NULL program tools.

Regression results from the Box- Behnken designed were shown Table (9). The more significant is the corresponding coefficient reflected the high magnitude of the *t*- value and low magnitude of the *p*- value. The variable with the largest effect was the moisture level- the linear effect- and the squared term of sodium nitrate, the results obtained in this study are obviously lower than those reported by Aguilar and Gutiérrez-Sánchez(1), Aguilar *et al.* (28), Kar and Banerjee (35) and Kar *et al*. (36). Liu *et al*., (37) reported that maximum production of tannase by *Aspergillus spp*.UCP1284 (SSF) using a factorial design (2^3) was 12.26U/g dry substrate with initial moisture content of 40% and using 2.0% of tannic acid.

(R^2) was calculated as 0.92 for tannase activity which indicates that the statistical model can explain 92.1 % of variability in the response (a value of R2 > 0.75 indicated the correctness of the model). The aptness of the model can be tested by the value of coefficient (R^2) and correlation coefficient (R), the closer the value of R to 1, the better the correlation between the measured and the predicted values.

Verification of the optimization methods: A verification experiment was done using the predicted optimal conditions for tannase biosynthesis while the basal medium was used as control. Under the optimization condition 12.24 U/gds tannase biosynthesis was reached. The result indicated that the optimization conditions accelerated about 5.91 fold times more tannase yield (12.24/g) of the basal medium (2.07 U/g). The two steps of optimization resulted in a formula of the following composition (g/l): pomegranate rind, 7g/flask; KH_2PO_4, 2.0; $MgSO_4.7H_2O$,0.52; KCl, 0.52; $ZnSO_4\,8H_2O$,0.01 and $FeSO_4\,8H_2O$,0.01 at initial pH 5.5 after 72 h incubation with moisture content of 1:7 under solid state fermentation.

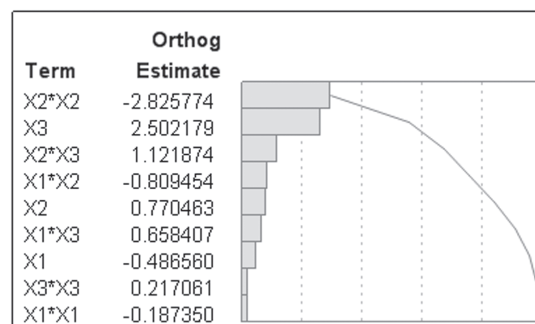

Fig. 2. Pareto Plot of Transformed Estimates

Conclusions

The present investigation suggests that agro residues such pomegranate rind are one of the best and most cost effective alternatives for the costly pure tannic acid for industrial production of microbial tannase. The culture conditions for the production of tannase enzyme from *Mucor circinelloides* isolate F6-3-12 were evaluated and standardized. These conditions were: solid-state fermentation, incubation temperature of 30 °C, fermentation time of 72 h. The first step response surface methodology resulted in a 2.8 fold increase in tannase production and 2 fold after the second step. As the range of application of this enzyme is very wide there is always a scope for novel tannase with better characteristics, which may be suitable in the diverse field of applications. By using cheaper and easily available substrates and use of dox medium as moistening agent without need of any mineral salt, we have tried to lower down the input cost for enzyme production which is one of the reasons that limit the use of tannase at the industrial level and thus it can be possible to use this enzyme in large scale in beer, food, fruit and leather industries.

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CRISPR: Genome -Editing and Beyond

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Abstract

The clustered regularly interspaced short palindromic repeats (CRISPR)/Cas system has been seized upon with a fervor enjoyed previously by small interfering RNA (siRNA) and short hairpin RNA (shRNA) technologies and has enormouspotential for high-throughput functional genomics studies. Editing via (CRISPR)–CRISPR-associated (Cas) constitutes a next-generation method for programmable and high throughput functional genomics. CRISPR–Cas systems are readily reprogrammed to induce sequence-specific DNA breaks at target loci, resulting in fixed mutations via host-dependent DNA repair mechanisms. Bacteria and archaea acquire resistance to invading viruses and plasmidsby integrating short fragments offoreign nucleic acids at one end of theCRISPR locus. CRISPR loci are transcribedand the long primary CRISPRtranscript is processed into a library of small RNAs that guide the immune system to invading nucleic acids, whichare subsequently degraded by dedicated nucleases.

Key words: CRISPR, bacterial immunity, gene editing

Introduction

Microbes have devised various strategies that have helped them to survive exposure to foreign genetic elements. Considerable proportions of bacterial and archaeal genomes consist of genes derived by horizontal gene transfer among related or unrelated species via transduction, conjugation, and transformation (1). This has forced microbes to establish an array of defense mechanisms that allows them to recognize and distinguish foreign DNA from self DNA. These systems maintain genetic integrity, yet occasionally allow exogenous DNA uptake and conservation of genetic material that gives an advantage for the bacteria to adapt in an environment. Recently, an adaptive microbial immune system has been identified as clustered regularly interspaced short palindromic repeats (CRISPR) that protects bacteria againstforeign genomes (2).

Clustered regularly interspaced short palindromic repeats are segments of prokaryotic DNA containing short repetitions of base sequences. Each repetition is followed by short segments of “spacer DNA” from previous exposures to a bacteriophage virus or plasmid. The latest analysis shows that CRISPR loci are composed of 21- to 48-base pair (bp) direct DNA repeats interspersed with nonrepetitive nucleotides of 26 to 72 bp called spacers. Repeats and spacers are conserved within a given loci. Each CRISPR locus is defined by the sequence of the repeat and the typical repeat is defined as the most frequent within aCRISPR locus.The number of repeats within a CRISPR varies between strains of the same microbial species. Two to 375 repeats have been found per locus. The number and the sequence of the spacers also vary between strains of the same microbial species(3).There are no open reading frame is present within CRISPR loci. Upstream of the CRISPR locus is a leader region containing 20 to 534 bp which

contain high adenine and thymine content(4). Within this leader region lies a promoter which helps in the transcription of the CRISPR. Finally, a few CRISPR-associated (*cas*) genes are almost always found in the vicinity of the CRISPR region. Adjacent to these CRISPR repeats and the spacer DNA is a set of conserved CRISPR-associated (*cas*) genes that encode the Cas proteins (5).

A CRISPR/Cas system, also named CRISPR-associated system, usually contains between 4 and 20 different *cas*genes. These genes are found both upstream or downstream of the repeat/spacer region. CRISPR along with Cas proteins, forms the CRISPR/Cas systems. Six "core" *cas* genes have been identified. Besides the *cas1* to *cas6* core genes, subtype-specific genes and genes encoding "repeat-associated mysterious proteins" (RAMP) have been identified and grouped into subtypes functionally paired with particular CRISPR repeat sequences (6).

CRISPRas an adaptive immunity in prokaryotes: Prokaryotes belonging to the most varied groups contain a peculiar type of DNA, repetitive in nature which was recognized in 2000 as a family (7), distinguished by the regular spacing of the recurrent motif and consequently defined as short regularly spaced repeats (SRSR).Clustered regularly interspaced short palindromic repeats (CRISPRs) are a novel class of repetitive () DNA (8) that has been identified in 88% of the archaea bacterial genomes and 39% of the eubacterial genomes that has been sequenced so far (9).

It has been found that some of the spacer sequences match with the fragments of extra-chromosomal elements (mainly from plasmids and virus genomes) and thus has led to the hypothesis that the CRISPR–Cas system might be a novel defense system that is able to protect a host cell against invading alien nucleic acid (10,11). As reviewed here, over the course of the past few years this hypothesis has been confirmed experimentally (12), and thus three distinct stages are recognized in the CRISPR defense mechanism: (i) adaptation of the CRISPR via the integration of short sequences of the invaders as spacers; (ii) expression of CRISPRs and subsequent processing to small CRISPRguide RNAs; and (iii) interference of target DNA by the crRNA guides. Recent analyses of key Cas proteins indicate that, despite some functional analogies, this fascinating prokaryotic immune system shares no phylogenetic relation with the eukaryotic RNA interference system (13).

Acquisition of new spacer sequences : The acquisition of new spacers either viral or plasmid fragments occurs at the leader (L) side of the CRISPR (14). The leader sequence includes a binding site for proteins (probably Cas proteins) that are responsible for repeat duplication and/or spacer acquisition (15). Both sense and anti-sense spacer sequence orientation turned out to be functional, however, only the leader strand is transcribed. This indicates that the mechanism at the level of mRNA does not operate via classical anti-sense mechanism, suggesting that DNA is the target. After disruption of the gene encoding, acquisition of new spacers seemed to be affected strongly suggesting its role in CRISPR adaptation (16).

It has been suggested that the spacer acquisition can also be due to non-homologous recombination. It was also reported that the mRNA were the source of the new spacers. The presence of a gene codingfor a putative reverse transcriptase (RT) the lies within thevicinity of the *cas* genes explained the presence of spacers that correspondsto coding and noncoding strands. However, manyCRISPR/Cas loci lack such a gene (17). Thus, the most likely source for the new spacers is thought to be dsDNA (18,19).

Adaptation to plasmids and predatory phages byspacer acquisition has been shown to occur readily inseveral species. In the course of studies of phage therapy, M102 phages wereintroduced into rats, for the prevention of tooth decayto eliminate *Streptococcus mutans*,which is the mainly responsible for dental cavities.Following this, bacterio phagein sensitivemutants were isolatedthat had added an

M102matchingspacer sequence toone of the two CRISPR arrays in this species (20). Similaradaptation can be induced in*S. thermophilus* cultures bychallenging the cultures by phage (21).

The studieshave revealed that the new spacers are inserted at theleader end of the CRISPR array and most of the integration shappens to be at the first position in the cluster. The loss of one or more repeatspacer sequences has also been observed, which suggests that CRISPRs do not grow unchecked (22). Commonly single repeatspacer unit gets added, but up to four new units have been detected (23).

CRISPR expression : Expression of the CRISPR regions was first described small RNA profiling studies of the archaea *Archaeoglobus fulgidus*and*S. Solfataricus* (24, 25). It has beensuggested that longer transcripts (termedpre-crRNA) of repeats and spacers, potentially covering the entire CRISPR, wereprocessed to small crRNAs. It has been observedthat in E. coli K12, the leader strand of the CRISPR region is transcribed and processed (12). In the expression studies of CRISPRsfrom the bacterium *Staphylococcus epidermidis* (26), andthe archaeon *P. furiosus* (27) similar type of observations has been recorded.

In contrary to this, it has been reported that the transcriptionhas been observed not only for the leader strand but also forthe complementary strand of CRISPRs in the archaealgenus *Sulfolobus*.CRISPR transcription initiates at the end of the locus that contains theleader sequence, and the CRISPR promoter might evenreside within the leader itself. Since transcription is constitutive and unidirectional in nature but one possible exception to unidirectionalityhas been reported (28).

The first biochemical insight into the molecular mechanismof CRISPR processing was obtained by analyzing E.coli strain K12 (12). This bacterium possesses a singlecluster of *cas* genes that encodes eight proteins of which three are well-conserved proteins, Cas1 (integrase/ nuclease), Cas2 (nuclease) and Cas3 (helicase/ nuclease). The other five-proteins viz; Cse1–Cse2–Cse4–Cas5e–Cse3 are more variable in nature. These variable proteins are also referred to asCasABCDE and constitutes a Cascade.It has been observed that in *Thermusthermophilus,*cyclic AMP regulator proteinupregulates *cas* genes (29,30) and thus the CRISPR response may be regulated bycAMP signal transduction. In *S. mutans*,increased expression of *cas* genes has been reported following the analysisof the transcriptome of a *clpP* protease mutant, which suggests theregulation of CRISPR loci (31).

CRISPR Interference **:** It has been seen that the interference stage of the CRISPR/Cassystem somewhat resembles the eukaryotic RNA interference(RNAi(32). But it is a misguided hypothesis that CRISPR mediates microbial immunity via RNA interference (17). RNAi silences the foreign invading nucleicacid sequence before or after it integrates intothe eukaryotic host chromosome, and/or prevent cellularprocesses through a small interfering RNAguide (33). On contrary to this, CRISPR-encoded immunity involves the enzymatic machinery. Although both the mechanismsinvolve a guide RNA in an inhibitoryribonucleoprotein complex, only Dicer, Slicer,and the RNA-induced silencing complex (RISC)may have analogous counterparts (34).

CRISPR systems, together with *cas* genes shows high diversity in mechanisms of adaptable immunity used by many bacteriaand archaea in protecting themselves from invading viruses, plasmids,and any other foreign nucleic acids (35). CRISPR interference occurs differentlyin different CRISPR systems. In one system, Cas6e/Cas6f cut at the junction of single-stranded RNA (ssRNA)and double-stranded RNA (dsRNA) formed by hairpin loops. In other systems,trans-activating (tracr) RNAs are used to form dsRNA which are cleaved by Cas9 and RNaseIII. In one of the systems, Cas6 homolog is usedfor cleaving of the direct repeats and hence donot require hairpin loop (36).

As a general theme of different CRISPR systems, there is biogenesisof crRNAs in which there is site specific cleavage of pre-crRNAprecursorsin the repeat sequences by Cas proteins followed by 32 splicing events. As a result, crRNAs have a well-defined52 endthat begins with ~8 nt of the upstream repeat sequenceand a more heterogeneous 32 end (26). In one of the CRISPR systems, sequences are incorporated from invading DNA between CRISPR repeat sequences encoded as arrays within the bacterial host genome. CRISPR repeat arrays are transcribed and subsequently processed into CRISPR RNAs (crRNAs), each containing a variable sequence transcribed from the foreign invading DNA, known as the "protospacer" sequence and part of the CRISPR repeat. Each crRNA hybridizes with a second RNA, known as the transactivating CRISPR RNA (tracrRNA) (37) which then forms a complex with the Cas9 nuclease (38).Twenty nucleotides at the 52 end of the protospacer portion of the crRNAdirect Cas9 to a specific target DNA site using standard RNA-DNA complementarity base-pairing rules, if they are adjacent to short sequences known as protospacer adjacent motifs (PAMs). Some CRISPR systems thatrecognizes other PAM sequences indifferent species of bacteria employs different crRNA and tracrRNA sequences have also been utilized for targeted genome editing (39,40,41).

CRISPR/Cas as a genome editing tool ; CRISPR/Cas9 has proved to be an efficient tool for genome editing of human cells. The "humanized" versions of *S. pyogenes* Cas9and *S. thermophilus* Cas9 werecoexpressed with custom-designed crRNAsor with tracrRNA co-expressed withcustom-designed crRNAs in human embryonickidney, chronic myelogenous leukemia, orinduced pluripotent stem cells as wellas in mouse cells (42). The end results in the target DNA were observed, demonstrating RNA-guided Cas9 hadstimulated gene editing by non-homologous endjoining repair or gene replacement by homologydirected repair. Multiplexing i.e. targeting with multiple crRNAswas also achieved successfully (43).

The therapeutic potential of CRISPR-Cas has already been demonstrated in many cases. In bacteriology, Cas9 has been employed as an antimicrobial agent and has been used to target antibiotic resistant in highly virulent strains of bacteria (17). A patient with cystic fibrosis showed functional repair of the CFTR gene in vitro in cultured intestinal stem cell organoids using CRISPR-Cas (44). Hydrodynamic injection of CRISPR components could correct a defective gene causing hereditary tyrosinemiain mice. This led to anexpansion of mutation-corrected hepatocytes in vivo and resultedin a rescued phenotype in adult mice (45). Germ linemediated editing of mice with Duchenne muscular dystrophyprevented the mice from the disease (46).

CRISPR-Cas9 is evolving as a promising technology inthe field of engineering and synthetic biology. Amultiplex CRISPR approach referred to as CRISPRmwas developed to facilitate directed evolution ofbiomolecules (47). CRISPRm generates quantitativegene assembly and DNA library insertioninto the fungal genomes byoptimizing CRISPR/Cas9, thus providing a strategy toimprove the activity of biomolecules. It has also been reported that short DNA oligonucleotides containing PAM sequences activates the enzymes by inducing Cas9 to bind ssRNAinaprogrammable fashion and thus providing ways to target transcripts without undergoing anyprior affinity tagging (48).

While CRISPR technology moves genome editingfrom the realm of the practically impossible to the possible but on the contrary moving from difficultto easy is still not achieved. Majority of applications require apure population of cells i.e. clones of single cell which is a labor and time intensive process. So, before planning any gene editing experiment all the requirements of the application should be taken care of. Asreported by Mohr et al. in 2016 (49), ideal ways of designing guideRNA (gRNA) for a particular event depends agreat deal upon the downstream purpose.

CRISPR with benefits

The key potential of the CRISPR-Cas system lies in its abilityto genetically modify an organism, leaving no foreignDNA behind and in its versatility and simplicity of programming.Unlike ZFNsand TALENs, which requires reprogramming i.e. editing of DNA-interacting domainslocated at different sites on the DNA-binding scaffolds, execution of the changes made by CRISPR-Cas systems only appliesto recombinantRNA sequences(50,51). Ease of use, low cost,high speed, multiplexing potential and equal or higher specificDNA targeting ability have increased its popularity at the scientific global level (52).

Engineering within cells using different combinations of mutations reflecting various stages of neoplastic disease can be done as multiple gene-targeting events within the same cell using CRISPR/Cas9 (53,54). So in order to identify stage-specific synthetic lethal interactions, cell lines so produced could be used in combination with siRNA or shRNA. Such combinations have already been performed in yeast and Drosophila (55,56).

The most eye catchingproperty of CRISPR is the ability to flexibly and precisely target Cas9 to essentially any genomic location. ForDNA targeting, Cas9 must recognise short PAM sequence and also there is requirement of base-paring of the 20 nt target sequence with the spacer region of the guide RNA (gRNA) (57). The strongest gRNAs (i.e., those resulting in highest fold repression by dCas9) have high level of complementarity to PAM-adjacent target, but targets where complementary factor is little less, may still be bound by the dCas9- gRNA complex (58,59). Repressive effect will be decreased if multiple mismatches occurs between the sgRNA and a potential target (60). By targeting different positions within the gene, repressive effect can also be adjusted (61).

The most important current application of CRISPRinterference is the production of phage-resistantstrainsof bacteria for the dairy industry (62). There is disruption in the generation of normal fermentation cycles once the dairy starter culture is infected with phagewhich ultimately leads to the low quality finished product (63). It has been observed that in *S. thermophilus,*the acquisition ofnew spacersis an exceptionaltool for the control of phage infection in the dairy industry,as it helps indifferentiating strains that are resistantto multiple bacteriophages but still retaining the samestarter culture properties.

An important advantage of CRISPRiover RNAi is the absence of CRISPR/Cas systems in eukaryotesfor various applications in whichcompetition with the endogenous pathways becomes difficult. In contrast to RNAi, which mostly to knocks down gene expression, CRISPR/ Cas9, on the other hand, permanently alter thegenetic code and up or down regulate geneexpression either transcriptionally or at the posttranscriptionallevel (64).

Concluding Remarks

Unlike other moments in scientific history, the CRISPR/Cassystem has opened an era of changes, which may spanfrom groundbreaking therapeutic applications to dauntingfears of irreversible perturbation of human evolution. The prokaryotic CRISPR/Cassystem is somewhat different as DNA rather than RNA is the prime target for interference. Although much more is still to be discovered, still it hasbecome quite clear that the CRISPR/Cas system is a specific technologyand is asuseful as the model to which it is applied and thusenabling diverse organisms to serve as models andaccelerating their manipulation is yet another reasonwhy CRISPR technology is so powerful.

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The Vaginal Microbiota in Women Health and Disease: Current Understanding and Future Perspectives - A Review

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Abstract

Vaginal Microbiota varies within individuals and between human populations. Colonization by Lactobacillus in the female genital tract is thought to be critical for maintaining genital health. Nevertheless, little is known about how genital microbiota influence host immune function and modulate disease susceptibility. This article discusses the advancement of new strategies for disease diagnosis and personalized treatments to promote health and improve the quality of women's lives. Previous research on the use of vaginal products for sexual intercourse available over the counter used by women has primarily focused on gels, creams and ointments composed by natural origin drugs/excipients and applied by means of an applicator. General concerns and misconceptions related to use of vaginal products were rare. More research is needed from the medical community to guide women clarifying therapeutic outcomes of vaginal products.

Key Words: Microbiome, Health, Lactobacilli, Genital Tract, Quality of Life, Immune regulation, Reproductive age

Introduction

The human body hosts complex microbial communities whose combined membership outnumbers our own cells by at least a factor of ten. Microorganisms that inhabit surfaces and cavities exposed or connected to the external environment at each body site includes ecological communities of microbial species that exist in a mutualistic relationship with the host(1) The kinds of organisms present are highly dependent on the prevailing environmental conditions and host factors and hence vary from site to site. Moreover, they vary between individuals and over time (2). Together, our ~100 trillion microbial symbionts (the human microbiota) endow us with crucial traits; for example, we rely on them to aid in nutrition, resist pathogens, and educate our immune system (3).

The microbiota normally associated with the human body have an important influence on human development, physiology, immunity, and nutrition (4). These microbial communities are believed to constitute the first line of defence against infection by competitively excluding invasive nonindigenous organisms that cause diseases. Despite their importance, surprisingly little is known about how these communities differ between individuals in composition and function, but more importantly, how their constituent members interact with each other and the host to form a dynamic ecosystem that responds to environmental disturbances. Major efforts are now underway to better understand the true role of these communities in health and diseases (5).

Vaginal microbiota form a mutually beneficial relationship with their host and have major impact on health and disease (4). The bacterial communities that reside in human vagina are an example of this finely balanced mutualistic association. In this relationship, the host provides

benefit to the microbial communities in the form of the nutrients needed to support bacterial growth. This is of obvious importance since bacteria are continually shed from the body in vaginal secretions, and bacterial growth must occur to replenish their numbers. Some of the required nutrients are derived from sloughed cells, while others are from glandular secretions. The indigenous bacterial communities, on the other hand, play a protective role in preventing colonization of the host by potentially pathogenic organisms, including those responsible for symptomatic bacterial vaginosis, yeast infections, sexually transmitted infections (STI), and urinary tract infections (6)(7)(8). Lactobacilli have long been thought to be the keystone species of vaginal communities in reproductive-age women. These microorganisms benefit the host by producing lactic acid as a fermentation product that lowers the vaginal pH to ~3.5–4.5 (9). While a wide range of other species are known to be members of vaginal bacterial communities, their ecological functions and influence on the overall community dynamics and function are largely undetermined. The vaginal ecosystem is thought to have been shaped by co-evolutionary processes between the human host and specific microbial partners, although the selective forces (traits) behind this mutualistic association are still not clear.

This review seeks to inform the scientific community about the background of new developments and update the progress of new strategies for disease diagnosis and personalized treatments to promote health and improve the quality of women’s lives. This cannot be accomplished without addressing a fundamental issue as to what constitutes a ‘normal’ and ‘healthy’ vaginal microbiota and understanding its function in health and diseases.

Dynamics of vaginal Microbiota

The female genital tract is anatomically made up of a succession of cavities (Fallopian tubes, uterine cavity, endocervix, and vagina) that correspond with the exterior through the vulvar cleft. This structure accommodates the menstrual flow and acts as the passage of foetus delivery; in addition, it allows sexual intercourse and also the entrance of pathogenic micro-organisms that are potentially harmful to the process of reproduction. Vaginal microflora undeniably presents one of the most important defence mechanisms for the reproductive tract by maintaining a healthy environment and preventing the proliferation of micro-organisms foreign to the vagina (10). The vagina and its distinctive microflora form a finely balanced ecosystem, with the vaginal environment controlling the microbial types present while the microflora in turn control the vaginal environment (11) (12).In obstetrics and gynaecology the bacterial flora of the female reproductive tract is thefocus of the study of infectious disease, as it is noted that many pelvic infections involve bacteria residing on the cervical-vaginal epithelium. The vaginal flora contains a large variety of bacterial species, including both aerobic and anaerobic organisms (Table 1 & Table 2) (13).

The development of the biota of the vagina is linked with maturation and deposition of glycogen in the vaginal epithelium from acidogenic and acidouric micro-organisms. An important distinction must be made between the flora of the vulva, vestibulum and that of the vagina proper. Immediately following parturition and during the first few days of puerperium, the vaginal flora is reasonably distinct from that of the vulval flora (14).

The means by which vaginal microbiomes help prevent urogenital diseases in women and maintain health are poorly understood. In a study directed by Ravel et al. (15) to understand vaginal microbiome of reproductive age woman, the vaginal bacterial communities of 396 asymptomatic North American women who represented four ethnic groups (white, black, Hispanic, and Asian) were sampled and the species composition characterized by pyrosequencing of barcoded 16S rRNA genes. The communities clustered into five groups: four were dominated by Lactobacillusiners, L. crispatus, L. gasseri, or L. jensenii, whereas the fifth had lower proportions of lactic acid bacteria and higher proportions of

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strictly anaerobic organisms, indicating that a potential key ecological function, the production of lactic acid, seems to be conserved in all communities(Fig. 1).

The normal microbial flora of the vagina plays a dynamic role in the prevention of genital and urinary tract infections in women. Thus, an accurate understanding of the composition and ecology of the ecosystem is crucial to understanding the aetiology of these diseases. Prior studies on the microbial flora of the human vagina indicate that micro-organisms normally present in the vagina play a pivotal role in preventing successful colonization by 'undesirable' organisms. This is inclusive of those organisms responsible for bacterial vaginosis, yeast infections, sexually transmitted diseases and urinary tract infections. Furthermore, epidemiologic studies have undoubtedly established that abnormal vaginal microbial communities and lower genital tract infections are considerably associated with an increased risk of HIV infection (11).

A healthy host-vaginal microbiome refers to an ecosystem in which a functional equilibrium is created through reciprocal and mutually beneficial interactions amongst the host and her resident micro-organisms. This healthy equilibrium functions to provide a barrier to both new colonization by pathogenic organisms and the overgrowth of organisms that are otherwise commensal (16). The innate immune system of the female reproductive tract is highly important in the prevention of ascending genital infections that can threaten healthy pregnancy and foetal development. The mucosa of the lower genital tract has to selectively support a habitat for resident commensal microbes (while simultaneously inhibiting the growth of potential pathogens) whereas the upper genital tract must remain aseptic (12).

Immune regulation of Female Genital tract

The vaginal innate immune system represents the first line of defence against foreign organisms and pathogenic microbes. Through its chief components, a natural balance is maintained and disease is averted. Many recent advances

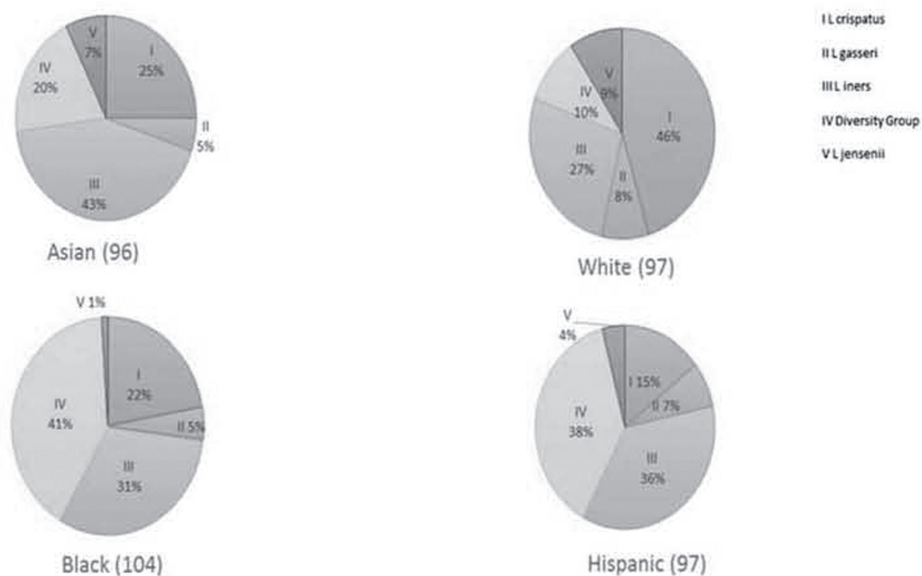

Fig 1. Representation of vaginal bacterial community groups within each ethnic group of women. The number of women from each ethnic group is in parentheses (adapted from Ravel et al. PNAS 2011; 108: 4680-4687)

have furthered our knowledge of this intricate equilibrium and the contribution of each element to the final homeostasis. Several adverse obstetric and gynaecologic conditions have been traced to abnormalities in the vaginal environment (17). Besides, female reproductive tract is immunologically unique in its requirement for tolerance to allogeneic sperm and, in the upper tract, to the conceptus. However, it must also be appropriately protected from, and respond to, a diverse array of sexually transmitted pathogens. Some of these infections can be lethal (e.g. Human Immunodeficiency Virus (HIV), Human Papilloma Virus (HPV)), and others (e.g. Chlamydia trachomatis and Neisseria gonorrhoeae) can have potentially devastating reproductive sequelae. Interactions between a host and a pathogen are complex, diverse and regulated, and are a function of the individual pathogen, and host immunity. Although there is undoubtedly commonality in the mucosal immune response, there is also evidence of a degree of site-specificity in immune mechanisms, dependent upon the function and anatomical location of an organ (18).

Events leading to a state of local immune-suppression (such as sexual intercourse or local induction of an allergic response) create suitable conditions for the proliferation of micro-organisms and also enable their transformation. These situations result in the rise of symptomatic vaginitis. Besides the protective effects of the endogenous vaginal flora, the protection against potentially pathogenic micro-organisms is also completed by the local components of both innate immunity and acquired immunity. The innate immune system is the most primitive branch of the immune system as it has been conserved throughout evolution (19) (10). One of its major characteristics is its ability to recognize molecular patterns associated with the pathogens (PAMPs) in the invading microbes, in place of recognizing specific antigens. Recognition of these molecular structures permits the immune system to distinguish between infectious non-self and non-infectious self (20).

Innate immunity acting of female genital tract (FRT) is represented by, firstly, soluble factors (such as mannose-binding lecithin [MBL], components of the complement, defensines, secretory leukocytes protease inhibitor [SLPI], nitric oxide), components in association with the membrane ('Toll-likereceptors') and, lastly, phagocytic cells (20). The recognition of a PAMP by a component of the innate immunity activates a sequence of events resulting in the release of pro-inflammatory cytokines and the activation of the acquired immune system, that is, the activation of lymphocytes T and B. A summary of the cellular origin, target cell lineages and function of each family of molecules is shown in Fig 2. It is important to note that, while the activation of the innate immune system occurs directly after the recognition of the pathogen, multiple days are required for the acquired immunity to become functional. The layer of the vagina constituted of epithelial cellsestablishes the initial contact point between micro-organisms and the host's genital tract. These epithelial cells possess 'Toll-like receptors' (TLR) on their surface and, thus, are imperative components of innate vaginal immunity. Eleven TLRs have already been identified, with each having a different specificity. The TLR1 and TLR2 complexes recognize lipoproteins and peptidoglycan present on the surface of gram-positive bacteria. Moreover, TLR3 is specific for the double DNA chain, which is an intermediate in the replication cycle of multiple viruses, while TLR4 recognizes the liposaccharide component of the gram-negative bacteria wall. Additionally, TLR5 reacts with flagellins, an important component of the bacterial flagella, and TLR9 has the capacity of differentiating DNA sequences containing the CpG dinucleotide in the non-methylated state (noting that in humans the DNA sequence is highly methylated, but the TLR9 reacts only with the CpG of bacteria which is non-methylated, thus, being specific for them). Vaginal cells also emit molecules with potent non-specific antimicrobial activity. A class of these molecules include positively-charged peptides, known as defensines, which rapidly bind negatively-charged bacterial surfaces. This binding action results in

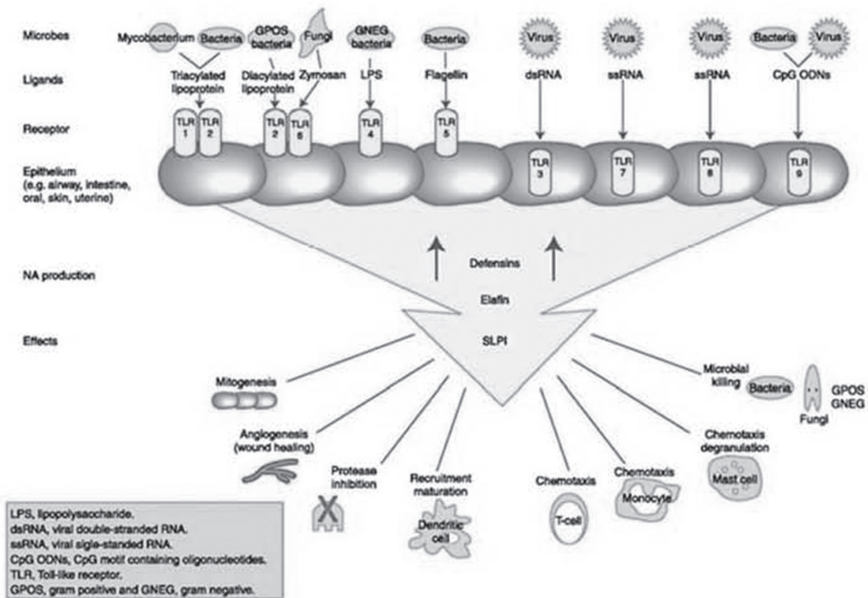

Figure 2 Summary illustrating the cellular origin, target cell lineages and function of the natural antimicrobial peptides, defensins and toll-like receptors(adopted from Andrew W Horne et al. Reproduction 2008;135:739-749)

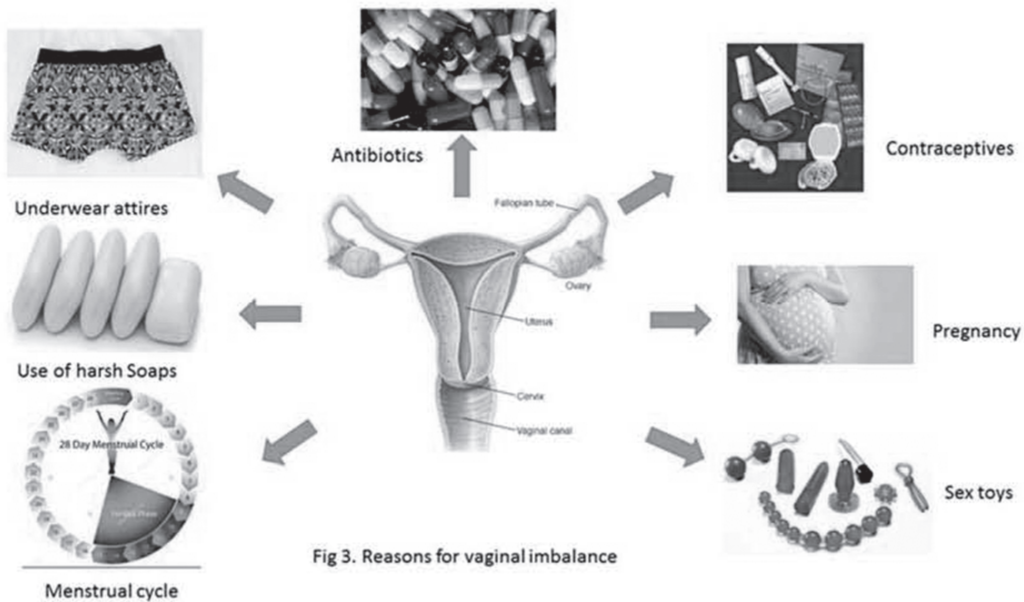

Fig 3. Reasons for vaginal imbalance

the disturbance of the micro-organism membrane and in cellular lysis. Another class of molecules is represented by the SLPI, which has the capability of inhibiting enzymes that break proteins (proteases), destroying both gram-positive and gram-negative bacteria and hindering the action of the human immunodeficiency virus. The production of SLPI additionally occurs in the uterine tube. Levels of this protein (SLPI)in the vagina are decreased in women with bacterial vaginosis,signifying that the SLPI, as a constituent of the innate immune system, plays a pivotal role in the vaginal homeostasis maintenance. Besides SLPI, other constituents of the innate immune system protecting against infections by retrovirus (particularly HIV) have been characterized in the female genital tract. Uterine or tubarian cells produce CCL20/ MIP3 alpha chemokine and in vitro studies with cell cultures show an increase in the production of this protein after stimulation with double chain of synthetic DNA homologue to the viral RNA chain. Antibodies with the ability of identifying and binding specific microbial antigens are located in the vagina through transudation of the systemic circulation. Following binding comes the microbial death by either complement-dependent mechanism oropsonisation. Furthermore, a constituent of the mucosa's immune system is found in the reproductive tract. Antibodies producing B-lymphocytes are present in both the endocervix and the vagina, locally producing both IgC and IgA classes of antibodies. The local antibodies amplification represents a rapid mechanism for fighting pathogenic micro-organisms, without having to wait for the commencement of systemic immune response. Antibodies formed locally and present in the vagina possibly differ from the systemic ones. It is also possible to identify antibodies in the cervical-vaginal secretion that are undetectable in peripheral blood (21).

Factors that impact on vaginal microbiota in women of reproductive age Lifestyle factors

Alcohol abuse: Sexual dysfunction is a condition that is caused by various different factors (Fig 3). Substance abuse, particularly alcoholism is known to be a factor for both men and women who are suffering from sexual dysfunction. Alcoholism is known to cause severe damage to the liver. A damaged liver can impact on the body's ability to regulate hormone distribution. In males, this can cause testicular atrophy, impotence and sterility. Women alcoholics can suffer from ovarian functionality issues, menstrual abnormalities and changes to sexuality (22).

Recent study by Loganantharaj et al.(23) to evaluate the immunological and virological changes in the genital microenvironment of females exposed to chronic alcohol suggest that chronic alcohol consumption negatively impacts the female genital micro-environment through the following mechanisms: increasing vaginal inflammation, decreasing beneficial bacterial species and increasing adverse bacterial morphotypes.

Underwear attires: Genital hygiene is the major component of women's health and is very important for the protection of reproductive health (24). Both the type and cleanliness of the underwear garment, as well as the frequency with which it is changed, are important factors affecting the risk of a woman obtaining a urinary infection. Nylon and synthetic underwear is unable to absorb perspiration as effectively as cotton underwear. This causes the perineum to remain humid and leads to an increased risk of genital tract infections. Frequent changing of underwear is recommended in preventing genital and urinary infections. Tight-fitting jeans and nylon underwear should be avoided as they tend to trap moisture and aid bacterial grow.

Harsh soap products: Washing the genital region more than once daily or using different commercial soap products may increase risk for vaginal infection by disturbing the genital flora and is therefore not recommended by the International Society for the Study of Vulvo-Vaginal Disease (25). Infection is most likely caused due to the reduced acidity of the vagina, either endogenously through hormones or exogenously through vaginal unhygienic practices (such as the usage of harsh

soaps or intra-vaginal cleansing “using fingers or douche”), poor menstrual hygiene and the usage of reusable cloth.

Antibiotics **:** The balance of the vaginal ecosystem depends on lactobacilli whose biofilm protects against microorganisms that are not normally present or are subdominant in vaginal flora. Lactobacillus species maintain the vaginal ecosystem in a healthy condition by production of antimicrobial substances. Depletion of lactobacilli in the vagina results in bacterial vaginosis (BV), where the normal flora is replaced by several bacterial pathogens, usually Gardnerella vaginalis and obligate anaerobes (26).Since the early 1980s, metronidazole has been used widely in the treatment of BV with good clinical results (27). Various preparations allowing a vaginal or oral administration and different regimens have been studied. In a clinical study conducted by Koumans et al.(28) the cure rates of women given antibiotics were higher (58%–100%) than the cure rates of women given placebo (5%–29%) when evaluated 4 weeks after treatment. Clindamycin is a second antimicrobial agent for the treatment of BV. This lincosamide antibiotic, a subclass of the larger family of macrolide antibiotics, has various treatment preparations including vaginal (ovule and cream) and oral. In a recent meta-analysis, intravaginal treatment (0.1%, 1%, 2% clindamycin cream twice daily for 5 days; 2% clindamycin cream at bedtime for 7 days) showed benefits to BV treatment with lower treatment failure compared with placebo (relative risk: 0.25; 95% confidence interval: 0.16–0.37)(29). Until recently, the mainstay therapy consisted of either metronidazole or clindamycin. A recent alternative has been the use of tinidazole. However, many clinical studies suggest that Probiotics should be seriously considered as part of the approach to disease prevention, and as an adjunct to antimicrobial treatment. Current recommendations do not advocate treatment of asymptomatic BV.

Hormonal fluctuations

Menstrual cycle : The vaginal microbial community plays a dynamic role in maintaining women’s health. Understanding the precise bacterial composition is challenging because of the diverse and difficult-to-culture nature of many bacterial constituents, necessitating culture-independent methodology (30). During a natural menstrual cycle, physiological changes could have an impact on bacterial growth, colonization, and community structure. The relative compositional stability of the vaginal microbiome is quite remarkable, given the variability in the host ecosystem associated with the menstrual cycle, sexual contact, and introduction of bacteria from the skin and external environment. In particular, the menstrual cycle creates an ever-changing vaginal environment, with ovulation, menses, and corresponding fluctuations of oestrogen and progesterone levels affecting bacterial attachment to the vaginal epithelium cervical mucus production, pH and redox potential and glycogen levels (31). Moreover, an increase in mucosal glycogen, superficial keratinization of the mucosa and a shift of vaginal pH from 7 to 4.5-6, due to lactic acid production from the glycogen by Doderlein’s bacilli (Lactobacilli), occurs at puberty or after oestrin. This results in an antigonococcal environment. During the menstrual cycle, hormonal deviationsinterfere in the substrate of different micro-organisms. These variations, with the addition of menstrual blood, lead to changes in vaginal pH. However, the levels of Lactobacillus remain constant throughout the menstrual cycle. The non-Lactobacillus bacteria levels increase during the proliferative phase of the menstrual cycle and the concentrations of Candidaalbicans become higher in the pre-menstrual period (32).Menstruation and sexual activity have been shown to have negative effects on the stability of the vaginal microbiota (33). The secretory phase of the menstrual cycle, which is characterized by high concentrations of oestrogen and progesterone, appears to be more stable in terms of microbial community composition.

Pregnancy : The vaginal microbiome in pregnancy plays an important role in both maternal and neonatal health outcomes (34). Pregnancy is accompanied by a shift in the bacterial community

structure of the vagina to a composition that is typically dominated by one or two species of Lactobacillus. These bacteria are believed to inhibit pathogen growth through secretion of antibacterial bacteriocins as well as the production of metabolites such as lactic acid that help to maintain a low, hostile pH (35).The maternal vaginal microbiome may also be an important source of pioneer bacteria for the neonatal gut microbiome, which have a profound effect on host system metabolism and immunity (36).Fluctuations in hormone levels in females can also cause the vaginal environment to adjust to one that is highly susceptible to infection and this can happen during pregnancy or while breastfeeding.

In pregnant woman, levels of oestrogen, mainly in the form of estriol, rise steadily over the course of pregnancy, with urine levels rising from a low in the first trimester of around 4 μg/24 hours to a high in the third trimester of up to 50,000 μg/24 hours (37). Increased oestrogen levels during pregnancy strengthen a Lactobacillus-dominant microbiota however, simultaneously, the occurrence of vulvovaginal candidiasis increases in comparison to that of non-pregnant women. The reason for this increase has been suggested to be the somewhat suppressed cell-mediated immunity in pregnant women which leads to an increased susceptibility to pathogens such as C. albicans (32). Oestrogen likewise stimulates the deposition of glycogen in vaginal epithelial tissue resulting in metabolized glucose within the vaginal epithelium and this is then converted to lactic acid via cellular metabolism (38). Recent data on changes in the vaginal microbiota during pregnancy are sparse and are meaningless unless the time of sampling is specified.

Use of Contraceptives

Currently, emerging scientific data (39) suggests that some commonly used contraceptives may increase risk of sexual HIV acquisition and transmission. There are several biologically plausible mechanisms by which hormonal contraceptives (HC) could increase HIV risk including disrupting epithelial barriers (thinning of the epithelium or altering epithelial integrity), causing changes in inflammatory responses that could in turn enhance HIV replication locally (40) or altering the vaginal microbiota which itself effects local immunity and genital inflammation.

Vaginal cells release molecules with potent non-specific antimicrobial activity. A class of these molecules, known as defensines, include positively-charged peptides that rapidly bind negatively-charged bacterial surfaces. This binding results in the disruption of the microorganism membrane and in cellular lysis (40). In women presenting with infections, HBD-1 and HBD-2 human defensines are produced by the vaginal epithelial cells. The production of HBD-2, but not that of HBD-1, is stimulated by oestrogens and inhibited by progesterone. This proposes that the usage of oral contraceptives may decrease the release of HBD-2, thereby increasing the susceptibility to infections. This suggests that the use of oral contraceptives may decrease the release of HBD-2, increasing, thus, the susceptibility to infections. Another class of molecules is represented by the SLPI, which possesses the capacity of inhibiting enzymes that break proteins (proteases), destroying gram-positive and gram-negative bacteria and blocking the action of the human immunodeficiency virus.

External influences such as spermicidal agents deplete the lactobacilli flora, especially strains producing hydrogen peroxide. The vaginal insertion of tampons, diaphragms and intrauterine devices (IUDs) can also disturb the microflora (41). A classic example was the usage of highly absorbent tampons to which Staphylococcus aureus strains producing toxic shock toxins, attached and resulted in morbidity and mortality. It is acknowledged that pathogen adhesion to IUDs can be associated with infections, however, when lactobacilli are the dominant organisms on IUDs, infections may not arise. Spermicides contain an ingredient called nonoxynol-9 which may cause a chemical irritation to both the vaginal and urethral mucosa as well as changes in the normal vaginal flora (42). Patients with recurrent Urinary tract infections (UTIs) should avoid diaphragms and

spermicide coated condoms, as well as other barrier agents containing nonoxynol-9 such as foam, suppositories, and sponges (43).Periodic condom usage has been shown to increases the risk of vaginal inflammatory states, but with normal microbiota [44].

Sexual devices

Vaginal lubricants : Vaginal lubricants in particular are widely available and frequently used by women in order to allow the minimizing of dyspareunia or to enhance sexual pleasure (45). A large number of women use these feminine hygiene products daily. For some it is part of their daily cleansing or bathing. Feminine hygiene products or methods may disturb the normal pH level of 4.5 in the vagina, which is vital for maintaining the healthy vaginal immune barrier environment. Through the change of pH or the direct bactericidal properties, these products and practices may affect the composition of the normal vaginal microbiome, which is important for the healthy mucosal environment and protection against yeast infection or other sexually transmitted pathogens.

Lactobacilli and, especially, L. crispatus are among the bacteria that are most common in healthy women and characteristic for the healthy vaginal environment (46). Despite their intrinsic antimicrobial potential however, vaginal lactobacilli fail to retain dominance in a considerable number of women, resulting in overgrowth of the vaginal epithelium by other bacteria, as observed, most typically, with anaerobic polymicrobial overgrowth in bacterial vaginosis, or less commonly, with overgrowth by streptococci, including group A and group B streptococci, by bifidobacteria, or by coliforms such as E. coli (47). Vaginal products remain harmful to the Lactobacillus bacteria and should therefore be utilised with caution. Some feminine hygiene products may change the vaginal immune barrier by negatively effecting epithelial cell integrity, survival of beneficial Lactobacillus species in the vaginal microenvironment, and changing the ability of the vaginal epithelial cells to produce any protective or inflammatory immune mediators, such as, IL-8(48).

Use of foreign devices (Sex toys) : The HIV/AIDS pandemic has elevated international responsiveness on ways to reduce the risk of STIs including HIV, through the use of condoms and potentially microbicides. It is documented that the use and effectiveness of such methods is not only a question of availability and knowledge, but is also closely linked to socio-cultural beliefs about health, hygiene and sexuality, often expressed through women's vaginal practices that are widespread in much of the world. Concern has also been raised about whether vaginal practices could have harmful effects such as increasing the susceptibility to sexually transmitted or reproductive tract infections (49).

Sexual practices involving the transmission of vaginal fluid such as sex toys (after use) allow the sexual transmission of some agents associated with bacterial vaginosis (50). Furthermore, sharing of sex toys is alsoassociated with decreased quantities of H2O2-producing lactobacilli and greater risk of colonization with G. vaginalis. In a cross sectional study by Mitchell et al. (51) on women reporting sex with women who provided information on sexual behaviours it was noticed that the use of sex toys is consistently associated with differences in vaginal ecosystem and increases the risk of bacterial vaginosis, either by decreasing colonization with protective H_2O_2-producing lactobacilli or by increasing the presence of bacterial associated species like Gardnerella vaginalis.

The menace of sharing sex toys (e.g. dildos, butt plugs and other objects meant to be inserted into the rectum and/or vagina) varies according to whether they are covered with condoms, and whether they are properly cleaned (i.e., disinfected) between use with different partners. Sex toys come into direct contact with rectal and/or vaginal mucosal membranes, which can both transmit HIV and are susceptible to infection (52). Shared an uncleaned dildo or other toy can transfer infectious fluids from an HIV-positive person to an uninfected person, and poses a high transmission risk. If sex toys are used with new, clean condoms

for each partner, or are properly cleaned (i.e., disinfected) before use by the uninfected partner, they pose a negligible risk of transmission. Note that using the same condom with both partners is effectively the same as not using one at all.

There is zero HIV transmission risk attached to using sex toys if they are not shared. However, sex toys are often used before or after other sexual activities. As with fingering, fisting and other forms of play, any tissue damage or inflammation that results from sex toy use can affect the risk of infection during other activities.

Vaginal infections : Many factors can disturb the natural balance in the vagina, and if they become too intense, the vaginal flora can no longer adapt, which may result in a vaginal infection. The number of lactobacilli in the vagina is usually then reduced and the pH is also often raised to values above 4.4. Vaginal complaints such as itching, burning and discharge, can be signs of a bacterial or yeast infection and thus of an imbalance in the vaginal flora (53). The scope of the article is very limited to discuss in detail the common vaginal infections and therefore bacterial vaginosis is discussed in brief.

Bacterial vaginosis : Bacterial vaginosis (BV) is the most common cause of vaginal infection in women of childbearing age. While the aetiology and transmissibility of BV remain unclear, there is strong evidence to suggest an association between BV and sexual activity (54). Bacterial vaginosis is characterised by imbalance of the vaginal microbiota with a notable reduction of lactobacilli species, an overgrowth of a mixture of mostly endogenous obligate anaerobic bacteria spp. and elevated pH level in the vagina. The total number of bacteria associated with BV is increased 100-1000 fold when compared to normal levels. Bacterial vaginosis is a polymicrobial syndrome resulting from a decreased concentration of protective lactobacilli and an increase in pathogenic bacteria (55). As a result, there is both a qualitative and quantitative change of the microbiota associated with BV. The presence of anaerobic bacteria gives both rise to amines and an elevated pH, which further encourages the growth of anaerobic bacteria. Among the bacterial spp. commonly identified in BV are Gardnerella vaginalis, Atopobiumvaginae, Prevotella spp., Mobiluncusspp., Mycoplasma hominis and Urea plasmaspp.The list of BV-associated bacteria is growing since new species are being revealed through the use of cultivation independent methods of detection (56) (Table 1). Bacterial vaginosis causes no complications in most cases, however it does present some serious health risks. Multiple studies have shown an association between BV and an increased susceptibility to STDs such as HIV-1, Herpes simplex virus, HPV, N. gonorrhoeae, and C. trachomatis. Bacterial vaginosis has also been associated with a higher risk of endometritis, pelvic inflammatory disease and post-operative (hysterectomy, legal abortion) infections (57). Potential mechanisms by which bacterial vaginosis might increase HIV transmission comprise effects on local immune mediators. Additionally, hydrogen peroxide produced by lactobacilli can inhibit HIV in vitro, and is absent in most women with bacterial vaginosis. If bacterial vaginosis is established as an important risk factor for HIV spread, its control will become an important public health issue in many countries. Approximately 50% of women are asymptomatic. Bacterial vaginosis is characterised by a thin, homogeneous white discharge, a vaginal pH of above 4.5, a positive amine test and the presence of clue cells detected microscopically. Bacterial vaginosis increases a woman's risk of acquiring HIV, increases complications in pregnancy and may also be involved in thepathogenesis of pelvic inflammatory disease. Bacterial vaginosis has also been associated with an increased incidence of non-gonococcal urethritis in male partners in one small study(58).

Prevention Recommendations for healthy Vaginal Microbiota : Probiotic therapy is an exciting avenue that has been pursued for the treatment of vaginal dysbiosis for many years (59). However, the use of probiotics for the treatment of vulvovaginal atropy, vaginal dryness, and

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Table 1. Prevalence of aerobic (facultative) isolates reported in vaginal flora studies published in the literature (adapted from Bryan Larsen, and Gilles R. G. MonifClin Infect Dis. (2001); 32:e69-e77)

	Prevalence in Vaginal Flora		
Aerobic Isolate	Low	Mean	High
Gram Positive Rods			
Diptheroids	3	40	80
Lactobacilli	18	60	90
Gram Positive cocci			
Staphylococcus aureus	0	2	25
Staphylococcus epidermidis	5	50	95
Staphylococcus species			
α-Hemolytic	8	20	38
β-Hemolytic	3	15	22
Nonhemolytic	0	20	32
Group D	2	28	45
Gram Negative Rods			
Escherichia coli	3	18	33
Klebsiella and Enerobacter species	0	10	20
Proteus species	0	5	10
Pseudomonas species	0	0.1	3

restoration of a healthy vaginal microbiota in women is a relatively new concept. Probiotics (including Lactobacillus spp.) potentially work through a variety of mechanisms to reinstate homeostasis by enhancing epithelial barrier function, commensal colonization, blocking adhesion of pathogenic bacteria, reducing pH, influencing antimicrobial peptide production/ secretion and overall mucosal immunity and vaginal health (60).

Both oral and vaginal routes of delivery have been pursued in clinical studies for reinstating vaginal homeostasis by delivery of Lactobacillus-based probiotic formulations and exhaustive efforts have likewise been made toward the administration of drugs, via alternative routes, that are poorly absorbed after the oral administration. The vagina as a route of drug delivery has been known since ancient times (61). In recent years, the vaginal route has been rediscovered as a potential route for systemic delivery of peptides and other therapeutically important macromolecules. However, successful delivery of drugs through the vagina remains a challenge, primarily due to the poor absorption across the vaginal epithelium. The rate and extent of drug absorption after intravaginal administration may vary depending on formulation factors, vaginal physiology, age of the patient and menstrual cycle. Suppositories, creams, gels, tablets and vaginal rings are commonly used vaginal drug delivery systems (62).

Vaginal drug delivery often ideal because it allows the use of lower doses, maintains steady drug administration levels, and requires less frequent administration than the oral route. With vaginal drug administration, absorption is unaffected by gastrointestinal disturbances, there is no first-pass effect, and use is discreet.

Conclusion

Research about the vaginal microbiome has advanced over the last decade. Light microscopy and advanced culture techniques revealed healthy vaginal microbiomes are dominated by Lactobacillus species. Next-generation sequencing technology has allowed scientists to

further categorize and understand the complexity and diversity of microbes that inhabit the vaginal cavity. Overall, Lactobacillus dominance is strongly correlated to vaginal health and homeostasis in both pre- and postmenopausal women. Disruptions in vaginal flora, such as BV, are related to a number of poor obstetrical and gynaecologic outcomes.

The U.S. Food and Drug Administration (FDA) and the European Medicine Agency (EMA), as well as most of the other regulatory bodies around the world, cautions women regarding the use of lubricants and other medical devices easily available over the counter (OTC) as these products lack extensive pre-clinical and clinical testing as otherwise required for drug products. There is lack of data on the safety of vaginal OTC lubricants, moreover different in vitro and in vivo animal studies indicate that water-based lubricants may induce changes to the vaginal environment and mucosa that can lead to toxic effects and, eventually, enhancement of the transmission of sexually transmitted pathogens, such as HIV.

With greater consciousness of the chemicals used in popular feminine hygiene products, the unique characteristics of the vaginal region, and the potential for health disparities among various groups of women through culturally determined use of these products, it is apparent that more studies are needed to understand their effects on vaginal microbiota.

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Conflict of interest

The authors declare no conflict of interest.

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Biological Actions of PPAR- γ in Health and Disease

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Abstract

Peroxisome proliferator-activated receptors (PPARs) are ligand activated transcription factors that modulate target gene expression in response to endogenous and exogenous ligands. Peroxisome proliferator-activated receptors are expressed in many tissues, including adipocytes, hepatocytes, muscles and endothelial cells. The PPARs, a family of nuclear receptors (NRs), are a set of three receptor sub-types encoded by distinct genes. The discovery of PPAR-specific ligands has led to a significant advancement in our understanding of the structure of these receptor proteins and molecular mechanisms of their ligand dependent activation. The nuclear receptor peroxisome proliferator-activated receptor (PPAR)-γ is a crucial cellular and metabolic switch that regulates many physiologic and disease processes.

Keywords: PPAR-gamma; inflammation; adipose tissue; insulin sensitivity; cancer.

Introduction

The peroxisome proliferator activated receptors (PPARs) are ligand-inducible transcription factors belonging to superfamily of nuclear harmone receptor (NHR) containing 48 members (1). These receptors are identified in the 1990 in rodents. These receptors are named because of its property of peroxisome proliferation. NHR also includes other members such as retinoic acid receptors (RARs), the thyroid hormone receptors (TRs) and the steroid receptors (1, 2). But, these agents are associated with no proliferation in the humans. Structurally, PPARs are similar to steroid or thyroid hormone receptor and are stimulated in response to small lipophillic ligands.

Isoforms of PPARs : Three subtypes of PPARs, classified as PPAR-α (NR1C1), PPAR-β/δ (NR1C2) and PPAR-γ (NR1C3), encoded by separate genes, were cloned from a Xenopus cDNAlibrary in 1992. PPAR-γ is further of three type's i.e PPAR-γ1, PPAR-γ2, PPAR-γ3 that differs at their 5' end and is generated by alternative promoter usage and splicing. Proteins produced from PPAR-γ1 and PPAR-γ3 mRNAs are identical, whereas, PPAR-γ2 protein contains an additional N-terminal region composed of 28 amino acids.

Structural features of PPARs: All three PPAR isoforms possess similar structural and functional features. Principally, four functional domains have been identified, called A/B, C, D and E/F (Fig. 1) The N- terminal A/B domain contains a ligand-independent activation function 1 (AF-1) (4), responsible for the phosphorylation of PPAR. The DNA binding domain (DBD) or C domain promotes the binding of PPAR to the peroxisome proliferator response element (PPRE) in the promoter region of target genes (5). The D site is a docking domain for cofactors. The E domain or ligand-binding domain (LBD) is responsible for ligand specificity and activation of PPAR binding to the PPRE, which increases the expression of targeted genes. Recruitment of PPAR co-factors to assist the gene transcription processes is carried out by the ligand-dependent activation function 2 (AF-2), which is located in the E/F domain (6).

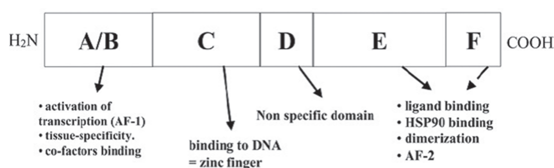

Fig. 1. Schematic representation of the functional domains of PPARs

PPARs are composed of four distinct functional regions. The A/B domain located at N-terminal with AF-1 is responsible for phosphorylation, the domain C is implicated in DNA binding, domain D is the docking region for cofactors and domain E/F is the ligand specific domain, containing AF-2, which promotes the recruitment of cofactors required for the gene transcription.

Mechanism of action : PPARs are the transcription factors i.e they regulate the transcription of genes in response to ligand binding as shown in figure 2 (7). After ligand binding, PPARs undergo specific conformational changes that allow for the recruitment of one coactivator protein or more. Ligands differ in their ability to interact with coactivators, which explains the various biologic responses observed.

RXR and heterodimerisation: Unlike the steroid hormone receptors, which function as homodimers, PPARs form heterodimers with the retinoid X receptor (RXR). Like PPARs, RXR exists as three distinct isoforms: RXRα, β, and γ, all of which are activated by the endogenous agonist 9-*cis* retinoic acid. No specific roles have yet been elaborated for these different isoforms within the PPAR: RXR complex. However, synthetic RXR agonists can activate the complex and thereby obtain antidiabetic outcomes similar to those seen with PPAR agonists in mouse models of type 2 diabetes The LBD domain facilitates the heterodimerisation of PPARs with the RXR and the resultant heterodimer subsequently binds to peroxisome proliferator response element (PPRE) with the recruitment of cofactors (1).

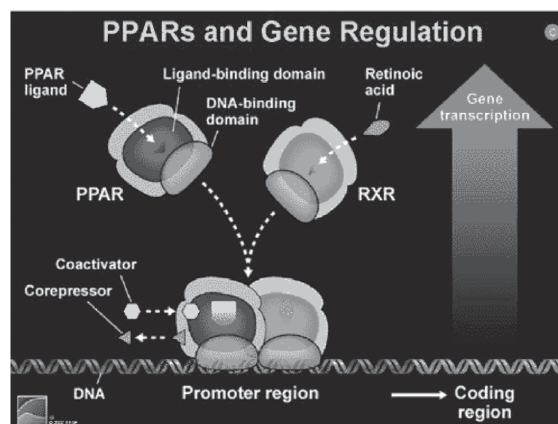

Fig. 2. Mechanism of action of PPAR-γ Agonist Peroxisome proliferator response elements (PPREs)

Peroxisome proliferator response elements (PPREs) are direct repeat (DR)-1 elements consisting of two hexanucleotides with the AGGTCA sequence separated by a single nucleotide spacer. The DR-1 pattern is specific for PPAR-RXR heterodimer.

Coactivatos/corepressors (cofactors) : Several cofactor proteins, coactivators or corepressors that mediate the ability of nuclear receptors to initiate or suppress the transcription process. They interact with nuclear receptors in a ligand-dependent manner (1). In the unliganded state, heterodimerised nuclear receptor associates with multicomponent co-repressors containing histone deacetylase activity, such as nuclear receptor co-repressor (NCoR) and the silencing mediator for retinoid and thyroid hormone receptor (SMRT). The deacetylated state of histone inhibits transcription. Alternatively, coactivators such as steroid receptor co-activator (SRC)-1 and the PPAR binding protein (PBP) with histone acetylase activity (8, 9) initiate a sequence of events which induces the gene transcription process upon ligand binding (2).

PPARs regulate gene transcription by two mechanisms i.e transactivation and transrepression. In transactivation, which is DNA-

dependent mechanism, PPAR forms a heterodimer complex with the retinoid X receptor (RXR) and recognizes specific DNA response elements called PPAR response elements (PPRE) in the promoter region of target genes. This results in transcription of PPARγ target genes which ultimately involved in diverse biological processes such as adipocytes proliferation, glucose and lipid metabolism. In transrepression, PPARs can repress gene transcription by negatively interfering with other signal-transduction pathways, such as the nuclear factor-*k*B (NF-*k*B) signaling pathway, in a DNA-binding–independent manner (1).

Peroxisome proliferator-activated receptor-gamma (PPAR-γ): Most widely used PPAR-g ligands are thiazolidinediones (10). First drug in this category is Troglitazone (Rezulin) then rosiglitazone (Avandia) and pioglitazone (Actos). Troglitazone is withdrawn from market due to hepatic toxicity. The PPAR- γ contains three isoforms, named as, PPAR- γ1, PPAR- γ2 and PPAR- γ3.

Table 1. Expression of PPAR isoforms

Isoform	**Tissue Expression**	**Glucose and Lipid lowering effect**
α	Skeletal muscle, Kidney, Heart, Liver, Monocytes, Macrophages, Vascular and endothelial smooth muscle	decreases acyl-CoA enzyme, thereby decreasing triglyceride concentration, increases uptake and oxidation of free fatty acids
β/δ	Skeletal muscle, Adipocytes, Macrophages, Lungs, Brain, Skin	enhances glucose tolerance and disposal improves lipid catabolism and fatty acid oxidation
γ 1	Kidney, Spleen, Pancreatic β-cells, Cardiac, skeletal and vascular smooth muscle	improves insulin resistance by producing additional adipocytes to better store the elevated fatty acids induces adipocyte expression and differentiation
γ2	Adipocytes	decreases hepatic glucose production
γ3	Colon, Macrophages, Adipocytes	redistributes lipids from visceral areas into subcutaneous fat decreases TNF and 11-b hydroxysteroid dehydrogenase 1 increases adiponectin, which increases insulin sensitivity inliver and skeletal muscle

PPAR-ligands: Although the nature of true endogenous PPAR ligands is still not known, PPARs can be activated by wide variety of endogenous or pharmacological ligands as shown in table 2. PPAR-α activators include variety of endogenously present fatty acids, LTB4 and hydroxyeicosatetraenoic acids (HETEs), and clinically used drugs, such as fibrates, a class of first-line drugs in the treatment of dyslipidemia. Similarly, PPAR-γ can be activated by a number of ligands, including docosahexaenoic acid, linoleic acid, the anti-diabetic glitazones, used as insulin sensitizers, and a number of lipids, including oxidized

LDL, azoyle-PAF, and eicosanoids, such as 5, 8, 11, 14-eicosatetraynoic acid and the prostanoids PGA1, PGA2, PGD2, and its dehydration products of the PGJ series of cyclopentanones (e.g., 15 deoxy-$\Delta^{12,14}$-PGJ2). PPARβ/δ activators include fatty acids and prostacyclin and synthetic compounds L-165,041, GW501516, compound F and L-783,483.

Table 2. PPAR Ligands

Endogenous ligand			**Biological effect**
PPAR-α	**PPAR-β**	**PPAR-γ**	
Palmitic acid Stearic acid Palmitoleic acid Oleic acid Linoleic acid Arachidonic acid Eicosapentaenoic acid	Docahexanoic acid Arachidonic acid Linoleic acid	Linoleic acid Arachidonic acid 15d-PGJ_2 9-HODE 13-HODE 15-HETE	Lipid and glucose metabolism
Exogenous ligand			**Implication**
PPAR-α	**PPAR-β**	**PPAR-γ**	
Agonists Gemfibrozil Clofibrate Fenofibrate Bezafibrate Ciprofibrate WY14643 GW7647 GW 9578 Nafenopin	**Agonists** L-165041 GW501516 GW0742	**Agonists** Thiazolidinediones JTT-501 KRP-297 NC-2100 MCC-555 CS-011	**PPAR-α agonists** Atherosclerosis, Inflammation **PPAR-β agonists** Infertility, Obesity, Dyslipidemia, Atherosclerosis, Hyperglycemia
Antagonist MK-886	**Antagonist** Sulindac	**Antagonists** GW-0072 BADGE GW-9662 SR-202 T0070907 GW-1929 CDDO-Me Diclofenac LG100641	**Antagonist** Cancer **PPAR-γ agonists** Cancer, NIDDM, Inflammation, Hypertension

Biological actions of PPAR-γ

Insulin sensitization: The mechanism underlying insulin-sensitising effects of TZDs are complex and not completely understood. Activation of PPAR-γ in insulin-resistant animals or humans results in an increase in the sensitivity of both the liver to insulin-mediated suppression of hepatic glucose production and insulin-mediated skeletal muscle glucose uptake. These invivo effects on insulin signaling are because of combined actions of PPAR-γ ligands on the adipose tissue and on liver and skeletal muscles.

Insulin resistance is one of the principle defects underlying the development of type-2 diabetes and Asian Indians are considered to be more insulin resistant. PPAR-γ has also been associated with several genes that affect insulin action (11, 12). Given that PPAR-γ is expressed predominantly in adipose tissue, the prevailing hypothesis regarding the net in vivo efficacy of PPAR-γ agonists involves direct actions on adipose cells, with secondary effects in key insulin-responsive tissues such as skeletal muscle and liver. PPAR-γ enhances the expression of a number of genes encoding proteins involved in glucose and lipid metabolism.

Tissue necrosis factor alpha (TNF-β), a pro-inflammatory cytokine that is expressed by adipocytes, has been linked to insulin resistance. In vivo investigations showed that PPAR-γ agonists improve insulin resistance by opposing the effect of TNF-α in adipocytes. Expression of the glucose transporter protein GLUT4 by PPAR- γ agonists in adipocytes is also pivotal in the process of glucose uptake.

On the other hand, resistin, a hormone secreted by adipocytes that elevates blood glucose levels, was inhibited by TZDs. It has been demonstrated that adipocyte-derived factors such as 11α-hydroxysteroid dehydrogenase 1 and adipocyte-related complement protein (Acrp) 30 (adiponectin) were influenced by PPAR- γ activation (13), improving insulin resistance and glucose homeostasis. Several in vivo studies showed that TZDs also promote insulin-stimulated glucose disposal in skeletal muscles by enhancing phosphatidylinositol (PI) 3-kinase activity and membranous protein kinase B/Akt (PKB/Akt) Ser-473 phosphorylation. However, as PPAR-γ expression is greater in adipose tissue than in skeletal muscle, it is unclear whether PPAR- γ exerts direct effects on skeletal muscles or alters expression of adipocyte genes that convey signals to skeletal muscles.

Adipocyte differentiation

Adipogenesis refers to the process of differentiation of the pre-adipocyte precursor cells into adipocytes that are capable of lipid filling, as well as the expression of hormones and cytokines (adipokines). PPAR-γ is expressed at high levels in adipose tissue and is central regulator og adipocytes gene expression and differentiation. In adipocytes, PPAR-γ regulates the expression of numerous genes involved in lipid metabolism, including aP2, PEPCK, acyl-CoA synthase, and LPL. PPAR-γ has also been shown to control expression of FATP-1 and CD36, both involved in lipid uptake into adipocytes. These genes have all been shown to possess PPREs within their regulatory regions (13).

In addition to the stimulation of adipocyte differentiation, activation of PPAR-γ also promotes apoptosis in mature lipid-filled adipocytes. This ligand-induced apoptosis in mature cells causes the stimulation of adipogenesis from pre-adipocyte precursors, resulting in an increased number of small, relatively insulin-sensitive adipocytes (14).

Atherosclerosis

Expression of PPAR-γ in endothelial cells, vascular smooth muscle cells (VSMCs) has raised questions regarding its effects on lipid metabolism. This has prompted research on its anti-inflammatory properties. Subsequently, its role in chronic inflammatory disorders such as atherosclerosis, arthritis and inflammatory bowel syndrome were also studied. PPAR-γ agonists were shown to have antiatherosclerotic effects in different animal models. Several mechanisms have been reported which counteracted the pro-

atherogenic activity of PPAR-γ. In endothelial cells, PPAR-γ activators inhibited the VCAM-1 and ICAM-1 expression, resulting in the reduction of monocyte accumulation in the arterial intima and also decreases the inflammatory cell recruitment by inhibiting the the chemokines IL-8 and MCP-1 (15, 16, 17). In human monocyte derived macrophages, PPAR- γ agonists inhibited MMP-9 gelatinolytic activity, an enzyme responsible for plaque rupture. The role of VSMCs in the progression of atherosclerosis is paramount and recent studies showed that they were key targets of PPAR-γ agonists. TZDs inhibited VSMC proliferation by decreasing phosphorylation of retinoblastoma protein and increasing levels of cyclin dependent inhibitor p27. In addition, PPAR-γ ligands inhibited the expression and activity of MMP-9 and VSMC migration, thus offsetting the PPAR-γ pro-atherogenic activity. Furthermore, PPAR-γ agonists inhibited angiotensin II type 1 receptor in vascular smooth muscle cells; this down-regulation is beneficial in atherosclerosis and hypertension. PPAR-γ agonists also play an important role in macrophage lipid homeostasis by inducing expression of several key genes including ABCA1, ABCG1, apolipoprotein E (apoE) and CLA-1/SR-BI. Therefore the metabolic syndrome which is clustering of cardiovascular risk factors with insulin resistance is chracterised by simultaneous presence of one or more of metabolic disorder such as glucose intolerance, hyperinsulemia, dyslipidemia, coagulation disturbances and hypertension can be effectively modulated by PPAR-γ agonists (1, 15, 18, 19).

Inflammation

Inflammation is a complex and dynamic process initiated by the body in response to tissue injury or infection. PPAR-γ agonists have been shown to be effective in number of inflammatory models such as ulcerative colitis, rheumatoid arthritis (20, 21), asthma (22, 23), allergic encephalomyelitis (24, 25, 26) and pulmonary inflammation (27, 28). Pro-inflammatory cytokines such as TNF-α, IL-1β and IL-6 and production of nitric oxide by inducible nitric oxide synthase (iNOS) plays a key role in all these inflammatory conditions. In animal model of asthma, activation of PPAR-γ induces a selective inhibition of eosinophil and lymphocyte influx, without affecting the neutrophil influx (29, 30). Various invitro and invivo studies have shown the efficacy of PPAR-γ agonists in acute and chronic inflammation (31, 32, 33, 34, 35). In vitro reports find PPARγ inhibition of monocyte chemoattractant protein-I directed chemotaxis. PPARγ agonists also inhibit chemokines (interleukin-8) in epithelial cells, leading to the suggestion of their use in inflammatory bowel diseases (36). Several lines of evidence suggest that PPAR-γ may exert anti-inflammatory effects by negatively regulating the expression of these pro-inflammatory genes that become induced during macrophage activation. PPAR-γ is expressed in monocytes, and particularly up-regulated upon activation (37). PPAR-γ ligands inhibit the induction of inducible nitric oxide synthase (iNOS), MMP-9, an scavenger receptor A gene transcription (37) and the production of TNF, IL-1β, and IL-6 (38, 39). Furthermore, PPAR-γ activation inhibits the transcriptional activity of cytokine induced pro-inflammatory transcription factors AP-1, NF-βB, and STAT1 transcription factors (37). In addition to effects on activation, PPAR-γ ligands induce apoptosis in old macrophages (40).

Immunoregulation

Various in vivo and in vitro studies have shown that PPAR-γ ligands are also capable of down-regulating most cells of the innate and adaptive immune system (40, 41, 42). This immunoregulatory effect of PPAR-γ ligands has led to numerous studies demonstrating the efficacy of PPAR-γ ligands in treating animal models of autoimmunity including experimental allergic encephalomyelitis, asthma, arthritis, colitis, and diabetes and leads to the excitement about the potential use of PPAR-γ ligands as therapeutic agents in human autoimmune diseases. PPAR-γ enhances the regulatory T-cells through PPAR-γ dependent and –independent mechanisms (43). This immunoregulatory effects of PPAR-γ ligands are believed to be mediated through down-regulation of antigen-presenting

cells and pathogenic T-cell function (44, 45, 46, 47, 48, 49).

Cardiovascular diseases : Peroxisome proliferator-activated receptor-γ (PPAR-γ), an essential transcriptional mediator of adipogenesis, lipid metabolism, insulin sensitivity, and glucose homeostasis, also recognized as a key player in inflammatory cells and in cardiovascular diseases (CVD) such as hypertension, cardiac hypertrophy, congestive heart failure, and atherosclerosis (7, 50, 51, 52). PPAR-γ agonists can lower blood pressure and this effect may be at least partially independent of their insulin-sensitizing effects (53, 54, 55, 56). PPAR-γ agonists have also been shown to inhibit hypertrophy of cultured neonatal rat ventricular cardiomyocytes induced by mechanical stress or angiotensin II, and cardiac hypertrophyinduced by aortic constriction in rats and mice. The inhibition on hypertrophy was accompanied by the inhibition on expression of embryonic genes, including atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP), skeletal α-Actin, as well as that of endothelin-1 that can induce cardiac hypertrophy(55, 56).

Cardiac remodeling after ischemic injury is one of the major causes that lead to heart failure. The remodeling process is characterized by myocyte hypertrophy and cardiac fibrosis. PPAR-γ agonists attenuate this remodeling process after ischemia in experimental animals (57, 58). Pioglitazone reduces cell growth, synthesis of collagen type I, and expression of matrix metalloproteinase-1 in cardiac fibroblasts undergone anoxia-reoxygenation or treated with angiotensin II, likely through inhibition of reactive oxygen species generation and NF-κB activation. So various evidences suggest that PPAR-γ activators impact the cardiovascular system through not only their lipid- and carbohydrate-lowering effects but also their anti-inflammatory and antioxidant actions (59-63).

Immune Response

PPARs are expressed in immune cells (48, 64) where they modulate the expression of cytokines and costimulatory molecules. In dendritic cells (DC), the major antigen-presenting cells capable of inducing T-cell-mediated immune responses against a wide range of antigens, PPAR-γ activators reduce the secretion of IL-12, a proinflammatory cytokine playing a key role in atherogenesis and the polarization of the immune response toward T_H1. PPAR-γ activation also affects playing a key role in atherogenesis and the polarization of the immune response toward T_H1 (65). PPAR-γ activation also affects the surface expression of costimulatory molecules, such as CD80 and CD86, and the synthesis of chemokines involved in the recruitment of TH1 cells, including RANTES and IP-10. In addition, PPAR-γ ligands reduce IL-10 secretion and inhibit the expression of the chemokine EB1 ligand and CCR7, both playing a pivotal role in DC migration to the lymph nodes. These effects are accompained by downregulation of LPSinduced These effects are accompained by downregulation of LPS induced nuclear localization of the RelB protein, a transcription factor of the NF-kB family controlling DC differentiation and function. These effects of PPAR-γ ligands in DCs can drive the local immune response by favoring the differentiation of TH2 cells, thus orienting the immune response toward a cytokines, including IFN-γ and TNF-α, and exert antiproliferative effects. PPAR-γ activation leads to decreased effects. PPAR-γ activation leads to a decreased production of IL-2 by negatively interfering with the T-cell specific transcription factor NFAT (66). Inhibition of inflammatory cytokine production and proliferation in T-cells is correlated with the suppression of activated transcription factor AP-1 and NF-kB. Moreover, PPAR-γ ligands can also control major histocompatibility complex class-(MHC) II mediated T-cell activation by inhibiting IFN-γ induced MHC-II expression in vascular cells (67-68).

Neurodegenerative diseases

PPAR-γ agonists are shown to be effective in number of neurodegenerative disorders such as Alzheimer disease (AD), Parkinson disease (PD), Amyotropic lateral sclerosis (ALS), Multiple sclerosis (MS) and Experimental allergic

encephalomyelitis (EAE). These neuro degenerative and neuro-immunological diseases occurs mainly due to activation of non-neuronal cells particularly the microglia and astrocytes (69, 70, 71, 72, 73). PPAR-γ receptors have been found to be expressed on the surface of microglia and PPAR-γ activation has been reported to inhibit the microglial activation (74, 75). The major hallmark of AD is the formation of amyloid plaques which are populated by abundant activated activated microglia and astrocytes along with increased expression of inflammatory enzymes such as inducible nitric oxide synthase (iNOS). *Invitro* and invivo studies have shown that the activation of PPAR-γ in microglial cells suppressed the inflammatory cytokine expression, iNOS and COX-2 expresssion. These effects results from the capacity of PPAR-γ to suppress proinflammatory genes through antagonism of transcription factor nuclear factor κB (NFκB), and, to a lesser extent, activator protein 1. PPAR-γ agonists also suppress the amyloid-β (Aβ)-mediated activation of microglia invitro thereby preventing the cortical or hippocampal neuronal cell death (38, 39, 74, 74).

PPAR-γ agonists are also effective in pathogenesis of PD. The pathological hallmark of idiopathic PD is the loss of dopaminergic neurons in the substantia nigra pars compacta (SNpc). Excitotoxicity, oxidative phosphorylation, generation of reative oxygen species are all thought to contribute to neuronal cell death. Pioglitazone decreased microglial and astrocyte activation, and also reduce the number of iNOS-posive cells in the striatum and SNpc (74, 75, 76). In ALS, pioglitazone is effective in transgenic animal model. Oral treatment of pioglitazone extended the survival of motor neurons in SOD-G93A transgenic mice and also delays the onset of disease. Number of activated microglia were also markedly reduced at the site of neurodegeneration by Pioglitazone (77, 78).

Proinflammatory cytokines plays a key role in the pathogenesis of MS and EAE, an established animal model of MS. PPAR-γ agonists exerts profound and long lasting anti-inflammatory effects in peripheral immune cells (37, 38, 39, 79) and in models of autoimmune disorders (24, 25, 27, 54) suggesting the use of these drugs in invitro and invivo models of MS. Moreover it has been demonstrated that expression of PPAR-γ increases in microglia and astrocyte during EAE, supporting a role this receptor in modulating inflammatory response in MS.

Cancer : The interest in studying the effects of PPAR-γ activation is derived from previous results suggesting that PPAR-γ ligands inhibited cell proliferation when inducing adipocytes differentiation. PPARγ is highly expressed in several human cancer cell lines, including liposarcoma, breasts (81, 82), colon (83, 84), lungs (85, 86), prostate (87, 88), bladder and gastric (89, 90). The PPARγ agonists such as TZDs and 15d-prostaglandin J2 (15d-PGJ-2) have demonstrated not only apoptosis and growth inhibition of numerous cancer cell lines in vitro, but have also shown tumour growth suppression *in vivo* rodent carcinoma models. PPAR-γ not only controls the expression of genes involved in differentiation but also negatively regulates the cell cycle. One possible mechanism is upregulation of tumor suppresor PTEN by PPAR gamma agonists (91, 92). PPAR-γ ligands were also shown to inhibit growth and at least for breast and prostate cancer cells to induce apoptosis. These observations suggest that induction of terminal differentiation by PPAR-γ agonists may represent a promising therapeutic approach to certain human malignancies (93-95).

Systemic sclerosis : Fibrosis is recognized as an important feature of many chronic diseases, such as systemic sclerosis (SSc), an autoimmune disease of unknown etiology, characterized by immune dysregulation and vascular injury, followed by progressive fibrosis affecting the skin and multiple internal organs. SSc has a poor prognosis because no therapy has been shown to reverse or arrest the progression of fibrosis, representing a major unmet medical need. Recently, antifibrotic effects of PPAR5-γ ligands have been studied *in vitro* and in vivo and some theories have emerged leading to

new insights. Aberrant PPAR-γ function seems to be implicated in pathological fibrosis in the skin and lungs. This antifibrotic effect is mainly related to the inhibition of TGF-β Smad signal transduction but other pathways can be involved (96, 97).

Risk associated with PPAR-γ : PPAR-γ agonists have been responsible for various therapeutic effects as well as side effects as shown in figure 3. The withdrawal of troglitazone has led to concerns of the other thiazolidinediones also increasing the incidence of hepatitis and potential liver failure, an approximately 1 in 20,000 individual occurrence with troglitazone. Because of this, the FDA recommends two to three month checks of liver enzymes for the first year of thiazolidinedione therapy to check for this rare but potentially catastrophic complication. The main side effect of all thiazolidinediones is water retention, leading to edema, generally a problem in less than 5% of individuals, but a big problem for some and potentially, with significant water retention, leading to a decompensation of potentially previously unrecognized heart failure. Therefore, thiazolidinediones should be prescribed with both caution and patient warnings about the potential for water retention/weight gain, especially in patients with decreased ventricular function (NYHA grade III or IV heart failure).

Though older studies suggested there may be an increased risk of coronary heart disease and heart attacks with rosiglitazone (98), pioglitazone treatment, in contrast, has shown significant protection from both micro- and macro-vascular cardiovascular events and plaque progression (99, 100). These studies led to a period of Food and Drug Administration advisories (2007 - 2013) that, aided by extensive media coverage, led to a substantial decrease in rosiglitazone use. In November 2013, the FDA

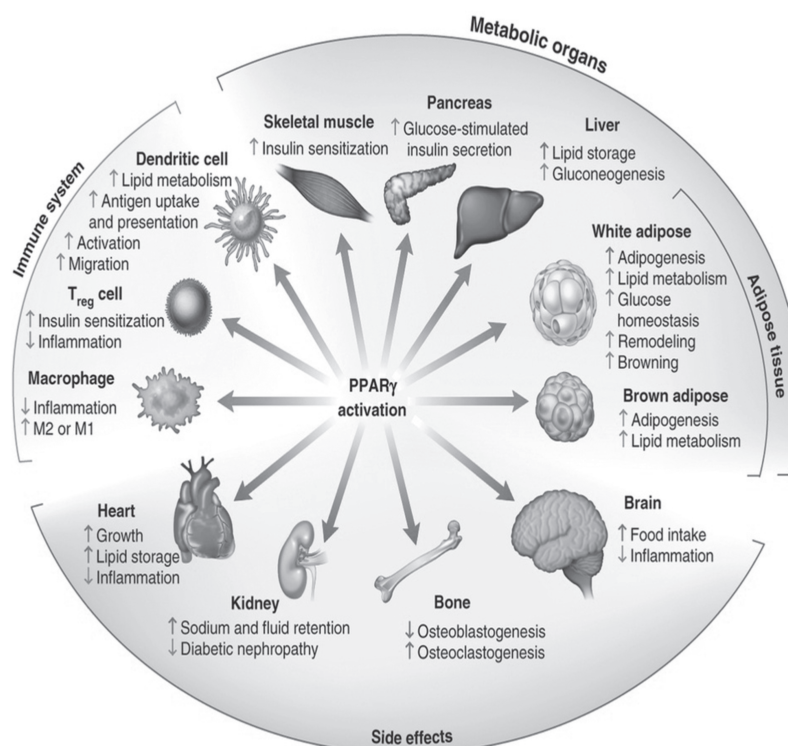

Fig. 3. Benefit and risk associated with PPAR-γ

announced it would remove the usage restrictions for rosiglitazone in patients with coronary artery disease (101). The new recommendations were largely based on the reasoning that prior meta-analyses leading to the original restrictions were not designed to assess cardiac outcomes and, thus, not uniformly collected or adjudicated. In contrast, one of the largest trials (RECORD trial) that were specifically designed to assess cardiac outcomes found no increased risk of myocardial infarction with rosiglitazone use, even after independent re-evaluation for FDA review (102).

A 2013 meta-analysis concluded that use of pioglitazone is associated with a slightly higher risk of bladder cancer compared to the general population. The authors of the same analysis recommended that other blood sugar lowering agents be considered in people with other risk factors for bladder cancer such as cigarette smoking, family history, or exposure to certain forms of chemotherapy (103).

Conclusion

PPARs are critical gene regulators in many metabolically active tissues, yet their functions are not fully established. PPAR gamma agonists convey beneficial effects as therapeutic agents for diabetes and atherosclerosis by lowering blood glucose, improving insulin resistance, inflammation, and lipid metabolism; however, adverse side effects limit their clinical use. Therefore, understanding how the PPAR gamma genes is regulated during disease processes will provide us the opportunity to design effective therapeutic modalities to treat disease by the inactivation, conjugation, and transport of toxic endogenous metabolites. Intensive research on this therapeutic target will likely lead to the development of safer and more effective PPAR agonists in the near future.

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NEWS ITEM

SCIENTIFIC NEWS

Potent Antagonistic Molecules to TB bacteria

Indian Institute of Science (IISc) Scientists have developed two new potent molecules that can severely impact the survival of mycobacteria, including Mycobacterium tuberculosis that causes TB. Unlike most antibiotics that target the bacterial metabolism by aiming at the cellular components, the novel molecules inhibit the stress response pathway of mycobacteria. The master regulator of stress pathway in case of mycobacteria is (p)ppGpp (Guanosine pentaphospahte or Guanosine tetraphosphate). Though a molecule that inhibits the (p)ppGpp formation has already been synthesised, the efficacy is not much. Very high concentration of Relacin molecule is needed to inhibit the pathway and, therefore, the efficacy is low. So we synthesised two new molecules — acetylated compound (AC compound) and acetylated benzoylated compound (AB compound) — by bringing about a modification in the base of the Relacin molecule, says Prof. Dipankar Chatterji. We found our compounds were targeting the Rel gene. The Rel gene makes Rel protein, which in turn synthesises (p)ppGpp. When the Rel gene is knocked out, the long-term survival of Mycobacterium smegmatis decreases and the molecules were able to inhibit biofilm formation by M. tuberculosis and M. smegmatis and also disrupt the biofilm already formed.

Zika virus vaccine

The Hyderabad-based Bharat Biotech developed Zika virus vaccine developed using African strain (MR 766) has shown 100% efficacy against mortality and disease in animal studies. Two doses (5 and 10 microgram) of the vaccine given through intramuscular route on days 0 and 21 to mice were found to protect the animals against Zika virus seven days after the second vaccination. The vaccine was found to confer 100% protection against infection caused by an Asian Zika virus strain as well as by the African Zika virus strain. All the animals that were not vaccinated died eight days after infection by the African strain of the virus and 12 days after infection by the Asian strain. All the mice that did not receive the vaccine, showed progressive morbidity before succumbing to infection. While all the animals that received the vaccine exhibited “undetectable” viral load, the amount of virus present in animals that did not receive the vaccine peaked four days after being infected with either the African or Asian Zika virus strain. The vaccine was developed using the African strain of the virus. It is important to prove that the vaccine developed with the African strain also protects against Zika infection caused by the contemporary Asian strains of Zika virus. Importing the contemporary Asian strains into the country was difficult, and hence the vaccine challenge studies with Asian strain had to be outsourced to a CRO in U.S. according to official of Bharath Biotech. A particular kind of mouse, AG129-which is highly immunocompromised and hence highly susceptible to virus infection was used for studying the protection conferred by the vaccine against Zika virus, disease pathogenesis and mortality. All the AG129 animals received the vaccine showed 100% protection against the virus, demonstrating the superior efficacy of the vaccine.

Relief to Dialysis patients

Scientists from University of California at San Francisco, USA, have developed first bionic kidney, which can replace damaged kidneys easily and effectively. The bionic kidney is a perfect replica of our kidneys. It consists of numerous microchips and is moved by the heart. Like the normal kidneys, it is able to filter waste and toxins from the bloodstream. The project was unveiled by Willian Vanderbilt Fissels and Shuvo Roy from the University of California, offering renewed hope for millions of kidney dialysis patients. Bionic kidney is made from renal cells. The first prototype is the size of a coffee cup and can balance the levels of sodium and potassium in the body while regulating blood pressure.The scientists have high hopes for the bionic kidney, that this device will be available for sale in next 2 years.

Vitamin-C in killing bacteria

A study by a team of researchers at the Indian Institute of Science, Bengaluru has found the molecular mechanism by which vitamin-C impedes and even kills Mycobacterium smegmatis, non-pathogenic bacteria that belongs to the same genus as the TB causing mycobacteria. The effects of vitamin-C on the stress response pathway has been studiedand the vitamin-C has been chosen, because its structure is similar to (p)ppGpp. It was hypothesised that the

vitamin-C should be competing to bind to the Rel enzyme and inhibiting (p)ppGpp synthesis. In vitro studies showed, significant inhibition of (p)ppGpp synthesis in the presence of vitamin-C. The inhibition level was seen to be increasing as the vitamin-C concentration increased. More the vitamin-C concentration the greater the possibility of vitamin-C binding to the Rel enzyme, thus inhibiting (p)ppGpp synthesis. At about 10 mM concentration, the synthesis of (p)ppGpp was completely inhibited. Using Mycobacterial cells, it was found that 1 mM of vitamin-C produced 50% inhibition in (p)ppGpp synthesis. Vitamin-C is able to get inside cells and inhibit (p)ppGpp synthesis. Source - Journal FEMS Microbiology Letters

Negative Mass

Scientists from Washington State University (WSU), cooled rubidium atoms to just above the temperature of absolute zero (close to -273oC), creating what's known as a Bose-Einstein condensate. They also synchronise and move together in what's known as a superfluid, which flows without losing energy. To create the conditions for negative mass, the researchers used lasers to trap the rubidium atoms and to kick them back and forth, changing the way they spin. When the atoms were released from the laser trap, they expanded, with some displaying negative mass. With negative mass, if you push something, it accelerates toward you, scientists described. Also described that it looks like the rubidium hits an invisible wall. The technique could be used to better understand the phenomenon, it was clear that it is the exquisite control we have over the nature of this negative mass, without any other complications. This heightened control also gives researchers a tool for exploring the possible relationships between negative mass and phenomena observed in the cosmos, such as neutron stars, black holes and dark energy. Source- Physical Review Letters journal.

Smart phone laboratory that can detect cancer

In a major step towards faster and convenient delivery of medical tests, Washington State University researchers have developed a low-cost, portable laboratory on a smart phone that can analyse several samples at once to catch a cancer biomarker, producing lab quality results. The research team created an eight channel smart phone spectrometer that can detect human interleukin-6 (IL-6), a known biomarker for lung, prostate, liver, breast and epithelial cancers. A spectrometer analyses, the amount and type of chemicals in a sample by measuring the light spectrum. The spectrometer would be especially useful in clinics and hospitals that have a large number of samples without on-site labs, or for doctors who practice abroad or in remote areas. The multichannel spectrometer can measure up to eight different samples at once using a common test called ELISA that identifies antibodies and colour change as disease markers, according to a study published in the journal Biosensors and Bioelectronics.

Early dinosaurs were similar to Crocodiles

Scientists have unearthed fossils of the earliest known dinosaur relative, a 245-million-years old giant reptile that walked on four legs like a crocodile.The six-foot-long, lizard-like carnivore, called Teleocrater rhadinus, was discovered in Tanzania. The finding fundamentally changes our ideas about the evolution of the prehistoric animals. It is the earliest member of the bird-like side of the family. It is not a direct ancestor of dinosaurs, but it’s the oldest known dinosaur cousin, researchers said. We used to think that many of the distinctive features of bird-line archosaurs evolved very quickly after they diverged from the crocodile line because early bird-line archosaurs like Marasuchus, Dromoeron, and Lagerpeton were small and very dinosaur-like.

Rampatri proved to be a Potential Source of anti Cancer drug

BARC scientists have developed two anti-cancer medicines from the fruit extract of the Rampatri plant, which may help destroy tumours and revive cells damaged by radiation. Rampatri plant, which is used as a spice in foods, belongs to the Myristicaceae family and is found in western coastal region of the country. Scientists at Bhabha atomic Research Centre (BARC) based in Anushaktinagar, Mumbai tested the medicines made from this plant on mice and found that they may help in treating lung cancer and neuroblastoma, a rare cancer found in children. In neuroblastoma, cancer cells grow in nerve cells of adrenal glands, neck, chest and spinal chord. The molecules of Rampatri fruit may destroy the cancer cells. Medicines developed from these molecules may also help in reviving cells destroyed due to radiation. Pre clinical trials have been done for both These fellowships are designed for Indian faculty and researchers who are in the early stages of their

again. The test results of the trial in Guinea were released Thursday in The Lancet. The vaccine was not ready in time to stop the outbreak, which probably began in a hollow, bat-filled tree in Guinea and swept Liberia and Guinea before being defeated. But the prospect of a vaccine stockpile has brought optimism among public health experts. “While these compelling results come too late for those who lost their lives during West Africa’s Ebola epidemic, they show that when the next outbreak hits, we will not be defenseless,” said Marie-Paule Kieny, World Health Organization’s assistant director-general for health systems and innovation and the study’s lead author. “The world can’t afford the confusion and human disaster that came with the last epidemic. “It’s certainly good news with regard to any new outbreak — and one will occur somewhere,” said Anthony S. Fauci, director of the National Institute for Allergy and Infectious Diseases, which makes many vaccines and did some early testing on this one. “But we still need to continue working on Ebola vaccines.”The Lancet study was done in 11,841 residents of Guinea last year. Among the 5,837 people who got the vaccine, none came down with Ebola 10 or more days later. There were 23 Ebola cases among the thousands of others not immediately vaccinated.

Indian Pepper may serve as a Potential Cancer Drug: The Indian long pepper, widely popular for spicing up food, may soon be used as a potential cancer treatment drug, according to a new study. The Indian long pepper contains a chemical that could stop your body from producing an enzyme that is commonly found in tumours in large numbers, according to the study in Journal of Biological Chemistry. UT Southwestern Medical Enter scientists have uncovered the chemical process behind anti-cancer properties of a spicy Indian pepper plant called the long pepper, whose suspected medicinal properties date back thousands of years. The secret lies in a chemical called Piperlongumine (PL), which has shown activity against many cancers including prostate, breast, lung, colon, lymphoma, leukaemia, primary brain tumours and gastric cancer. Using X-ray crystallography, researchers were able to create molecular structures that show how the chemical is transformed after being ingested. PL converts to hPL, an active drug that silences a gene called GSTP1. The GSTP1 gene produces a detoxification enzyme that is often overly abundant in tumours, the study said.

Scientists hear voice of ancient humans in baboon calls: The barks, yacks and wa-hoos of the Guinea baboons reveal distinct human-like vowel sounds. Baboon grunts and mating calls may hold secrets about human speech, according to a new study suggesting that the origins of human language could reach back as much as 25 million years. The barks, yacks and wa-hoos of the Guinea baboons reveal distinct human-like vowel sounds, according to the study published on Wednesday in the journal Plos One by scientists from six universities in France and Alabama. The authors from the Grenoble Alpes University studied the acoustics of 1,335 baboon sounds and the animals’ tongue anatomy. Researchers suggest that the human vocal system developed from abilities already present in ancestors such as the Guinea baboon. The scientists also found similar muscles in baboon tongues as human tongues which are key to our ability to make vowel sounds. “The evidence developed in this study does not support the hypothesis of the recent, sudden, and simultaneous appearance of language and speech in modern Homo sapiens,” the study says. “It suggests that spoken languages evolved from ancient articulatory skills already present in our last common ancestor ... about 25 million years ago.”

Study says Hindu Kush – Himalayan Water Supplies may be affected by ongoing Climate change: More than a glacial retreat in the Hindu Kush-Himalaya region (HKH), the shifts in rain and snow due to climate change are likely to have an impact on regional water supplies and groundwater recharge, a study said. The study was conducted by two Kathmandu-based experts from International Centre for Integrated Mountain Development (ICIMOD). It showed that at lower elevations, glacial retreat is unlikely to cause significant changes in water availability over the next couple of decades, but other factors, including groundwater depletion and increasing human water use, could have a greater impact. Higher elevation areas could experience altered water flow in some river basins if current rates of glacial retreat continue. "The shifts in the location, intensity, and variability of rain and snow due to climate change impacts will likely to have a greater impact on regional water supplies and groundwater recharge than glacial retreat," the study said. The Hindu Kush-Himalaya is one of the most dynamic, diverse, and complex mountain systems in the world, with several rivers and glacial systems making the region a "Third

Pole" of the earth, providing fresh water resources to more than 210 million people in the mountains and 1.3 billion people downstream. Scientific evidence shows that most glaciers in the Hindu Kush-Himalaya region are shrinking, but the consequences of this melt for the regional water systems, especially groundwater, is not clear.

OPPORTUNITIES
(Post-doctoral Fellowships)

IISER, Mohali: IISER Mohali invites applications at the level of Postdoctoral Research Associates. Interested applicants who have either a PhD degree or have submitted their PhD thesis can apply for these positions. It is preferred that the candidate has some overlap with the research interests of one or more faculty members at the Institute. This is a rolling advertisement and the applications will be reviewed from time to time. The salary for Postdoctoral Research Associates will be as per the MHRD norms. Applications should be sent by email todeanfaculty@ iisermohali.ac.in with the subject clearly stating "Application for Postdoctoral position at IISER Mohali".

IIT – Bhubaneswar: Candidates preferably below 35 years having a Ph.D. Degree and willing to carry out advanced research in the fields of Basic Sciences; Earth, Ocean & Climate Sciences; Electrical Sciences; Infrastructure; Mechanical Sciences; Humanities, Social Sciences & Management may apply for this position. For further information please contact to Assistant Registrar (Academic Affairs), Contact No – 0674-2576019, E-mail id – ar.acad@iitbbs.ac.in

IIT – Jodhpur: Candidates with consistently good academic record and interdisciplinary research potential, as full-time Institute Post-Doctoral Fellowships (PDFs) to carryout research in the following areas at the Institute: Computer Science & Engineering, Electrical Engineering, Mechanical Engineering, Biology and others may apply. For more details visit the website http://iitj.ac.in/PDFs.

IIT – Gandhinagar: Indian institute of Technology Gandhinagar is seeking applications for postdoctoral positions in Cognitive Science. Candidates of any nationality with a PhD degree ('all but defense' are eligible) in any discipline related to Cognitive Science can apply. We will also consider applications of exceptionally meritorious candidates who would like to switch their focus of research from another discipline into Cognitive Science. For more details visit the website http://cogs.iitgn.ac.in/

Indian Institute of Science: Applications are invited for a Research Associate/Post-Doctoral Fellow to work on a Wellcome Trust DBT-India Alliance funded project at the laboratory of Dr. Sridharan Devarajan, at the Centre for Neuroscience at IISc, Bangalore. Please send a CV with two references by e-mail to sridhar@cns.iisc.ernet.in. Short listed candidates will be called for an interview.

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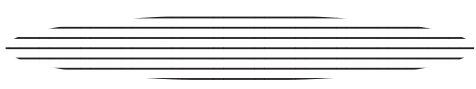

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