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CONTENTS

Current Trends in Biotechnology and Pharmacy
Vol. 10 (2) April 2016, ISSN 0973-8916 (Print), 2230-7303 (Online)

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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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Effect of Excipients on Disintegration, Viability and Activity of Fast Disintegrating Tablets Containing Probiotic and Starter Cultures

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Abstract

The effect of excipients on viability, activity and disintegration time of fast disintegrating tablets of probiotic and starter cultures were studied. Active ingredients (AI) of freeze dried cultures in three different combinations *viz*., *Streptococcus thermophilus* MTCC 5460 + *Lactobacillus helveticus* MTCC 5463(AI1); *Streptococcus thermophilus* MTCC 5460 + *Lactobacillus rhamnosus* MTCC 5462(AI2); *Streptococcus thermophilus* MTCC 5460 + *Lactobacillus bulgaricus* NCIM 2358 (AI3) were mixed with excipients such as spray dried lactose, super starch 200, sodium starch glycolate, PVP K-30, croscarmelos sodium, cros povidone at varying levels. Direct compression method with 1-2 kg/cm^2 pressure was used for tablet preparation. Tablets were assessed for their viability, activity and disintegration time. Viability of the tablets was measured as log cfu/ tablet and activity assessed based on its ability to form curd and its acidity and pH. Further, the interaction of active ingredients and excipients selected were studied using Fourier Transform Infrared (FTIR) Spectroscopy at a resolution of 2 cm^{-1}, over a frequency range of 4000 to 400 cm^{-1}. Excipients and their levels had a significant ($P < 0.05$) effect on disintegration time, viability and activity of tablets. For all the three active ingredients, tablets made using combination of excipients crospovidone, super starch 200 and PVP K-30 was found to be superior in terms of viability (>8.5 log cfu / tablet) and disintegration time (32.56 sec). Further, the Infrared spectra of active ingredients with excipients depicted the same characteristic peaks of probiotics in physical mixtures without any markable change in their position indicating no chemical interaction between probiotics and excipients used and hence proved their compatibility and suitability for preparation of probiotic and starter tablets.

Keywords : *Lactobacillus helveticus* MTCC 5463, Fast disintegrating tablets, Probiotic and starter cultures, Viability, Activity

Introduction

Apart from the traditional role in food fermentation and preservation, starter cultures belonging to the lactic acid bacteria (LAB) are increasingly finding application as functional starters because of their ability to provide health benefits (1-4). Dry dosage forms of these functional starters such as powder blends, capsules and tablets are widely used for direct vat food applications as well as for nutritional/ dietary supplementation. The viability and functionality of these cultures in such dosage

forms depends to a great extent on the composition of the dry matrix of dosage forms which in turn depends on the kind and level of excipients used. Among the various dosage forms, tablet forms are known to give better stability, proper distribution, accurate dosage as well as convenience to the consumers in terms of self-administration and compactness. Selection of excipients, which are compatible with the cultures and which could also act as protective agents during processing and storage are considered crucial in the development of formulations for dosage forms (5). A number of studies have highlighted the protective effect of excipients such as reducing agents and bulking agents in the freeze dried preparations of cultures (6-9). At the same time, the importance of excipients in the tablet formulation in the survival as well as functionality of probiotic tablets is also being reported. Some of these studies revealed the importance of excipients in preserving the high number of viable probiotics (10, 11), some dealt with the use of carrier agents in probiotic tablets for successful colonic delivery (12), still some studies highlighted the importance of matrix for providing gastric-protection features (13) and in maintaining suitable environment during storage (14, 15). Additionally, the incorporation of excipients has a huge influence on the flow properties and compaction behavior of tablet formulations during tablet making. Very few studies are being reported on fast disintegrating tablets of probiotic and starter cultures. Taking these important aspects into consideration, the present research work was carried out to study the effect of excipients on the disintegration time as well as viability and activity of fast disintegrating tablets of probiotics and starter cultures for application as inocula for product preparation or as food supplement. This study also provides a rationale for careful selection of suitable excipients for probiotic and starter culture tablets.

Materials and Methods

Microbial Cultures *: Streptococcus thermophilus* MTCC 5460, Probiotics *Lactobacillus helveticus* MTCC 5463 and *Lactobacillus rhamnosus* MTCC 5462 were obtained from SMC College of Dairy Science, AAU, Anand while *Lactobacillus delbrueckii* subsp. *bulgaricus* NCIM 2358 was obtained from NDRI, Karnal. The probiotic cultures are thoroughly studied for their probiotic potential (16, 17). All the cultures were propagated in sterilized reconstituted skim milk (12% Total solids) by incubation at 37°C for 24 h and stored at 5 ± 2 °C. During the course of study, prior to their use, the cultures were given three successive transfers in the paneer whey medium to activate them.

Preparation of freeze dried cultures *:* Freeze dried powders of the cultures were prepared using protocol optimized by Jani et al. (18). The active cultures were inoculated in the paneer whey medium at 2% rate and grown at 37°C for 24 h. The cells were harvested by centrifugation at 6000 rpm at 4 °C for 20min. Cell pellets were washed twice with saline (0.85% NaCl). The cells were then suspended in 12 % reconstituted skim milk added with 1% glycerol. The contents were mixed thoroughly and distributed in glass petriplates. The suspension was frozen at -20°C for overnight and freeze dried using freeze dryer model Virtis genesis 25XL. During the entire primary drying process, vacuum of 100 millitorr was maintained. Temperatures in the drying chamber were gradually increased from -40 °C to 30 °C over a period of 15-16 h looking to the process of drying.

Preparation of active ingredients (AI) : Freeze dried cultures in three different combinations, *viz*., (a) *Streptococcus thermophilus* MTCC 5460 +*Lactobacillus helveticus* MTCC 5463; (b) *Streptococcus thermophilus* MTCC 5460 +*Lactobacillus rhamnosus* MTCC 5462; (c) *Streptococcus thermophilus* MTCC 5460 +*Lactobacillus bulgaricus* NCIM 2358) were mixed with reducing and bulking agents for preparing the active ingredients (AI) to be used for tablets. L-Ascorbic acid and spray dried lactose were used as the reducing and bulking agents respectively. The rate of culture, reducing agent and bulking agent in the AIs were fixed as

Vol. 10 (2) 108-117 April 2016, ISSN 0973-8916 (Print), 2230-7303 (Online)

20, 20 and 60% (w/w) respectively based on study done by Panchal *et al*. (19). The AIs were studied for culture–excipient compatibility and micromeritic characteristics.

Estimation of viability, activity and micromeritic properties of active ingredients: Serial dilutions of the active ingredients were made using 2% peptone water as dilution blank. For preparing the initial dilution, 0.1 g powder was reconstituted in peptone water and incubated at 37°C for 2 h to recover cell injuries. Subsequent dilutions were then prepared in peptone water and appropriate dilutions were pour plated using respective selective agar medium. MRS medium was used for lactobacilli and M17 medium was used for streptococci. Once the initial agar layer was set, a second layer (5-8 ml) of the same medium was made to maintain facultatively anaerobic conditions. The plates were then incubated at 37±2°C for 72 h. Colony counts were taken with the help of colony counter and the count was expressed as log (cfu/g) (20). To test the activity, all four freeze dried cultures and their AIs were checked for their ability to form curd. The inoculation rate was 0.1g/100ml milk. After inoculation of the samples, the skim milk flasks were incubated at 37°C for overnight (15h). The curd was analyzed for titratable acidity, pH and viable count. Active ingredients (AI) were evaluated for bulk density, tapped density, angle of repose, carr's index and hausner's ratio to evaluate micromeritic properties (21) using following formulae.

Angle of repose (è) = tan-1 (h/r) ; Where, h= height of pile , r = radius of pile

$$\text{Carr's index} = \frac{\text{tapped density } (\rho t) - \text{bulk density } (\rho b)}{\text{tapped density } (\rho t)} \times 100$$

Hausner's ratio = ñt / ñb; Where, ñt = tapped density ; ñb = bulk density

Microbiological analysis of curd : Eleven gram of curd sample was aseptically weighed and transferred to 99ml phosphate buffer dilution blank to obtain 1:10 dilution. Subsequently, 1 ml of above dilution was used for making further dilutions in 9 ml phosphate buffer tubes. Suitable dilutions were prepared and poured in a set of sterile Petri dishes in duplicates and poured with suitable agar for enumeration of total bacterial count.

Preparation of culture tablets : All the excipients were passed through 80 mesh sieve. Required quantities of AIs and excipients except lubricant and glidant were mixed thoroughly in a double cone blender. The powder blend was mixed with lubricant glidant mixture. This powder mixture was compressed into tablets by direct compression method with 1-2 kg/cm^2 pressure using ten station rotary tablet machine (Rimek, RSB4-1, Karnavati Engg. Pvt. Ltd., Ahmedabad, India) using round tooling at rotational speed of 50 rpm.

Analysis of tablets for disintegration, hardness, viability and activity : For estimating the disintegration time, the tablets were put in the water maintained at 37°C and the time of complete dissolution was noted. Hardness of tablets was measured using Monsanto hardness tester. For estimating the viability of tablets, serial dilutions of the tablets were made using 2% peptone water as dilution blank. For preparing the initial dilution one tablet was dissolved in 10ml of peptone water. Subsequent dilutions were then prepared in peptone water and appropriate dilutions were pour plated using respective selective agar medium. Once the initial agar layer was set, a second layer (5-8 ml) of the same medium was made to maintain facultatively anaerobic conditions. The plates were then incubated at 37±2°C for 72 h. Colony counts were taken with the help of colony counter and the count was expressed as log cfu/tablet. For checking the activity of the cultures in the tablets, one tablet was put in 100ml sterile reconstituted skim milk (12%Total solids) and dissolved completely. It was then incubated at 37±2°C for overnight (15h). The curd formed was evaluated for sensory characteristics by expert panel of judges using nine point hedonic scales.

Vol. 10 (2) 108-117 April 2016, ISSN 0973-8916 (Print), 2230-7303 (Online)

Active ingredients-excipients compatibility study using FTIR spectrometer : Transmission spectra of active ingredients and the selected excipients and their physical mixtures were measured using FTIR spectrometer in order to detect the existence of interactions between probiotics and physical mixtures. The samples were first ground gently in a mortar and mixed with potassium bromide before being compressed into tablets. Scans were obtained at a resolution of 2 cm^{-1}, over a frequency range of 4000 to 400 cm^{-1}.

Statistical analysis *:* The values of each attribute under study were subjected to statistical analysis using Completely Randomized Design with equal number of observations using the model proposed by Steel and Torrie (22).

Results and Discussion

The overall composition of formulations used for preparation of tablets comprised of active ingredients and various excipients (Table-1). The formulations varied in terms of presence or absence of excipients such as spray dried lactose, sodium starch glycolate, poly vinyl pyrolidone (PVP) K-30, cross carmelos sodium and crosspovidone. Talc and magnesium stearate were used as glidant and lubricant to facilitate tablet making.

Viability, activity and micromeritic properties of active ingredients: Viability, activity and micromeritic properties of all the three active ingredients are shown in Table-2. A significant (*P <0.05*) difference in the counts of *Lactobacillus helveticus* MTCC 5463, *Lactobacillus rhamnosus* MTCC 5462 and *Lactobacillus delbrueckii* subsp. *bulgaricus* NCIM 2358 was observed in the active ingredients. The viable counts of all strains in all the three active ingredients were found to be > 9.0 log cfu/g. This satisfied the requirement of high level of probiotic and starter culture cell count in the tablet and is a must for ensuring functionality of the tablet. In this study, the effect of higher viable counts were reflected in the rate of acid development during curd preparation, which was taken as a measure of activity of active ingredients. The curds obtained were of uniform quality. The set curds were sufficiently firm to hold their shape when poured. There was no whey separation and the curds were organoleptically acceptable. All the three active ingredients exhibited good viability and activity and the results were in line with the viability of strains of lactobacilli and streptococci observed by earlier workers especially in freeze dried preparations (18, 19, 23, 24).

Micromeritic properties are considered important for the flow properties and the compaction behavior required during dosage form making. The interpretations regarding the relationship between flow, angle of repose and carr's index were drawn as per Indian Pharmacopeia (IP)/United States Pharmacopeia (USP). Values for micromertitic properties of active ingredients are shown in Table 2. Values for angle of repose differed non-significantly between the active ingredients where as in case of the values for carr's index, a significant (*P <0.05*) difference was observed between AI1 (52.37 ± 1.26), AI2 (55.43 ± 0.17) and AI3 (58.29 ± 1.11). The values for Hausner's ratio also differed significantly (*P <0.05*) between the active ingredients. Angle of repose for all the cultures was found to be in the range of 30.00 to 40.00, indicating a fair to passable flow property. Also the values for Carr's index varied between 11.00 to 20.00, again indicating fair to passable flow property of the active ingredients. The micromeritic properties indicated the suitability of the formulations for tablet preparation in terms of flow characteristics.

Disintegration time and hardness of tablets: Tablets of all the three active ingredients prepared using different formulations exhibited a significant (*P<0.05*) difference in the disintegration time (Fig. 1). This may be mainly because of difference in the kind and level of excipients used for preparation of formulations. Cultures did not show any significant effect on the disintegration time. Except for tablets prepared using formulations F4 and F5 all other tablets showed a longer disintegration time (>120 sec). The mean

Vol. 10 (2) 108-117 April 2016, ISSN 0973-8916 (Print), 2230-7303 (Online)

disintegration time for tablets made using F4 and F5 was 37 sec and 33 sec respectively. These two formulations contained super disintegrants croscarmelos sodium and crospovidone along with Polyvinyl pyrrolidone (PVP) K-30. The combination of these excipients might have facilitated the faster disintegration. Nagashima *et al*. (25) in their study on development of effervescent tablets reported a disintegration time of 1 minute for the tablets prepared using the effervescent formulation (25). Superdisintegrants such as crospovidone use a combination of swelling and wicking action for fast disintegration of tablets. The unique, porous particle morphology facilitates wicking of liquid into the tablet and particles to generate rapid disintegration. Due to its high crosslink density, crospovidone swells rapidly in water without gelling (26). The hardness values of tablets of different formulations varied between 1.44 ± 0.56 kg/cm^2 to 1.21±0.11 kg/cm^2.

Viability and activity of tablets: The individual viable counts of strains in the tablets of AI1, AI2, AI3, prepared using different formulations showed significant (*P <0.05*) difference (Table 3). This clearly indicated that the excipients do have an influence on the viability of cultures. Between the formulations F4 and F5, the tablets prepared using F5 showed a significantly higher viability (>8.5 log cfu/tablet) for culture strains MTCC 5460 and MTCC 5463. The activity of the tablets was measured by preparing curd using the tablets and incubating overnight at 37±2°C. Tablets from different formulations had a significant (*P <0.05*) effect on the acidity and pH of the curd samples (Table-4). This may be because of the inherent difference in the milk fermentation ability of the cultures used. The current study results showed that combination of excipients crospovidone, super starch 200 and PVP K-30 was found to be superior in terms of viability (>8.5 log cfu / tablet) and disintegration time (32.56 sec) of probiotic and starter culture tablets. Not many literatures are available regarding fast disintegrating culture tablets. But our study results corroborated with a few of other study results, which have shown the importance of excipients on functional and technological properties of probiotic tablets. The importance of excipients in preserving the high number of viable probiotics in a gastric juice resistant tablet was reported by Stadler and Viernstein (10). The study used hydroxyl propylmethylcellulose acetate succinate (HPMCAS) as well as alginate, apple pectin and Metolose® as matrix forming components. They reported an improved gastric resistance of probiotics with higher amount of HPMCAS. Nagashima *et al*. (25) studied the effects of excipients on the viability of probiotic microorganisms in the effervescent tablet formulation containing *Lactobacillus acidophilus* and *Saccharomyces boulardii.* They suggested that the ingredients with negative effects on viability may decrease the concentration of microorganisms (25). e Silva *et al*. (27) in their work on development of probiotic tablets using microparticles selected the composition of tablets containing several excipients such as 5% sodium croscarmellose or 5% sodium croscarmellose and 20% sodium alginate or 5% sodium croscarmellose and 20% cellulose acetate phthalate (CAP) based on their higher resistance to acid and their ability to confer protection to probiotic bacteria in the tablets (27). Lone and Dhole (28) in their study on formulation and evaluation of probiotic tablets containing anti-inflammatory drug reported that the combination of HPMCAS and sodium alginate together increased acid tolerance of probiotic lactobacilli strains added in matrix tablets compared to other matrices (28).

Active ingredient-excipient compatibility using FTIR: Transmission spectra of AI1, AI2, AI3, starch 200, PVP K30 and crospovidone and their physical mixtures were measured using FTIR spectrometer to detect the existence of interactions between probiotics and physical mixtures. This is essential for careful selection of most appropriate excipients and in designing a stable and effective dosage form. The method allows pointing out the implication of the different functional groups of active ingredient and

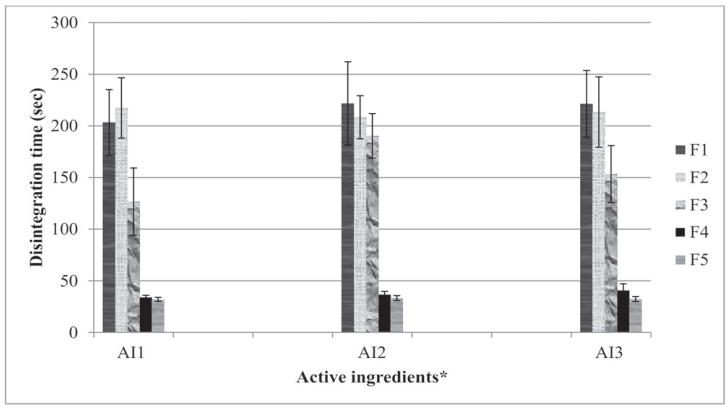

Fig. 1. Disintegration time of tablets prepared using different formulations *(AI1= MTCC 5460+MTCC 5463; AI2=MTCC 5460+MTCC 5462; AI3=MTCC 5460 +NCIM 2358)
F = formulations.

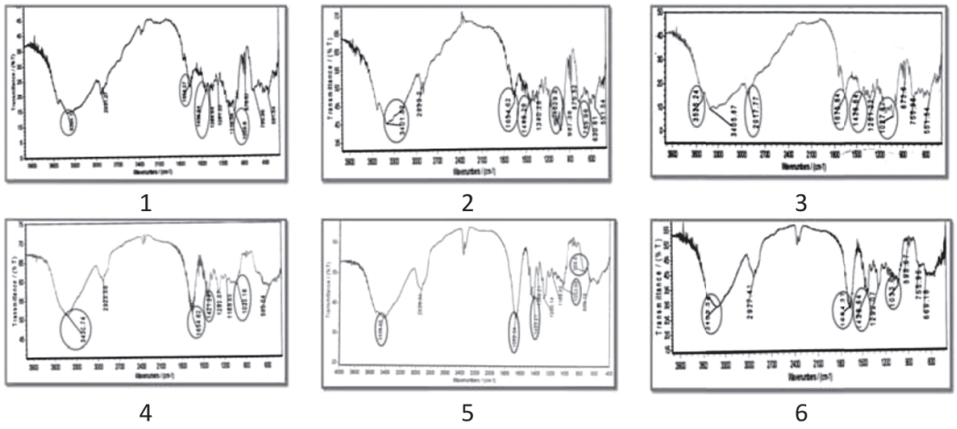

Fig. 2. FTIR spectra of active ingredients and excipients [1 and 4 (AI1 and AI1+excipients), 2 and 5 (AI2 and AI2+excipients), 3 and 6 (AI3 and AI3+excipients)]

Table 1. Formulations used for preparation of tablets using direct compression method

Ingredients	Formulations				
	F1 (mg)	F2 (mg)	F3 (mg)	F4 (mg)	F5 (mg)
Active ingredient	100	100	100	100	100
Spray Dried Lactose	195	195	195	-	-
Super Starch 200	100	100	100	160	160
Sodium Starch Glycolate	50	-	-	-	-
PVP K-30	-	-	-	15	15
Cross Carmelos sodium	-	50	-	15	-
Cross povidone	-	-	50	-	20
Talc	20	20	20	7	7
Magnesium Stearate	15	15	15	3	3
Total weight of Tablet (mg)	480	480	480	300	305

Table 2. Viability, activity and micromeritic properties of active ingredients

Active ingredients*	Viability (log cfu/g)		Activity		Micromeritic properties		
	MTCC 5460	MTCC 5463/ MTCC 5462/ NCIM 2358	Acidity (% Lactic Acid)	pH	Angle of repose (Degrees)	Carr's index (%)	Hausner's ratio
AI1	9.31 ± 0.07	9.26 ± 0.01^{b}	0.92 ± 0.02^{b}	4.37 ± 0.08^{a}	36.79 ± 2.82	12.37 ± 1.26^{a}	1.83 ± 0.52^{b}
AI2	9.28 ± 0.04	9.17 ± 0.05^{a}	0.95 ± 0.03^{b}	4.32 ± 0.03^{a}	35.28 ± 0.73	15.43 ± 0.17^{b}	1.97 ± 0.48^{b}
AI3	9.25 ± 0.11	9.19 ± 0.05^{a}	0.78 ± 0.02^{a}	4.53 ± 0.02^{b}	36.17 ± 1.11	18.29 ± 1.11^{c}	0.46 ± 0.01^{a}
C.D.	NS	0.05	0.03	0.06	NS	1.17	0.49

Each observation is mean±SD of four replications

*(AI1= MTCC 5460+MTCC 5463; AI2=MTCC 5460+MTCC 5462; AI3=MTCC 5460 +NCIM 2358)
$^{a-c}$ Superscript letters following numbers in the same column denote significant difference ($p<0.05$)
NS = Non significant

Table 3. Viability of the tablets prepared from different formulations using direct compression method

Formulations	Viability (log cfu/tablet)					
	AI1		AI2		AI3	
	MTCC 5460	MTCC 5463	MTCC 5460	MTCC 5462	MTCC 5460	NCIM 2358
F1	8.41 ± 0.19^{ab}	8.75 ±0.20^{bc}	8.33 ± 0.17^{ab}	8.64 ± 0.11^{b}	8.62 ±0.07^{b}	8.63 ± 0.06^{c}
F2	8.54 ± 0.01^{b}	8.40 ± 0.10^{ab}	8.72 ± 0.08^{c}	8.69 ± 0.05^{b}	8.59 ±0.09^{b}	8.48 ± 0.06^{b}
F3	8.63 ± 0.21^{b}	8.56 ± 0.36^{abc}	8.75 ± 0.11^{c}	8.62 ± 0.20^{b}	8.62 ± 0.04^{b}	8.62 ± 0.13^{bc}
F4	8.23 ± 0.12^{a}	8.22 ± 0.06^{a}	8.20 ± 0.15^{a}	8.17 ± 0.07^{a}	8.20 ± 0.27^{a}	8.21 ± 0.08^{a}
F5	8.63 ± 0.12^{b}	8.81 ± 0.04^{c}	8.52 ± 0.09^{bc}	8.70 ± 0.07^{b}	8.64 ± 0.06^{b}	8.73 ± 0.03^{c}
CD (0.05)	0.27	0.35	0.23	0.20	0.25	0.14

Each observation is mean±SD of four replications

(AI1= MTCC 5460+MTCC 5463; AI2=MTCC 5460+MTCC 5462; AI3=MTCC 5460 +NCIM 2358)
$^{a-c}$ Superscript letters following numbers in the same column denote significant difference ($p<0.05$)

Table 4. Activity of the tablets prepared from different formulations using direct compression method

Active ingredients*	Formulations				
	F1	F2	F3	F4	F5
			Acidity (% Lactic acid)		
AI1	0.73 ± 0.07	0.86 ± 0.04	0.99 ± 0.02	0.84 ± 0.04	0.92 ± 0.03
AI2	0.92 ± 0.04	1.02 ± 0.02	1.09 ± 0.02	0.91 ± 0.02	1.00 ± 0.02
AI3	0.81 ± 0.05	0.88 ± 0.02	1.00 ± 0.01	0.84 ± 0.02	0.98 ± 0.01
CD (0.05) C=0.025, F=0.032; CxF=NS					
			pH		
AI1	4.78 ± 0.45	4.52 ± 0.02	4.30 ± 0.11	4.24 ± 0.02	4.47 ± 0.04
AI2	4.48 ± 0.05	4.21 ± 0.05	4.14 ± 0.02	4.47 ± 0.02	4.23 ± 0.02
AI3	4.55 ± 0.01	4.45 ± 0.09	4.24 ± 0.02	4.51 ± 0.01	4.42 ± 0.01
CD (0.05) C=0.093, F=0.120; CxF=NS					

Each observation is mean±SD of four replications, C type of active ingredients, F kind of formulations. NS = Non significant
*(AI1= MTCC 5460+MTCC 5463; AI2=MTCC 5460+MTCC 5462; AI3=MTCC 5460 +NCIM 2358)

excipient molecules by analyzing the significant changes in the shape and position of the absorbance bands (29). The infrared (IR) spectra of the AIs and their physical mixtures with starch 200, PVP K30 and Cross Povidone are shown in Fig.2. The main absorption bands of probiotics are -OH stretching at 3394-3525 cm^{-1}, C=O stretching at 1654-64 cm^{-1}, C-O-C symmetric stretching at 1021-29 cm^{-1}, CH_3- stretching at 1421-38 cm^{-1} and 775 cm^{-1} for mono substituted ring . Figure 2.0 depict the characteristic peaks of probiotics in physical mixtures (for AI1 and AI2) without any markable change in their position indicating no chemical interaction between probiotics and polymer used. In the binary mixture of AI3 and all excipients, an IR peak at 3525 and 3405 cm^{-1} observed in pure probiotics samples. This peaks disappeared and another peaks such as 3409 and 2927 cm^{-1} were found, indicating a slight incompatibility between AI3 and excipients but no adverse effects were observed in the tablet characteristics and its viability and activity, hence proving their compatibility and suitability for preparation of probiotic and starter culture tablets.

Conclusion

The type, level and combinations of excipients used for preparing the tablet formulation had a significant effect on the viability, activity and disintegration time of culture tablets. The combinations of excipients were found to be the important factors than the effect of individual excipients. But the extent of such effects, especially in case of a culture preparation, may vary from strain to strain in the same composition or in different compositions. Hence proper selection of the excipients and their concentrations which do not exert any adverse effect on the viability and activity of the cultures used in the formulations and at the same which will also facilitate ease of tablet preparation are of utmost importance.

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Antioxidative System as Influenced by High Temperature stress in *Brassica juncea* (L) Czern & Coss

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Abstract

The present investigation was undertaken to study the changes in antioxidative enzymes, lipid peroxidation and metabolites under heat stress and its revival in *B. juncea* genotypes (Thermo-tolerant BPR-542-6 and thermo-susceptible RGN-152). The activities of antioxidative enzymes *viz.* superoxide dismutase (SOD), ascorbate peroxidase (APX), glutathione reductase (GR), peroxidase (POX) and catalase (CAT) increased under high temperature stress upon revival. SOD and CAT started decreasing but activity of POX and GR still continued increasing in both the genotypes. But APX enzyme exhibited differential behaviour on revival which increased in tolerant genotype but decreased in susceptible genotype. Lipoxygenase (LOX), malondialdehyde (MDA) and hydrogen peroxide (H_2O_2) which are the indicators of cell membrane damage increased under heat stress in both the genotypes but increase was higher in susceptible genotype. On recovery, LOX, MDA and H_2O_2 content decreased in both the genotypes. Ascorbic acid and carotenoids content increased under high temperature stress but during recovery, ascorbic acid continued increasing while carotenoids started decreasing in both the genotypes.

Key words: Antioxidant, Ascorbate peroxidase, catalase, glutathione reductase, superoxide dismutase, peroxidase.

Abbreviations: superoxide dismutase (SOD), ascorbate peroxidase (APX), glutathione reductase (GR), peroxidase (POX) and catalase (CAT), Lipoxygenase (LOX), malondialdehyde (MDA), hydrogen peroxide (H_2O_2), hydroxyl radical (OH^-), singlet oxygen ($^1O_2$)

Introduction

The rise in temperature due to global warming has implication on world wide crop production systems (Wahid *et al*., 2007). Indian mustard [*Brassica juncea* (L) Czern & Coss], is the major oilseed *rabi* crop of north-western India. High temperature prevailing at sowing time reduces germination, emergence and survival of seedling resulting in loss of productivity (Wilson *et al.,* 2014). There are increasing evidences that high temperature increased the generation of reactive oxygen species (ROS) such as superoxide radical ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), hydroxyl radical (OH^-) and singlet oxygen ($^1O_2$) (Kumar *et al*., 2012). The equilibrium between the production and scavenging of ROS may be perturbed under adverse abiotic stresses thereby results into reduction in crop yield. These overproduced ROS under stress conditions react directly with lipids, proteins and nucleic acids which cause lipid peroxidation mediated membrane injury, protein degradation, enzyme inactivation, pigment bleaching and disruption of DNA strands (Davis, 1987). Plants have developed the scavenging mechanism of ROS

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categorized as enzymatic and non-enzymatic defense system. When ROS increases, chain reactions start in which superoxide dismutase (SOD), a metalloenzyme catalyzes the dismutation of O_2^- radical to molecular O_2 and H_2O_2. The H_2O_2 is then detoxified either by catalase/peroxidase or in ascorbate glutathione cycle which involves oxidation and reduction of ascorbate and glutathione through ascorbate peroxidase (APX) and glutathione reductase (GR) action (Noctor and Foyer, 1998). Catalase (CAT) reduces H_2O_2 into H_2O and O_2, whereas peroxidase (POX) decomposes H_2O_2 by oxidation of co-substrate such as phenolic compounds. *Brassica juncea* is an important oilseed crop of winter season and its early sowing implies many important advantages. But high temperature prevailing at the sowing time imposes severe limitation on early germination pattern and subsequent seedling establishment and yield of *Brassica juncea*. Keeping in view the burning problem of global warming which may result into yield loss, the present investigation was proposed to understand the response of tolerant and susceptible genotypes of *B. juncea* to high temperature stress and its revival with main emphasis to study the role of antioxidative enzymes in imparting tolerance to *Brassica* crop.

Materials and Methods

Plant material and growth conditions : Thirty six genotypes of *Brassica juncea* were procured from National Research Centre of Rapeseed–Mustard, Sewar, Bhartpur (Rajasthan) for the study. Seeds of thirty six genotypes were sown in trays having sandy loam soil. Each tray was filled with 7 kg soil which was previously homogenized with enough water to bring the soil to field capacity (150 ml water/kg soil). Each tray filled with soil was uniformly marked into 6 rows and 6 spots in each row. Five seeds of each genotype were sown in each spot. Seedlings were allowed to grow at optimum temperature (25±0.5°C), relative humidity (70%) and 16 hrs light, 8 hrs dark cycle for five days. Screening of *Brassica juncea* to high temperature was done by adopting the method of Chhabra *et al*. (2007). Seedlings were exposed to threshold high temperature (45±0.5°C) and 30% relative humidity continuously. Time taken to 50% mortality was recorded in all the genotypes and genotypes were screened for high temperature tolerance. Experiment was repeated five times and average values were recorded. After subjecting the *B. juncea* seedlings to heat stress, the seedlings were revived by placing the trays at 25±0.5°C for 24 hrs.

Antioxidative system analysis : For the extraction of MDA and H_2O_2, 1.0 g seedlings each from control, stressed and revived plants were taken and ground in 5 ml of chilled 0.8 N $HClO_4$ and centrifuged at 10,000 x g for 25 min. The clear supernatant was decanted carefully and was used for further estimation of metabolites. Malondialdehyde was estimated by the method of Heath and Packer (1968). The concentration of malondialdehyde was calculated using the extinction coefficient of 155 mM^{-1} cm^{-1}. Hydrogen peroxide was estimated by Sinha (1972). The quantity of H_2O_2 was determined from the standard curve of H_2O_2 (10-160 mmoles). Ascorbic acid was extracted from the seedlings by homogenizing in 5 ml of 5% (*w/v*) metaphosphoric acid in glacial acetic acid and homogenate was centrifuged at 10,000 x g for 25 min. The supernatant thus obtained was used for the estimation of ascorbic acid. Ascorbic acid content was estimated by the method of Roe (1964) which is based on the reduction of 2, 6-dichlorophenol indophenol (2, 6-DCPIP) dye by ascorbic acid. For carotenoids extraction, 30 mg of the fresh leaves were cut into small discs and dipped in test tubes containing 3 ml dimethyl sulfoxide (DMSO). The tubes were kept overnight at room temperature. The carotenoids extracted in the DMSO were estimated by recording its absorbance at 480, 645 and 663 nm respectively (Wellburn, 1994).

Extraction medium for SOD, APX, CAT, GR and LOX consisted of 0.1 M phosphate buffer (pH 7.5) containing 5% (w/v) polyvinyl polypyrrolidone (PVPP), 1 mM EDTA, and 10 mM β -mercaptoethanol as described by Chawla *et*

Vol. 10 (2) 118-125 April 2016, ISSN 0973-8916 (Print), 2230-7303 (Online)

al. (2013). For POX the extraction was done in 0.01 M phosphate buffer (pH 7.5) containing 3% (w/v) PVPP. The homogenate was prepared by grinding 1 g of tissue in 5 ml of ice cold extraction medium in pre-cooled mortar and pestle. The homogenate thus prepared, was centrifuged at 10,000 x g for 15 min at 4°C. The supernatant was carefully decanted and used as the crude enzyme preparation. Lipoxygenase was assayed spectro-photometrically at 234 nm by the method of Catherine *et al*. (1998). One unit of LOX was defined as amount of enzyme required to produce 100 mmoles of conjugated dienes produced min^{-1}

Superoxide dismutase activity was determined by quantifying the ability of the enzyme to inhibit light induced conversion of nitroblue tetrazolium (NBT) to formazan (Nishikimi *et al*., 1972). One enzyme unit was defined as the amount of enzyme which could cause 50 per cent inhibition of the photochemical reaction. Method of Nakano and Asada (1981) was employed to assay APX. One unit of APX corresponded to 1.0 O.D. change per min. Glutathione reductase activity was determined by the method of Halliwell and Foyer (1978). One enzyme unit was defined as 100 mmole of NADPH oxidized per minute. Peroxidase activity was assayed at 37°C as described by Shannon *et al*. (1966). The unit of POX activity was defined as 1.0 μmole of H_2O_2 utilized per min. Catalase activity was measured according to the method of Sinha (1972). One unit of enzyme activity was

Table 1: Screening of *B. juncea* genotypes for tolerance to high temperature stress.

S.No	Genotypes	Time*	S.No	Genotypes	Time*
1	BPR-542-6	5:15	19	RH-0204	3:45
2	NRCDR-02	5:15	20	RH-0115	3:45
3	RB-50	5:15	21	JMM-0702	3:30
4	BPR-54-9	5:00	22	CS-54	3:30
5	RH-0305	5:00	23	BPR-541-4	3:30
6	NRCDR-601	4:45	24	NPJ-92	3:15
7	NPJ-118	4:45	25	RH-0119	3:15
8	BPR-543-2	4:30	26	CS-5000-1-1-1-4	3:15
9	ONK-1	4:30	27	NPJ-112	3:15
10	NPJ-116	4:15	28	NPJ-113	3:15
11	BPR-537	4:15	29	BPR-541-2	3:00
12	BPR-541-3	4:15	30	BPR-542-14	3:00
13	BPR-540-6	4:15	31	BPR-549-2	3:00
14	CS-3000-1-1-1-5	4:15	32	SKM-531	2:45
15	RGN-145	4:00	33	RH-0116	2:45
16	RH-0204	3:45	34	HUJM-05-05	2:30
17	BPR-541-5	3:45	35	NPJ-119	2:30
18	RGN-73	3:45	36	RGN-152	2:30

*Time (hrs:min) taken to 50% seedling mortality

defined as the amount of enzyme which catalyzed the oxidation of 1 mmole H_2O_2 per minute under assay conditions.

Statistical analysis : The data were analyzed statistically using completely randomized design and the significance was tested at 5% level of critical difference using OPSTAT software (CCS HAU).

Results and Discussion

Thirty six genotypes were screened for high temperature (45±0.5°C) tolerance by recording the time taken to 50% seedling mortality (Table 1).

On the basis of time taken to 50% seedling mortality, tolerant genotype (BPR-542-6) and susceptible genotype (RGN-152) were selected. The stressed and revived seedlings were used for further studies. High temperature is known to affect membrane linked processes due to alteration in membrane fluidity and permeability (Larkindale and Knight, 2003). This loss of membrane integrity is due to lipid peroxidation which may occur either by chemical oxidation or by enzyme such as lipoxygenase. Therefore, lipoxygenase plays a central role in promoting oxidative injury by generating free radicals through action on PUFA (polyunsaturated fatty acid). The hydroperoxides thus formed decompose into oxy, peroxy radicals including malondialdehyde and 4-hydroxy alkanals which are sensitive markers of lipid peroxidation.

The changes in LOX activity, MDA and H_2O_2 content during high temperature stress and after recovery in thermo-tolerant (BPR-542-6) and thermo-susceptible (RGN-152) genotypes have been presented in Figure 1. LOX activity increased significantly in both the tolerant and susceptible genotype under heat stress however, increase was higher in susceptible genotype i.e. RGN-152 (65.48%) as compared to that in tolerant genotypes. On revival, the LOX activity decreased significantly when compared to the stressed seedlings but activity remained higher over the control. Similar results were found for MDA and H_2O_2 in both genotypes. Malondialdehyde, a product of lipid peroxidation has been considered as an indicator of degree of oxidative damage. The results presented are in agreement with those reported by Wilson *et al.* 2014 under high temperature stress. The decline in lipid peroxidation in terms of MDA content after recovery in the present study is also in accordance with the observation of Cai *et al.* (2005) who observed decrease in MDA content after recovery from drought in coffee plants. Increase in H_2O_2 content was observed in both the genotypes under high temperature stress however, the level of increase was more in thermo-susceptible genotype. It increased from 32.25 to 46.50 µmoles g^{-1} fresh weight in RGN-152. During the recovery period, H_2O_2 content decreased with respect to stressed seedlings but was still higher than their respective control in both genotypes.

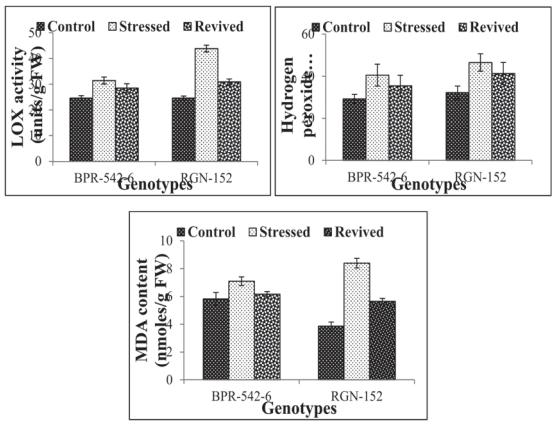

Fig. 1: Effect of high temperature stress and its revival on LOX, H_2O_2 and MDA content in *B. juncea* seedlings.

Parallel increase in LOX, MDA and H_2O_2 content observed under high temperature stress conditions in both the genotypes with significant higher increase in susceptible genotype indicates that higher production of reactive oxygen species in susceptible genotypes lead to more disruption of the membrane integrity. Such observations have also been reported by Almeselmani *et al.* (2006) who found lower H_2O_2 content and lipid peroxidation in tolerant wheat genotypes under

Vol. 10 (2) 118-125 April 2016, ISSN 0973-8916 (Print), 2230-7303 (Online)

high temperature stress. On revival, decrease in LOX, MDA and H_2O_2 content over stressed condition explains the decrease in reactive oxygen species, but the seedlings are still under oxidative stress.

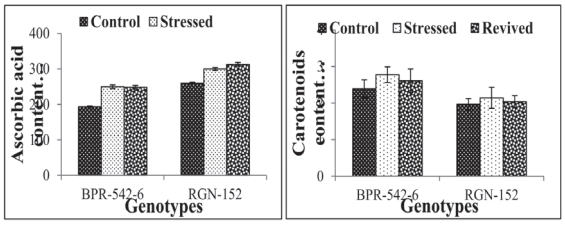

Fig.2: Effect of high temperature stress and its revival on ascorbic acid and carotenoid content in *B. juncea* seedlings.

Changes in ascorbic acid and carotenoid content in thermo-tolerant and thermo-susceptible genotypes due to heat stress are presented in Figure 2. Ascorbic acid content increased significantly in both the genotypes under stress but per cent increase was found more in tolerant genotype i.e. 29.31% (BPR-542-6) as compared to the susceptible genotype i.e.15.38% (RGN-152). On revival, the ascorbic acid content still continued to increase in both the genotype however increase was found significant in tolerant genotype. Carotenoid act as antioxidant by reacting with lipid peroxidation products and scavenging singlet oxygen (Pandhir and Sekhon, 2006). The basal level of carotenoids has been found more in tolerant genotype than susceptible genotypes. The carotenoids increased under stressed condition in both the genotypes however the increase was significantly higher in tolerant genotype (~16%) as compared to susceptible genotype (~ 9%).When the stress was relieved, the carotenoids started to decline but were found still higher in tolerant than susceptible genotype.

The results presented in Figure 3 demonstrate that the activity of SOD, APX, GR, POX and CAT increased due to heat stress in tolerant and susceptible genotype. The increase in SOD activity was significantly higher in tolerant genotype i.e. BPR-542-6 (106.57%) as compared to susceptible genotypes RGN-152 (81.73%). On revival, the activity decreased significantly as compared to stressed seedlings but remained still higher than the control. An increase in SOD activity under high temperature stress has also been reported by Almeselmani *et al*. (2006) in wheat genotype. Higher increase in SOD activity under stress in tolerant genotypes in the present study may be responsible for imparting tolerance to these genotypes.

APX activity increased under heat stress in both the thermo-tolerant and thermo-susceptible genotype but the level of APX activity was more in tolerant genotype under stressed conditions. The basal activity of APX was also found higher in thermo-tolerant genotype i.e. BPR-542-6 (1.60 units g^{-1} fresh weight) when compared thermo-susceptible genotype i.e. RGN-152 (0.95 units g^{-1} fresh weight.). But the response of APX activity during recovery period was different in tolerant and susceptible genotypes; the activity continued to increase in thermo-tolerant genotype but decreased in susceptible genotype. Increase in APX under stressed condition may be due to up regulation of ascorbate-glutathione cycle in response to high temperature stress (Ma *et al*., 2008). In the present study, higher basal level of APX in tolerant genotypes and an increase throughout stressed and revival conditions explain that glutathione ascorbate cycle is more efficiently operating in tolerant genotype thereby imparting more tolerance to the seedlings.

GR activity under stress and revival is similar to the activity of POX. GR activity increased in both the genotypes under stressed condition but the increase was significantly higher in tolerant genotype i.e. BPR-542-6 (52.36%) as compared to susceptible genotype RGN-152 (21.07%). The GR activity continued to increase under revival condition in both the genotypes. Locatto *et al*. (2009) also observed an increase in GR activity when the cell suspension of tobacco was exposed to 55°C. However initial increase and then decrease in GR activity was

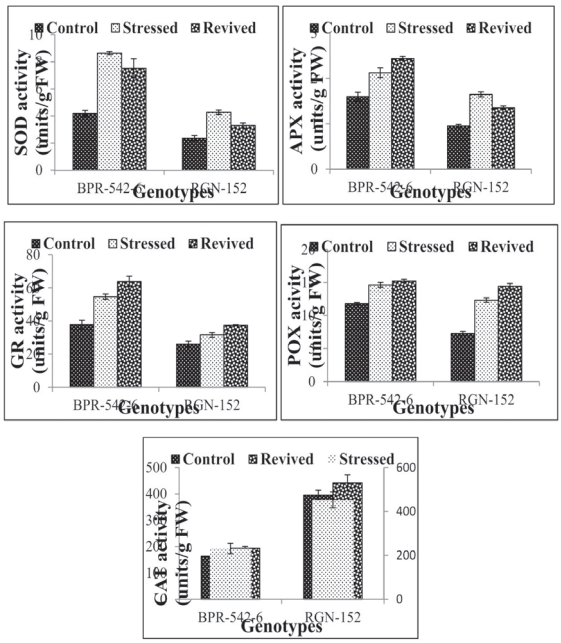

Fig. 3: Effect of high temperature stress and its revival on antioxidant enzymes in *B. juncea* seedlings.

also observed by Ma *et al.* (2008) in apple leaves after increasing the duration of high temperature stress.

The peroxidase activity increased in both the genotypes under high temperature stress and the activity remained higher during the recovery period than the control condition. Basal level of POX activity was more in thermo-tolerant genotype (BPR-542-6) (11.79 unit's g^{-1} fresh weight). The POX activity was found significantly higher in tolerant genotype under stressed condition i.e. 14.64 units g^{-1} fresh weight (BPR-542-6). The results obtained in present investigation are in accordance with those observed by Kaur *et al*. (2009) and Wilson *et al*. (2014) in *B. juncea* where an increase in POX activity was observed in tolerant and susceptible genotypes following heat stress.

Catalase activity increased under heat stress in both the genotypes but increase was more in tolerant genotype i.e. 41.32% (BPR-542-6) than in susceptible genotype i.e. 14.48% (RGN-152). The basal level of CAT was found maximum in susceptible genotype RGN-152 (395.37 unit's g^{-1} fresh weight) and minimum in BPR-542-6 (163.53 unit's g^{-1} fresh weight). After relieving the stress, CAT activity decreased significantly but remained higher than the control in both the genotypes. Increase in CAT activity under heat stress was also observed by Kaur *et al*. (2009) in *B. juncea* species. Our results are in accordance with the observations of Cai *et al*. (2005) in mulberry, Almeselmani *et al.* (2006) in wheat genotypes who also observed more increase in CAT activity in tolerant genotypes under heat stress.

Conclusion

Significant increase in antioxidant enzymes during stress and continuous increase in activities of POX, GR and APX on revival in tolerant genotypes might be responsible for imparting tolerance to the seedling of *B. juncea.* The differential response of these genotypes to heat stress as a result of variation in the activities of their anti-oxidative enzymes suggests that by manipulation of the enzyme activities through genetic engineering may impart tolerance to the genotypes.

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Evaluation of *in vitro* Release Pattern of Curcumin Loaded Egg Albumin Nanoparticles Prepared Using Acetone as Desolvation agent

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Abstract

The objectives of this study was to investigate the *in vitro* release profile of curcumin-loaded egg albumin nanoparticles using acetone as a desolvation agent and to study the effect of egg albumin concentration on curcumin release from nanoparticles. An initial burst release of curcumin which is adsorbed on the surface of nanoparticles and further sustained release of entrapped curcumin up to 72 h was observed in all particles. Release data was fitted in various mathematical models and the mechanism of curcumin release was analyzed using Korsmeyer-Peppas model. First order release model showed highest correlation coefficient (>0.96) and indicated that the release was concentration dependent. The Korsmeyer-Peppas release exponent (n) values of less than 0.45 proved the Fickian diffusion of curcumin from all nanoparticles.

Key words: Curcumin, Egg albumin, Acetone, Nanoparticles, *In vitro* release

Introduction

Curcumin is a polyphenol compound derived from the rhizome of turmeric (*Curcuma longa* L.) and is responsible for the yellow colour of turmeric. It is an effective ingredient which has been used in traditional medicine for many centuries in India (1). Curcumin has a wide range of pharmacological applications such as anti-inflammatory, anti-HIV, antimicrobial, antioxidant and anticancer with low or no intrinsic toxicity (2). But the application of curcumin as therapeutic agent is limited due to its low solubility in water and instability to alkaline conditions, liability to heat and light. Hence, methods to improve the solubility of curcumin in aqueous solution and to provide the controlled delivery in physiological pH conditions are essential to use it as a therapeutic agent.

Nanotechnology has been introduced into several aspects of the food science, including encapsulations and delivery systems, which protect and deliver functional ingredients. Nanocarriers can improve the solubility and stability of encapsulated drugs (3). Albumin based carrier systems could be used for drug encapsulation, because different drug binding sites are present in the albumin molecules (4). The high solubility of albumin (up to 40 %w/v) at pH 7.4 makes it an attractive macromolecular carrier capable of accommodating a wide variety of drugs. Desolvation of albumin with organic solvents followed by chemical cross-linking is a common method for the preparation of albumin based nanoparticles. Organic solvents such as acetone and ethanol have been used as desolvation solvents for the preparation of protein nanoparticles. It was reported that acetone used as a desolvation agent produced smaller albumin

nanoparticles than those obtained using ethanol (5). Ovalbumin is a non-toxic biodegradable protein, derived from chicken eggs. Ovalbumin has been studied as carrier systems for a wide variety of drugs, including proteins and enzymes (6). This study was conducted with the aim to evaluate the effect of egg albumin concentration on *in vitro* release properties of curcumin-loaded egg albumin nanoparticles prepared using acetone as desolvation agent.

Materials and Methods

Egg albumin powder (minimum 80.5% protein) was obtained as a gift from M/s SKM egg products, Erode, Tamil Nadu. Curcumin (minimum 95%) was obtained from M/s Synthite Industries Ltd, Kolenchery, Kerala. Glutaraldehyde (25% w/v solution) was purchased from M/s Astron Chemicals (India), Ahmedabad. All other reagents used in the experiments were of AR grade.

***Preparation of curcumin loaded egg albumin nanoparticles*:** Based on the earlier research studies, acetone was selected as desolvation agent and intermittent addition of acetone was chosen to obtain smaller size nanoparticles. Since curcumin is stable only at a pH< 6.5 (7), egg albumin was chosen as a drug carrier and was made into solution with a pH range of 5 - 7 and at 5 - 15% (w/v) concentration. Curcumin at 0.25% (w/v) of egg albumin solution was selected for encapsulation. The experimental runs were obtained by using a two-factor, five-level central composite design (CCD), with egg albumin solution concentration (EAC, X_1) and pH of egg albumin solution (pH, X_2) as independent variables. Curcumin loaded egg albumin nanoparticles were prepared according to the experimental runs (Table 1) using acetone as desolvation agent since it could produce smaller particles than ethanol. To evaluate the effect of egg albumin concentration on *in vitro* release profile of nanoparticles (Table 1, 13 combinations) was studied in phosphate-buffered saline (PBS).

Curcumin nanoparticles were prepared using egg albumin as the polymer by desolvation technique. For the preparation of curcumin particles the method described by Weber *et al.* (8) was followed with some modifications. Egg albumin solution of various per cent (w/v) concentrations and pH as per the experimental design was prepared in distilled water at room temperature (28±2°C). pH of the solution was directly measured using a digital pH meter (HANNA instruments, Woonsocket, Rhode Island) and it was adjusted by adding 0.1N HCl or sodium chloride solution. Subsequently, 0.25% (w/v based on egg albumin solution) curcumin was dissolved in 20 ml acetone, which was added intermittently into the aqueous egg albumin solutions under magnetic stirring at 500 rpm at room temperature. Each five minutes interval 2ml of curcumin dissolved acetone was added in egg albumin solution and this addition was continued until the solution became just turbid. This resulted in the formation of an opalescent suspension spontaneously at room temperature. After this desolvation process, 0.11 ml of 8% (v/v) glutaraldehyde in water was added to the turbid solution to induce particle crosslinking. Then this solution was stirred continuously at 500 rpm at room temperature for 1 h. The complete cross linking process of the colloidal suspension was performed over a time period of 24 h. After that formed nanoparticles were purified by three cycles of centrifugation (3000 rpm, 30 min) and re-dispersion with distilled water to remove unreacted chemicals and desolvation agents. Purified pellets were transferred to a Teflon plate and dried in an oven at 50°C for 6 h. The resulting curcumin-egg albumin particles were gently pulverized using the mortar and pestle to obtain the dried fine nanoparticles (9).

***In vitro release studies of curcumin loaded egg albumin nanoparticles*:** The *in vitro* drug release profiles of curcumin-egg albumin nanoparticles were determined by the method suggested by Jithan *et al*. (10) as follows. These experiments were conducted in dark conditions as curcumin extensively degrades in the presence of light. The release medium was

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phosphate-buffered saline (PBS 0.1M, pH 7.4), which contained 1% ascorbic acid and 0.1 butylated hydroxytoluene to prevent further degradation of curcumin. One hundred milligrams of nanoparticles were re-dispersed in 200 ml of the release medium (PBS). The entire system was kept at 37±1°C under stirring at100 rpm. At the desired time intervals of 0, 2,4, 6, 8, 24, 48 and 72 h, 20 ml of release medium was removed and replaced with the same volume of fresh medium. The samples withdrawn were filtered using 0.2 μ sterile filter and the amount of curcumin in release medium was determined by UV-VIS spectrophotometer at 425nm. All measurements were performed in triplicate. The release was quantified as follows:

$$Release\ (\%) = \frac{Released\ curcumin}{Total\ curcumin}\ x\ 100 \quad \text{.... (1)}$$

***Release kinetics*:** To study the release mechanism of curcumin from prepared nanoparticles, data obtained from *in vitro* release studies were fitted to zero order, first order, Higuchi, Hixson-Crowell and Korsmeyer-Peppas kinetics models and the models are as follows:

Zero Order Model:

$$Q_t = Q_0 + K_o t \ldots\ldots\ldots\ldots\ldots\ldots (2)$$

Where Q_t is the amount of curcumin released in time t, Q_0 is the initial amount of curcumin in the solution and K_0 is the zero order release constant. Data obtained from *in vitro* drug release studies were plotted as cumulative amount of drug released versus time.

First Order Model (11):

$$ln\ M_t = ln\ M_0 + K_1 t \ldots\ldots\ldots\ldots\ldots\ldots (3)$$

Where M_t is the cumulative amount of curcumin released at any specified time point and M_o is the initial amount of curcumin in the prepared particles. The data obtained are plotted as log cumulative percentage of drug remaining vs. time.

Higuchi Model (12):

$$Q_t = K_H t_{1/2} \ldots\ldots\ldots\ldots\ldots\ldots (4)$$

Where Q_t is the amount of curcumin released in time t, K_H is the Higuchi dissolution constant. The data obtained were plotted as cumulative percentage drug release versus square root of time.

Hixson-Crowell Model (13):

$$Q_0^{\,1/3} - Q_t^{\,1/3} = K_{HC} t \ldots\ldots\ldots\ldots\ldots\ldots (5)$$

Where, Q_t is the amount of curcumin released in time t, Q_0 is the initial amount of curcumin in prepared particles and K_{HC} is the rate constant for Hixson-Crowell rate equation. Data obtained from *in vitro* drug release studies were plotted as cube root of drug percentage remaining in matrix versus time.

Korsmeyer-Peppas Model (14):

$$M_t / M_\infty = K_K\, t^n \ldots\ldots\ldots\ldots\ldots\ldots (6)$$

Where Mt / M" is a fraction of curcumin released at time t, K_K is the release rate constant and n is the release exponent. The n value is used to characterize different release for cylindrical shaped matrices. Data obtained from *in vitro* drug release studies were plotted as log cumulative percentage drug release versus log time.

Plotted release data were fitted using a linear equation using Microsoft Excel and the correlation coefficient (R^2) was obtained for each graph. Selection of model for the release data was based on the comparison of correlation coefficients (15).

Results and Discussion

Nanoparticles size: Curcumin loaded egg albumin nanoparticles were prepared using various combinations of factors (Table 1) and the particle size were ranged from 205.3 to 250.1 nm. Particle size results were reported in authors' previous study of preparation of curcumin loaded egg albumin nanoparticles using acetone (16).

***In vitro release pattern of curcumin loaded egg albumin nanoparticles*:** *In vitro* release studies of curcumin loaded egg albumin

nanoparticles prepared using acetone as desolvation agent was done and the curcumin release was analyzed for 72 h. Cumulative curcumin release per cent of all nanoparticles at specific time intervals are presented in Table 1. An initial burst release of curcumin which is adsorbed in surface of nanoparticles and further sustained release of entrapped curcumin up to 72 h was observed in all particles. The burst release of curcumin may be due to the surface associated curcumin bound weakly to the surface and curcumin entrapped nearer to the surface of the nanoparticles. The remaining amount of curcumin which is encapsulated within the structure was released in a controlled manner for the entire period of study. The highest cumulative curcumin release of 60.41% was observed in the nanoparticles prepared using the egg albumin concentration of 2.92% (w/v) and pH of the solution of 6. Egg albumin solution concentration of 17.07% (w/v) and the solution pH of 6 showed the lowest curcumin release of 36.6% after 72 h. Sadeghi *et al*. (17) studied the *in vitro* release of curcumin from curcumin-BSA nanoparticles and reported that BSA can be used as a carrier to improve controlled release of curcumin. Release of curcumin from nanoparticles was found to be 70% after 72 h and remaining 30% of curcumin was strongly entrapped inside the nanoparticles. This was observed mainly due to the physical attachment of 70% of curcumin on surface of BSA nanoparticles.

Fig. 1 shows the *in vitro* release profile of curcumin loaded egg albumin prepared using various combinations of factors. Maximum amount of curcumin was released from the nanoparticles prepared using lower concentration (2.92% w/v) of egg albumin. This might be due to the fastest dissolution of egg albumin and rapid diffusion of curcumin from nanoparticles of smaller size. It was observed that increase in egg albumin concentration decreased the curcumin release rate due to slower dissolution of egg albumin and diffusion of curcumin at higher egg albumin concentrations. At higher polymer concentration, the viscosity of the gel matrix was increased, which resulted in a decrease in the effective diffusion coefficient of the drug, (18) and was more likely to be resistant to drug diffusion

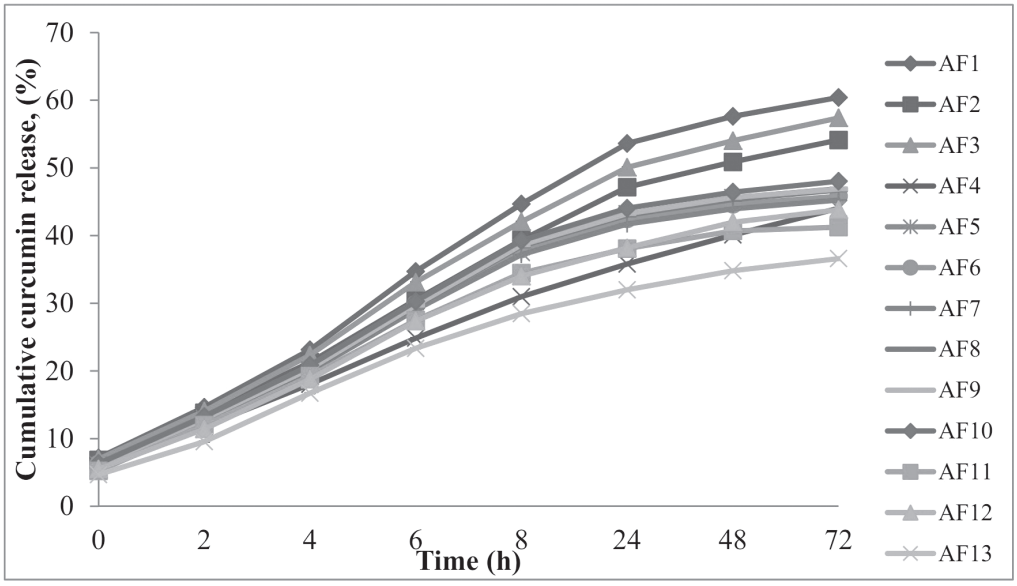

Fig. 1. Zero order in vitro release kinetics of all formulations of curcumin loaded egg albumin nanoparticles

and erosion (19). This indicates that the egg albumin concentration is an important factor affecting the curcumin release rate from nanoparticles. Chinna Gangadhar *et al*. (20) evaluated the effect of polymer concentration on the release rate of indomethacin from microspheres and found that the increase in the polymer concentration decreased the rate and amount of drug release.

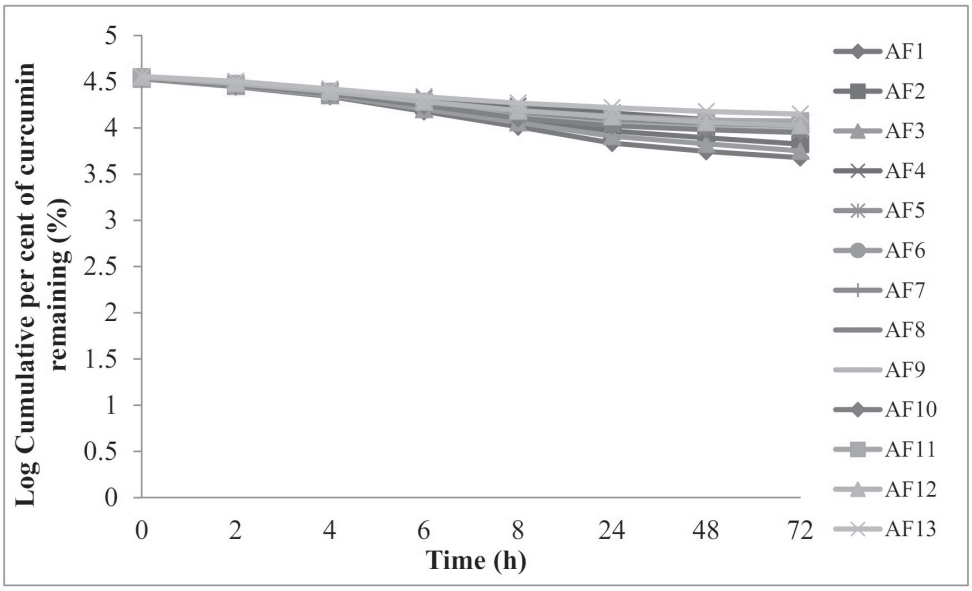

Fig. 2. First order in vitro release kinetics of all formulations of curcumin loaded egg albumin nanoparticles

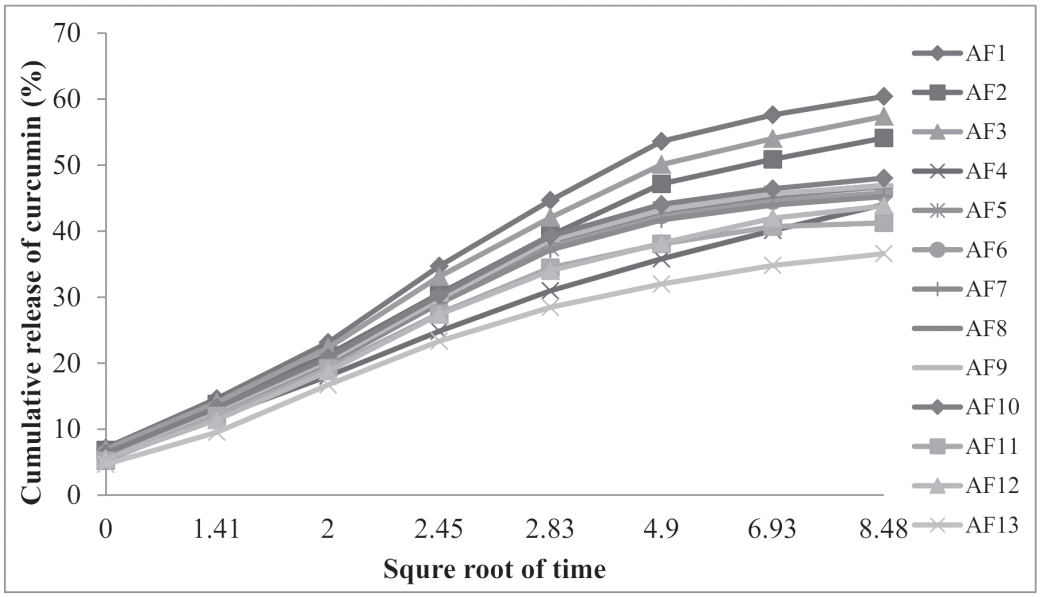

Fig. 3. Higuchi plot for in vitro release kinetics of all formulations of curcumin loaded egg albumin nanoparticles

In vitro release pattern of curcumin loaded egg albumin nanoparticles

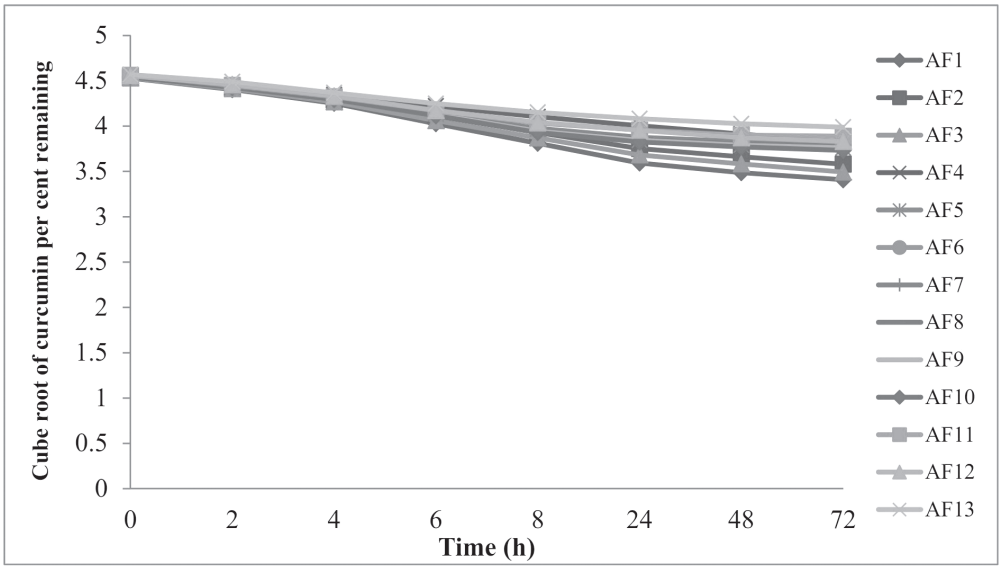

Fig.4. Hixson-Crowell plot for *in vitro* release kinetics of all formulations of curcumin loaded egg albumin nanoparticles

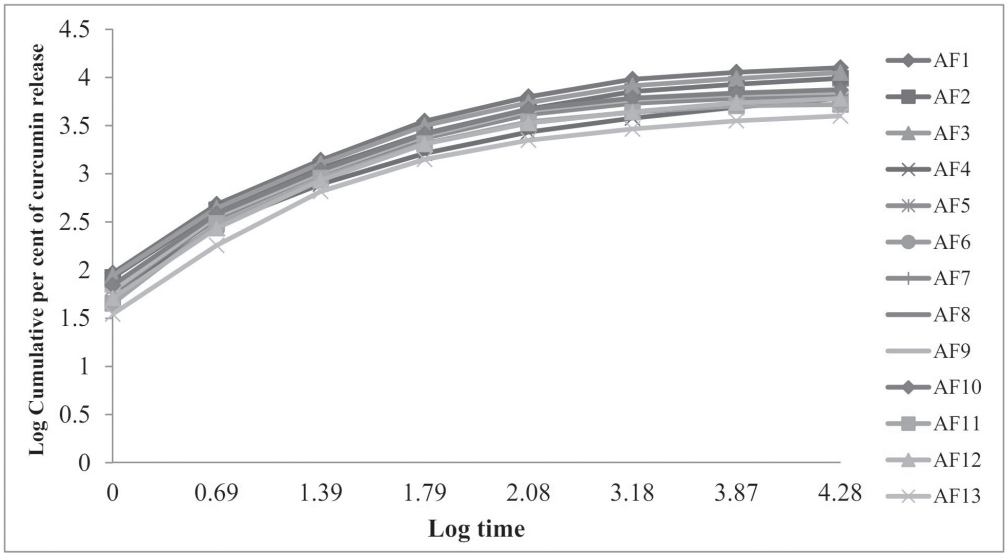

Fig. 5. Korsmeyer-Peppas plot for in vitro release kinetics of all formulations of curcumin loaded egg albumin nanoparticles

In vitro release data was analyzed mathematically using zero-order, first-order, Higuchi, Hixson-Crowell and Korsmeyer-Peppas equations to describe the overall release of curcumin from all formulations. The plotted release data for zero-order, first-order, Higuchi, Hixson-Crowell and Korsmeyer-Peppas models are shown in Fig. 1, 2, 3, 4 and 5, respectively. By fitting all models to release data, correlation coefficients, release rate constants and release

Table 1. *In vitro* release data of curcumin loaded egg albumin nanoparticles prepared using acetone as desolvation agent

Formulation code	Factors (EAC/pH)	Cumulative curcumin release (%)							
		0 h	2 h	4 h	6 h	8 h	24 h	48 h	72 h
AF1	**2.92/6**	7.16±0.19	14.63±0.39	23.13±0.69	34.7±0.83	44.67±2.05	53.61±1.61	57.62±2.64	60.41±1.81
AF2	**5.0/5**	6.85±0.25	13.82±0.50	21.27±0.56	30.51±0.77	39.35±1.18	47.16±1.25	50.89±1.53	54.12±1.43
AF3	**5.0/7**	6.98±0.25	14.07±0.42	22.34±0.80	33.1±1.02	42.06±1.26	50.09±1.81	54.02±1.62	57.39±2.07
AF4	**10/4.58**	5.66±0.17	11.81±0.42	18.04±0.83	24.85±0.54	30.97±0.93	35.8±1.64	40.1±1.20	44.03±2.02
AF5	**10.0/6**	6.12±0.18	12.97±0.47	20.16±0.60	29.23±0.40	37.5±0.99	43.22±1.30	45.59±1.21	46.93±1.41
AF6	**10.0/6**	6.25±0.12	12.87±0.59	19.92±0.60	29.89±0.60	38.01±1.37	42.46±1.27	44.62±1.61	45.83±1.37
AF7	**10.0/6**	5.91±0.18	12.66±0.38	19.93±0.40	29.04±0.53	37.19±1.12	41.71±0.83	43.94±1.32	45.24±0.90
AF8	**10.0/6**	5.82±0.15	12.8±0.38	20.19±0.60	29.66±0.73	38.46±1.02	43.03±1.29	45.33±1.20	46.74±1.40
AF9	**10.0/6**	5.97±0.21	12.9±0.34	20.32±0.54	29.69±0.61	38.62±1.39	43.23±1.14	45.64±1.64	46.87±1.24
AF10	**10.0/7.41**	6.31±0.19	13.27±0.48	20.84±0.75	30.29±0.55	39.27±1.41	44.05±1.59	46.42±1.67	48.03±1.73
AF11	**15/5**	5.23±0.14	12.01±0.43	19.24±0.58	27.51±0.69	34.46±1.58	38.08±1.14	40.69±1.86	41.25±1.24
AF12	**15/7**	5.51±0.20	11.43±0.52	18.79±0.50	27.37±0.68	34.02±1.02	38.19±1.01	41.97±1.26	43.82±1.16
AF13	**17.07/6**	4.68±0.17	9.55±0.29	16.22±0.60	23.34±0.77	28.47±0.85	31.99±1.15	34.81±1.04	36.6±1.32

EAC – egg albumin solution concentration (%w/v); pH – pH of egg albumin solution

In vitro release pattern of curcumin loaded egg albumin nanoparticles

Current Trends in Biotechnology and Pharmacy
Vol. 10 (2) 126-135 April 2016, ISSN 0973-8916 (Print), 2230-7303 (Online)

Table 2. Release kinetics parameters obtained from model fitting of *in vitro* release data of curcumin loaded egg albumin nanoparticles prepared using acetone as desolvation agent

Factors (EAC/pH)	Zero order		First order		Higuchi		Hixson-Crowell		Korsmeyer- peppas		
	R^2	K_o	R^2	K_1	R^2	K_H	R^2	K_{HC}	R^2	K_K	n
2.92/6	0.947	6.95	0.986	-0.133	0.976	8.20	0.985	-0.174	0.882	8.12	0.292
5.0/5	0.954	6.09	0.990	-0.108	0.982	7.17	0.989	-0.146	0.889	7.71	0.281
5.0/7	0.951	6.53	0.990	-0.120	0.979	7.68	0.988	-0.159	0.883	7.91	0.287
10/4.58	0.967	4.81	0.999	-0.076	0.991	5.59	0.997	-0.106	0.885	6.56	0.271
10.0/6	0.943	5.54	0.973	-0.089	0.959	6.26	0.970	-0.122	0.856	7.31	0.275
10.0/6	0.935	5.44	0.964	-0.086	0.948	6.09	0.959	-0.118	0.849	7.45	0.270
10.0/6	0.939	5.39	0.967	-0.084	0.952	6.01	0.963	-0.116	0.845	7.20	0.273
10.0/6	0.938	5.60	0.968	-0.089	0.952	6.27	0.963	-0.122	0.844	7.14	0.279
10.0/6	0.938	5.60	0.967	-0.089	0.952	6.28	0.963	-0.123	0.847	7.26	0.277
10.0/7.41	0.94	5.68	0.970	-0.092	0.955	6.38	0.966	-0.125	0.851	7.57	0.273
15/5	0.937	5.02	0.962	-0.074	0.945	5.46	0.957	-0.103	0.824	6.71	0.272
15/7	0.951	5.09	0.983	-0.079	0.968	5.78	0.979	-0.110	0.857	6.57	0.278
17.07/6	0.951	4.26	0.979	-0.061	0.965	4.77	0.975	-0.087	0.846	5.66	0.274

EAC – egg albumin solution concentration (%w/v); pH – pH of egg albumin solution

exponent (n) values were obtained for all nanoparticles prepared using acetone as a desolvation agent and values are shown in Table 2. Release kinetics analysis showed that first order release model obtained highest correlation coefficient (>0.96) and indicated that the release was concentration dependent. Hixson-Crowell model showed the R^2 values of greater than 0.95 and describes that a change in surface area or diameter of particles due to erosion with progressive release of curcumin as a function of time. Correlation coefficient values of all particles were found to be greater than 0.94 for Higuchi model. It described that the curcumin release pattern was by the diffusion controlled release. *In vitro* release data were also fitted to Korsmeyer-Peppas equation with the R^2 values of greater than 0.82 and release exponent values (n) of all formulations were found to be less than 0.29. The 'n' values of less than 0.4 confirmed the Fickian diffusion mechanism of curcumin from all nanoparticles. This Fickian diffusional release might be occurred by the usual molecular diffusion of the curcumin due to a chemical potential gradient. *In vitro* release studies revealed that the prepared nanoparticles could release the entrapped curcumin in a sustained manner through diffusion mechanism. Shaikh *et al*. (21) prepared the biodegradable curcumin nanoparticles with a view to improve its oral bioavailability and evaluated the *in vitro* release of curcumin from nanoparticles. A biphasic pattern of release was observed and the *in vitro* release was primarily by diffusion and followed Higuchi release pattern.

Conclusion

In vitro release results showed that the curcumin release rate decreased with the increase in egg albumin concentration. *In vitro* release data was fitted in mathematical models such as zero-order, first-order, Higuchi, Hixson-Crowell and Korsmeyer-Peppas and the highest correlation coefficient was observed for first order model in all formulations followed by Hixson-Crowell model. This indicated the concentration dependent release of curcumin and release exponent values of less than 0.4 proved the diffusion controlled mechanism of release. Results suggested that curcumin-loaded egg albumin nanoparticles could release the entrapped curcumin in a sustained manner. Due to this controlled delivery of curcumin in physiological pH conditions the bioavailability of curcumin could be increased and the rapid elimination can be avoided.

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Biosynthesis of Partial Glycerides via Enzymatic Esterification of *Mucor racimosus* Lipase for Edible and Industrial Purposes

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Abstract

The present study is mainly focused on the screening of seven microorganisms for the production of most efficient lipase enzyme for the formation of glycerides (mainly monoglycerides and diglycerides). It was found that *Mucor racimosus* lipase enzyme was superior to all the other tested microbial lipases for the production of partial glycerides and triglycerides. The experiments were designed on the esterification reaction between mixed free fatty acids and glycerol as well as that of between the fatty acids oil and glycerol. The latter deals with the preparation of partial glycerides namely monoglycerides and diglycerides as well as triglycerides. These compounds can be used as emulsifiers for different industries. Enzymatic esterification parameters were carried out at a various incubation temperature, incubation period, enzyme concentration and substrate concentration to reveal the optimum conditions of esterification reaction. The highest esterification percent (89%) was achieved at the temperature of 30°C and incubation period of 24 hours using the enzyme concentration of 2.5 mg/ml with the substrate concentration of 0.002M oleic acid and 0.12M glycerol.

Key words: Lipase, *Mucor racimosus*, esterification, monoglycerides, diglycerides, triglycerides.

Introduction

Lipolytic enzymes are one of the most important groups of biocatalysts for biotechnological application (1, 2). It is known that lipases (EC 3.1.1.3) hydrolyze triacylglycerols to fatty acids, di-acylglycerols, monoacyl glycerols and glycerol whatever under certain conditions, catalyze reverse reactions such as esterification and trans-esterification (3, 4). Moreover, lipases are glycerol ester hydrolases (EC: 3.1.1.3), which hydrolyze ester linkages of glycerides at water-oil interface (5, 6). Much interest in microbial lipase production has been increased due to its characteristics features of broad substrate specificity, versatile molecular structure, and stability in organic solvents (7, 8). Secreted lipases from various fungi and bacteria recently have attracted considerable attention due to its vast biotechnological potential (9, 10). It has a wide range of enzymatic properties and substrate specificities, making them very useful for industrial applications, such as the processing of fats and oils, additives, detergents, cosmetics, paper manufacturing, and pharmaceuticals (11-13). Moreover, it has been also used medical applications as in digestive aids, malignant tumors, gastrointestinal disturbances and dyspepsia therapy (12, 13).

Monoacylglycerol (MAG) constitutes the major type of food emulsifiers in many food

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systems like bakery products, margarine, and dairy products and used as a basic starting material to prepare several other derivatives with modified functional properties (14, 15, 25). MAGs have been used as surface-active agents in many consumer and industrial cleaning products such as detergents, shampoos, lotions, and toothpaste or as raw materials for the synthesis of chemical compounds such as alkyd resins (15, 16, 18, 26). Moreover, it is also used as texturizing agents and hair care additive. MAG is also used as binders in tablets and as emollients for transdermal, slow-release drugs in the pharmaceutical industry (25). Owing to the fact that the edible oil contained at least 80% of 1, 3-DAG, the later has been used as cooking oil with health benefits (27). DAG is also used as precursors for the organic synthesis of prodrugs for the treatment of lymphoma and Parkinson's disease in medicine (28, 29).

MAG can be prepared by either direct esterification of glycerol with fatty acids or glycerolysis of glycerol with oils or fats (indirect esterification). Recently many approaches have been investigated in the enzymatic synthesis of MAG (monoacylglycerol) and DAG (diacylglycerol). These are selective hydrolysis, esterification of fatty acids or transesterification of fatty esters with glycerol, and glycerolysis of fats or oils (19-24). Lipases can be used as biocatalysts for the glycerolysis process which have many advantages over the chemical process such as mild reaction condition, high catalytic efficiency and stereo- and positional specificities (15, 17, 18).

The aim of this study to screen seven different microbial lipases from different microorganisms for the most efficient enzyme in the esterification reaction. The most efficient microbial lipase was selected and the results were implemented on glycerol/ olive oil mixed fatty acids as a general trend for the utilization of vegetable oil fatty acids in the production of glycerides namely monoglycerides, diglycerides and triglycerides. Moreover, preparative thin layer chromatography and gas liquid chromatography are also applied for the evaluation of esterification and hydrolytic products.

Materials and Methods

***Microorganisms and culture conditions*:** All the microbial cultures (*Mucor racemosus*, *Asp niger* NRRL3, *Asp. niger* 7H , *Asp niger* (oil), *Asp. niger* 16H , *Asp. terreus* 2H and *Asp.terreus* 1H) were obtained from National Research Centre (NRC) ,Cairo, Egypt and Northern Regional Research Laboratory (NRRL). The microbial cultures were grown on potato dextrose agar (PDA) medium. The culture was inoculated 100 ml of the fermentation medium in 250 ml Erlenmeyer flasks and incubated at 30 °C in shaking incubator 200 rpm.

***Production, evaluation and partial purification of lipases*:** Productions of lipases from different microorganisms were carried out according to Akhtar *et al*. (30), while the determination of the lipase activity was evaluated as per Parry *et al.* (31). Partial purification of lipase from different microorganisms was done as the method described by Abbas *et al.* (32).

***Screening of different partially purified lipases on the esterification reaction*:** The seven different active hydrolyzing lipases belonging to the previously mentioned microorganisms are tested for the esterification reaction. The model esterification medium consists of; 0.2 ml of oleic acid (95 %) and 1.8 ml of glycerol. Each fungal enzyme which contains the equal amount of protein was dissolved in 1 ml of tris buffer (pH 7.5, 0.2 M) and then added to the reaction mixture followed by incubation at 30°C for 24 hours in rotating shaker (New Brunswick Scientific Edison, N.J., U.S.A.) at 200 rpm. The esterification processes were carried out under various following conditions.

***Effect of enzyme concentration*:** The reaction mixtures were contained the oleic acid (0.5ml) and 95% glycerol (9ml) with different

concentrations (0.5 – 20mg) of enzyme solution diluted in in 1ml of tris buffer (pH7.5 & 0.2M). The reaction mixtures were incubated at 30^{0}C for 24 hours using a rotating shaker at 200 r.p.m. The reaction was stopped by adding 10ml of chloroform and the sample was taken for investigating the tri oleoyl glycerol, (T.O.G), partial glycerides (monoalkylglycerol) (M.O.G.), dioleoyl glycerol (D.O.G.) and non-reacted oleic acid. The chloroform was evaporated by rotary evaporator under reduced pressure.

Effect of substrate concentrations (Glycerol and oleic acid): The influence of varying concentrations of glycerol (0.1, 0.12, 0.14, 0.16 M) with a constant concentration of oleic acid (0.002 M) was carried out. Another varying concentration of oleic acid (0.001, 0.002, 0.003 and 0.0045 M) with a constant concentration of glycerol (0.12 M) was also studied under the previously mentioned esterification conditions using 2.5 mg. of partially purified lyophilized lipase diluted in 1 ml of tris- buffer (0.2 M, pH 7.5) and incubated at 30° C for 24 hours at 200 rpm. The estimation was calculated on the basis of 100 mg of the products.

***Effect of incubation time and temperatures*:** The effect of various incubation periods (12.0, 24.0, 36.0, 48.0 and 72.0 hours) and incubation temperatures (25–45°C) was also studied. The reaction mixture was studied using 0.12M glycerol and 0.002 M oleic acid under the previously mentioned esterification conditions. The esterification products were evaluated and estimated as previously described as above.

***Toxicity test of Mucor racemosus strain*:** Extractions of mycotoxins (AFs) were carried out according to Munimbazi *et al.* (33) while determination and derivatization of aflatoxins (B1, B_2, G_1 and G_2) were carried out according to the method described by Deabes *et al*. (34, 35).

Evaluation of chemical esterification reaction: The esterification products from oleic acid/ glycerol medium were evaluated using preparative T.L.C as previously described and the estimation was calculated on the basis of 100 mg of the products, as usual. The optimal esterification optimal conditions on mixed olive oil fatty acid / glycerol were carried out using saponification method (36).

***Evaluation of enzymatic esterification reactions products*:** After the preparation of fatty acids from olive oil, certain amount of mixed fatty acid (equivalent to 0.002 M (0.5/g) of oleic acid) was mixed with glycerol (0.12 M) (1.0 /g) at optimum concentration of partially purified *Mucor racemosus* lipase under the previously mentioned optimum conditions to obtain the (monoacylglycerol, diacyl-glycerol, non-reacted fatty acids and triacylglycerol). 100 mg of esterified products were fractionated by preparative thin layer chromatography (TLC) (coated with silica gel E-Merck, 200U). The resulting products were applied as bands (alongside the standard compounds) on each preparative silica gel plate (300 microns) and the plate was developed with the solvent system (diethyl ether : hexane : glacial acetic acid) (70 : 30 : 1 v/v/v). The fractions corresponding to the mono oleoyl-glycerol, dioleoyl glycerol, unesterified fatty acid and triole oy glycerol were visualized with iodine vapor and then scrapped off the plate. The scrapped fractions were extracted two times with moistened diethyl ether. The filtrate was taken by Pasteur pipette and evaporated gently to obtain a residue and weight of each fraction was determined (37).

Results and Discussion

From the results of screening analysis of seven active lyophilized lipase enzymes, it seems that the seven lipases perform esterification reaction at different rates between oleic- acid and glycerol. Monoolein, diolein and triolein, were the main product of the reaction which was isolated by preparative T.L.C. on the basis of 100 mg of the products. The lipase purified from *Mucor racemosus* was superior to all other microorganisms and has shown maximum esterification percentage of 59.14 %. The two isolates of Aspergillus *terreus* (1H, 2H) have

shown high yield production of monoolein of 21.78 % and 21.8 %, *respectively*. On the other hand, higher yields of diolein (24.11, 21.03, 26.16, 28.99 and 24.30%) were obtained by *Aspergillus niger* 7H, *Aspergillus niger* 16H, *Mucor racemosus*, *Aspergillus niger* NRRL3 and *Aspergillus niger* (Oi1), respectively (Fig.1). Moreover, *Mucor racemosus* was found to be superior to all other microorganisms in performing the esterification reaction.

The detailed studies on various factors (enzyme concentration, substrate concentration, incubation period and incubation temperature) affecting the rate of esterification were also conducted. The optimal concentration of the partially purified from *Mucor racemosus* was determined as 2.5 mg/ml for the esterification reaction. Generally, it was observed that the enzyme concentration of *Mucor racemosus* lipase was proportional to the esterification percent until the concentration of 5.0 mg/ml after which the esterification percentage was slightly decreased (Fig.2). All these results were in agreements with previously published reports (38, 39, 40).

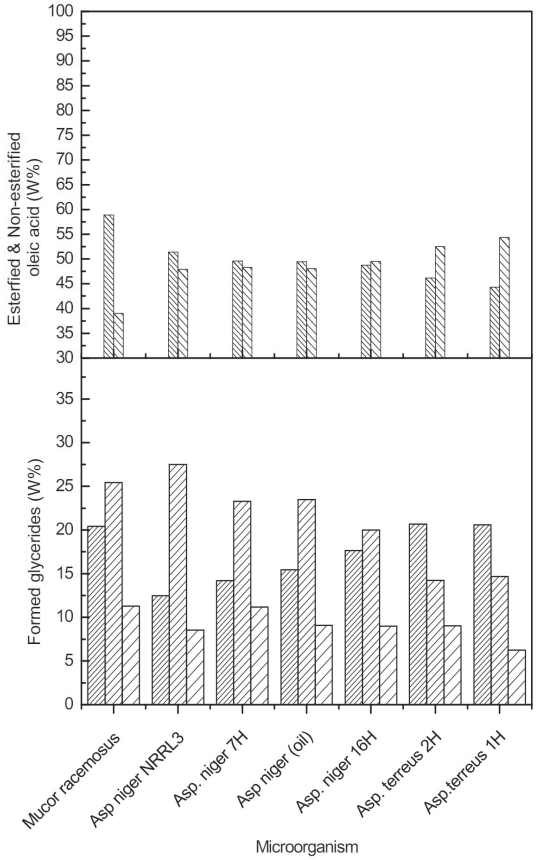

Fig. 1. Screening of active partially purified lipases from different fungi for the esterification reaction between glycerol and oleic acid.

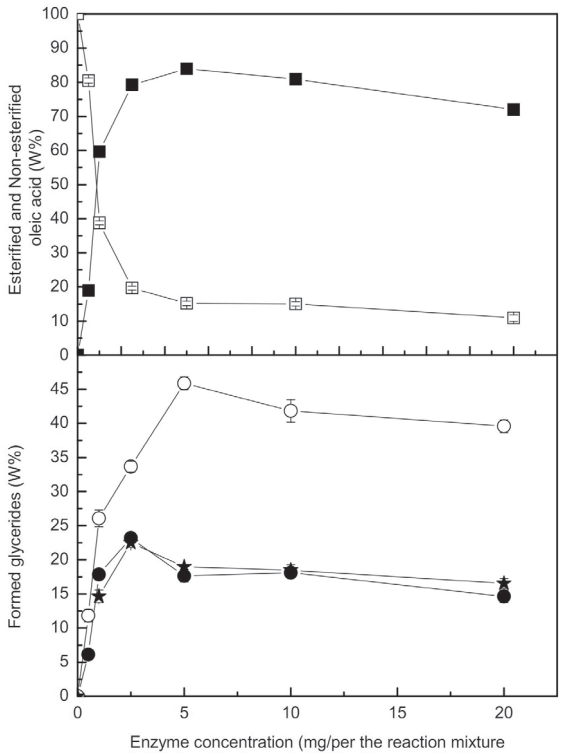

Fig. 2. Effect of the concentration of partially purified *Mucor racemosus* lipase on the esterification reaction. The estimation of products(Ï%) monoolein, (Ë%) diolein, (*) Triolein, (%) esterified oleic acid and ($_{«\%}$) non-esterified oleic acid were evaluated on the basis of 100 mg of the esterification products.

The optimal substrate concentration was estimated by taking various molar ratio molar ratio of glycerol to oleic acid. of With respect to the (A), 1: 0.008, 1:0.16, 1: 0.25 and 1:0.375 in which glycerol is in a constant concentration and oleic acid is in a variable concentration. The other molar ratio (B) of oleic acid: glycerol : 1:50, 1:60, 1:70 & 1:80 in which the oleic acid is constant while the glycerol is at variable concentrations (B). The optimum esterification percent was amounted to 89.50 % with a high level of diolein (48.30 %) and moderate amounts of monoolein and triolein (21.20 % and 20.0%, respectively) (Fig.3). This may be attributed to the possibility of the enzyme to enhance the formation of diolein. It is noteworthy to mention that some authors used high concentrations of enzyme 15 % (w/w) at a molar ratio of 1:2 for 24 hours and reached > 80 % of esterification level [41]. The presented data were confirmed with earlier reports (40) who observed that large amounts of glycerol increased the velocity of esterification and attained a higher degree of esterification. On the other hand, they reported that large amounts of glycerol repressed synthesis of diacylglycerol and increased the content of monoacylglycerol and the degree of esterification reached to 89.9 % after 48 hours.

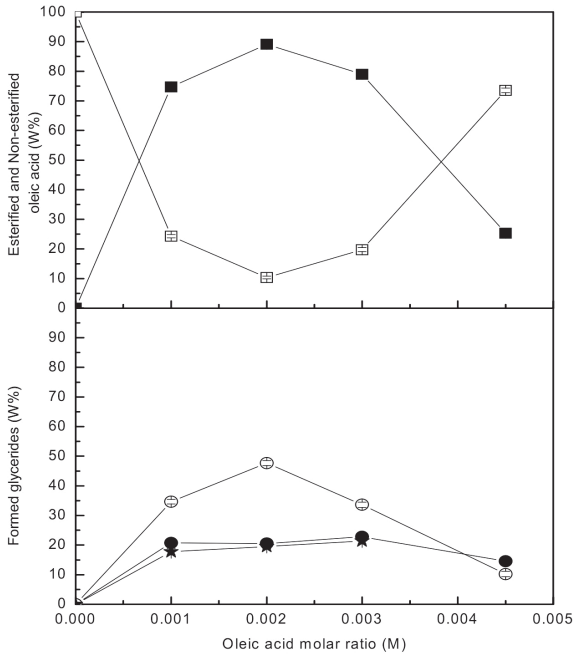

Fig. 3a. Effect of oleic acid concentrations (at fixed glycerol concentration) on the esterification reaction using *Mucor racemosus* lipase.
The estimation of products, (Ï%) monoolein, (Ë%) diolein, (*) Triolein, (%) esterified oleic acid and („%) non-esterified oleic acid, were evaluated on the basis of 100 mg of the esterification products.

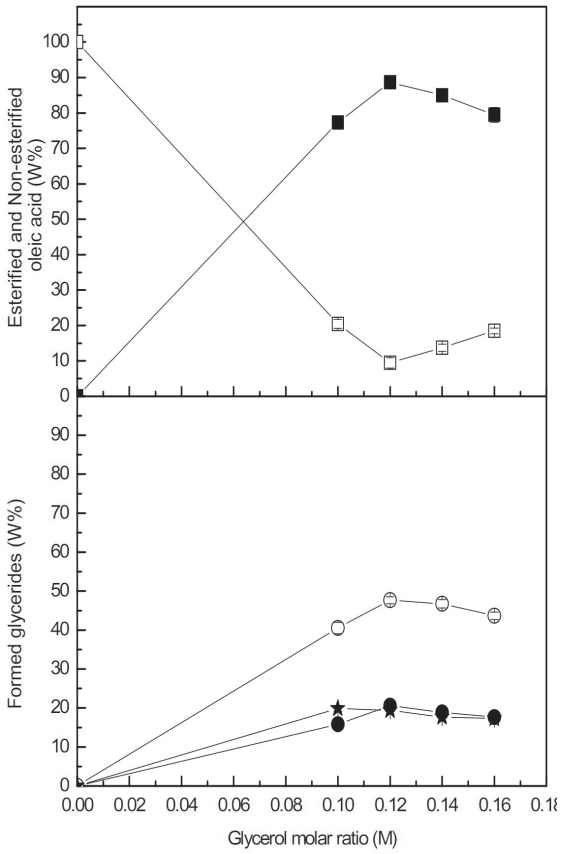

Fig. 3b. Effect of substrate concentrations (glycerol) at fixed oleic acid concentrations on the esterification reaction using *Mucor racemosus* lipase.
The estimation of products, (Ï%) monoolein, (Ë%) diolein, (*) Triolein, (%) esterified oleic acid and („%) non-esterified oleic acid, were evaluated on the basis of 100 mg of the esterification products.

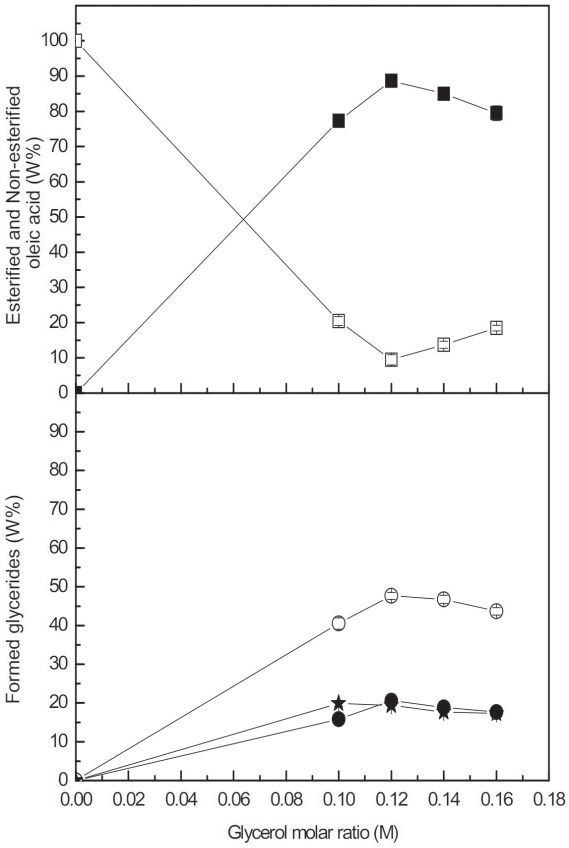

Fig. 4. Effect of different incubation periods on esterification reaction using *Mucor racemosus* lipase. The estimation of products (Ï%) monoolein, (Ë%) diolein, (*) Triolein, (%) esterified oleic acid and („%) non-esterified oleic acid were evaluated on the basis of 100 mg of the esterification products

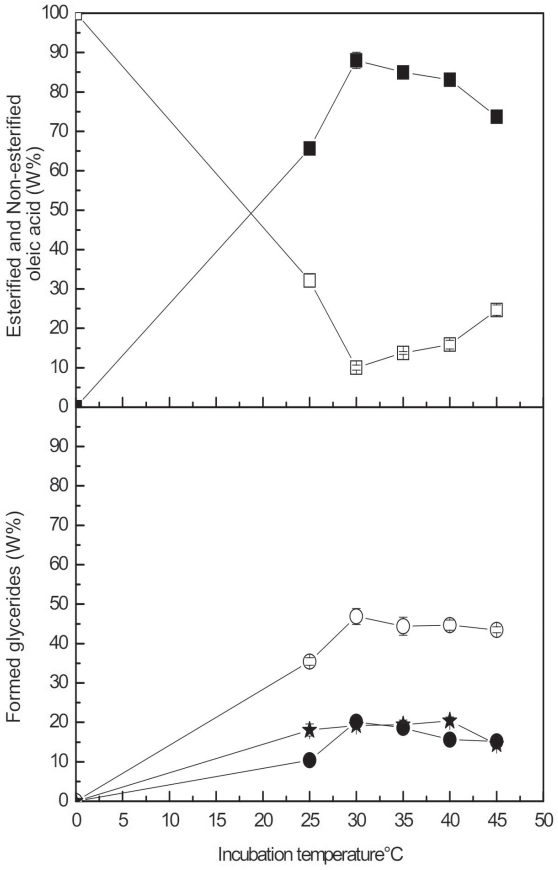

Fig. 5. Effect of different incubation temperatures on esterification reaction using *Mucor racemosus* lipase. The estimation of products (Ï%) monoolein, (Ë%) diolein, (*) Triolein, (%) esterified oleic acid and („%) non-esterified oleic acid were evaluated on the basis of 100 mg of the esterification products.

The effect of different incubation periods starting from 12 to 72 hours was also studied. The optimum values of esterification percent (89.5 and 88.7 %) were obtained after 24 and 36 hours, respectively (Fig. 4). However, the esterification percent after 24 hours was preferable to that after 36 hours which was attributed to the possible short time of 24 hours that led to similar esterification capacity. Thus, much time was saved in the esterification reaction. Accordingly, 24 hours incubation period is selected as the optimum incubation period. These results were supported by finding of earlier reports (40) who reported that the optimum incubation period 24 hours for the production of monoacylglycerol and diacylglycerol by *Penicillium camembertii* lipase with the esterification to 86.1%.

Referring to the effect of temperature on the esterification of oleic acid with glycerol, the esterification capacity amounted to (89.50%) at 30°C (Fig. 5). There was a drastic decrease in the esterification value reaching to 66.69 % at 25°C with lowering the temperature while the differences in the esterification capacities

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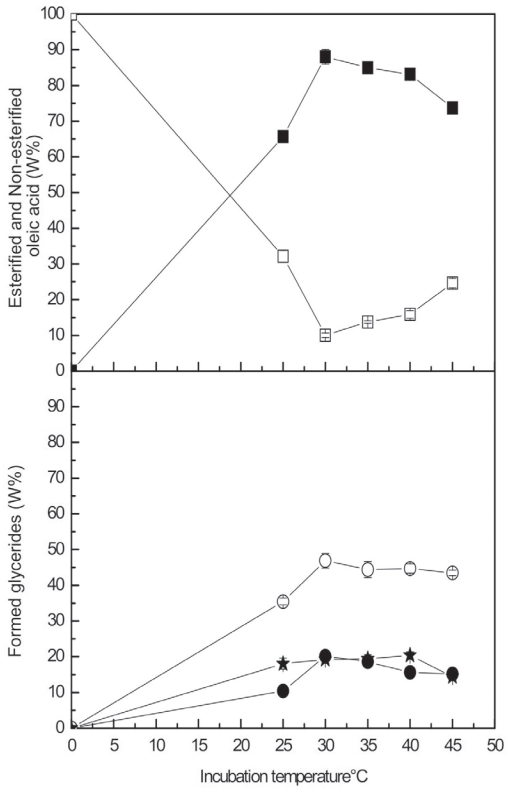

Fig. 6. Quantitative fractionation via preparative thin layer chromatography (TLC) of the enzymatically esterified products of *Mucor racemous* (monoolein, diolein, residual (non-esterified) oleic acid and triolein)
to-Scaled Chromatogram of sample
Auto-Scaled Chromatogram of standard
Auto-Scaled Chromatogram of sample

between 30°C and 40°C was very less. These results are approximately supported by earlier studies (40) who found that decrease in the temperatures hindered the synthesis of diacylglycerol and monoacylglycerol and almost the same amounts of monoacylglycerol and diacylglycerol were formed by *Penicillium camembertii* lipase after 24 hours in the reaction at 30°C and 40°C reaching degree of esterification of 86.1%.

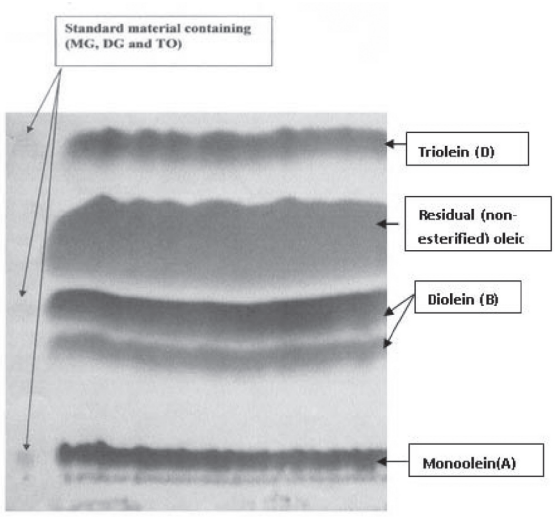

Fig. 7. HPLC auto-scaled chromatogram to show the detection of aflatoxins in *Mucor racemosus*

As a trial to apply the results of glycerol / oleic acid esterification, it was planned to use olive oil mixed fatty acid of olive oil as a representative of sources of a fatty acid of natural origin. Mixed free fatty acids from olive oil have the following composition as determined by G.L.C. (palmitic acid, 12%; stearic acid, traces; oleic acid, 81.1%; linoleic acid, 5.34% and linolenic acid traces) (Table-2a). It was noted that, esterified fatty acids (oleic acid, 71.1 % palmitic acid, 5.0 %, linoleic acid, 3.0 % and unknown acid, 0.33 %) amounted to 79.4% while the non-esterified fatty acid (palmitic acid, 7.0%, oleic acid, 10.0%; linoleic acid, 2.0% unknown acid, 1.17% and stearic acid, traces) amounted to 20.17 % (Table-2b). Thus, lipase enzyme probably selects the unsaturated fatty acids (oleic acid and linoleic acid) more than saturated fatty acid (palmitic acid) to form partial glycerides and triglycerides. Thus, the affinity of the reaction was arranged in descending order. Oleic acid > linoleic acid > palmitic acid. Generally, *Mucor racemosus* lipase enhanced the formation of monoglyceride (24.20 %), diglyceride (41.8 %) and low amounts of residual triglyceride (14.0 %)

Table 1. Enzymatic esterification of prepared mixed fatty acids of olive oil with glycerol determined by T.L.C (w%)

Monooleoyl glycerol	Dioleoyl glycerol	Trioleoyl glycerol	Esterified fatty acid*	*Non-esterified fatty acid
24.20	41.8	14.00	80.00	20.00

Table 2a. Composition of non-esterified fatty acids (non-reacted) determined as methyl esters by G.L.C analysis (w%)

Palmitic acid	Stearic acid	Oleic acid	Linoleic acid	Linolenic acid	Unknown	Total
7.0	Traces	10.0	2.0	Traces	1.17	20.17

Table 2 b. Composition of enzymatically esterified fatty acids (80.0%) calculated by substracting non-esterified fatty acids from the original mixed fatty acids of olive oil (w%)

Palmitic acid	Stearic acid	Oleicacid	Linoleic acid	Linolenic acid	Unknown
5.0	Traces	71.1	3.0	Traces	0.33

Table 3. Detection of mycotoxins (Aflatoxins) in *Mucor racemosus* by Toxicity test

Sample	Mycotoxins Aflatoxin			
	G_1	B_1	G_2	B_2
	ND	ND	ND	ND

ND = Non detected

from the esterification reaction between the prepared mixed fatty acids of olive oil with glycerol (Fig. 6; Table-2). Although Aspergillus *niger* (oil) exhibited maximum lipolytic activities, *Mucor racemosus* has been selected for the studies due to its non-pathogenicity as confirmed by the toxicity test as shown in Fig. 7; Table-3 (34)

Conclusion

It can be concluded that the value added products namely, monoacylglycerol and diacylglycerol, produced from esterification reactions, are useful in the field of biotechnology which is mainly used as emulsifiers in different industries, particularly in the food industry. In other words, *Mucor racimosus* lipase has the ability to produce appreciable amounts of unsaturated monoglycerides, diglycerides of oleic acid and linoleic acid.

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Selection of Micronutrients for Vancomycin Production by *Amycolatopsis orientalis* using Plackett-Burman Design

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Abstract

Micronutrients significantly influence both growth and production of the product by any microorganism. Taking this fact into consideration different micronutrients were screened for Vancomycin production using statistical design like Plackett-Burman. About 11 micronutrients including trace elements were independently screened in both chloride and sulphate forms using 12 experimental design of Plackett-Burman. The fermentative production of Vancomycin was studied for 10 days by bioassay method. The vancomycin yields were subjected to statistical analysis. Significant micronutrients in both chloride and sulphate form were short listed based on regression coefficients and t-values. The micronutrients significant in sulphate form was lithium sulphate and in chloride form was magnesium chloride. These were short listed for further optimization studies as optimized production medium is a necessity for any commercial production. This screening strategy not only short listed the efficient diverse nutrients but also improved the antibiotic yields.

Key words: *Amycolatopsis orientalis*, Vancomycin, Plackett-Burman, Micronutrients,

Introduction

Vancomycin belongs to a group of glycopepties and is the 1st clinically effective drug against the resistant forms of *S. aureus* (1). Vacomycin is a bactericidal drug highly effective against infections like infective endocarditis caused by *S.aureus* (2,3). It acts by inhibiting one or both of the two sequential enzymatic reactions involved in cell wall synthesis namely peptidoglycan elongation or transglycosylation and cross linking or transpeptidation (4). With increased prevelance of multiple antibiotic resistant strains in the 21st century its use was resurrected after its first clinical use in 1958. It is used to treat infections caused by different bacteria like penicillin-resistant strains of *Streptococcus pneumonia* (5), *Bacillus anthracis, Bacillus cereus* (6) and *Corynebacterium diphtheria* (7). Intraventricular application of vancomycin is an effective therapeutic regimen for treatment of shunt associated staphylococcal ventriculitis (8). It is also used to combat Gram-positive bacterial infections in intensive care patients (9). Being a drug of such importance and usage, its indigenous production being a necessity, an attempt for the same was made in the present study. Development of fermentative technology for indigenous production was aimed. The study concentrated on the screening of different micronutrients in sulphate and chloride forms using 12 experimental design of Plackett-Burman (10). This is an efficient statistical methodology that is used to screen up to n-1 variables in just n number of experiments hence saves both time and materials.

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Materials and Methods

Microorganism, its physical and physiological conditions (Medium used): *Amycolatopsis orientalis* 43491 was grown and maintained on ISP 2 medium slants or yeast-malt agar slants (4g/L of glucose, 10g/L of malt extract and 4g/L of yeast extract at pH 7.2). The inoculum for the fermentation was prepared in two stages where in the first stage sporulating culture from slant was inoculated into a shake flask of the medium and incubated at 28^0C for 3 days at 220 rpm. The culture grown was used for further study for Screening of different nutrients.

Bioassay: The flasks were incubated for 12 days. The fermented broth was collected and assayed every alternate day (2^{nd}, 4^{th}, 6^{th}, 8^{th}, 10^{th} and 12^{th} day) until the maximum broth potency had been passed. The samples were collected aseptically, centrifuged at 5000g for 15 min. The supernatant was filtered through a 0.45µm Millipore filter. Bioassay plates were prepared by pouring 25ml of preseeded agar having 1 ml of sensitive culture containing 10^5cells/ml. After the agar was set wells were made with a 6mm diameter cork borer. The filtrate was bioassayed using the sensitive organism, strain *Bacillus subtilis* ATCC 11774 (11). The zones of inhibition developed were measured, and the concentration of the antibiotic was determined using a graph of the standard antibiotic vancomycin. The bioassay results were compared with those of high-performance liquid chromatography and were tallied (12).

Statistical Analysis: Different micronutrients in sulphate and chloride forms were tested by a 12 experimental design taking care of their concentrations and status (ingredients added as such to prevent any precipitation). In each case the nutrients were added according to the pattern of the design. The fermentation was carried out for 12 day and the broth samples collected were bioassayed. 'Indostat' software package was used to analyze the results for antibiotic production yield. Significant nutrients were selected based on the highest regression coefficients and t-values and were ranked first and so on. Those with probabilities less than 0.005 were considered to be significant. The significant nutrients were shortlisted for further optimization studies.

Results

Different micronutrients in both sulphate and chloride form when screened by 12 experimental design of Plackett-Burman (1946) showed that the antibiotic yields varied in different flasks on different days. For micronutrients in sulphate form the yields ranged from 100-1440 µg/ml and for those in chloride form 430-1400 µg/ml. The antibiotic bioassay data when subjected to statistical analysis yielded regression coefficients and t-values. The probabilities of the experiments were almost same they being 0.00011 for sulphates and 0.00012 for chlorides, thus the experiments were highly significant. Nutrients with highest positive regression coefficients and their corresponding t-values were ranked first and so on, on different days. Different sulphate forms were screened using the 12 experimental design of Plackett-Burman (1946) and their regression coefficients and t-values are as indicated in (Table 1) and the ranking of the ingridients varied with fermentation time as indicated in the figure (Fig 1). Lithium sulphate ranked 1^{st} among all followed by calcium sulphate and ferric Sulpahte. Similarly different chlorides were screened using the same design and ranking of chlorides of trace elements varied with fermentation time. The effect of chloride ingredients varied on different days as indicated by the regression and t-values in (Table 2). While considering the ranking of chlorides on different days two chlorides were considered as there was variation indicated in (Fig 2). On 4^{th} and 6^{th} days ferric chloride ranked 1^{st} followed by cadmium chloride and magnesium chloride for 4^{th} day and cadmium chloride and mercuric chloride for 6^{th} day. On 8^{th} and 10^{th} days magnesium chloride ranked 1^{st} followed by nickel chloride and mercuric chloride for 8^{th} day and mercuric chloride and manganese chloride for 10^{th} day. The nutrients were further selected for 2^{nd} step screening based on either ranking like

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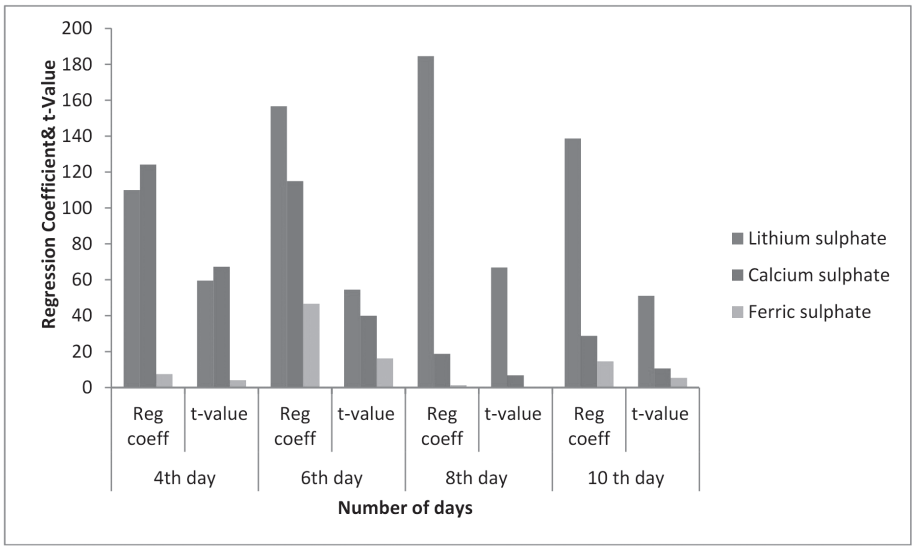

Fig 1: Variation in the Regression coefficients and t-Values of the Selected and Short Listed Micronutrients of Sulphate forms for Vancomycin Production on 4th, 6th, 8th and 10th Day.

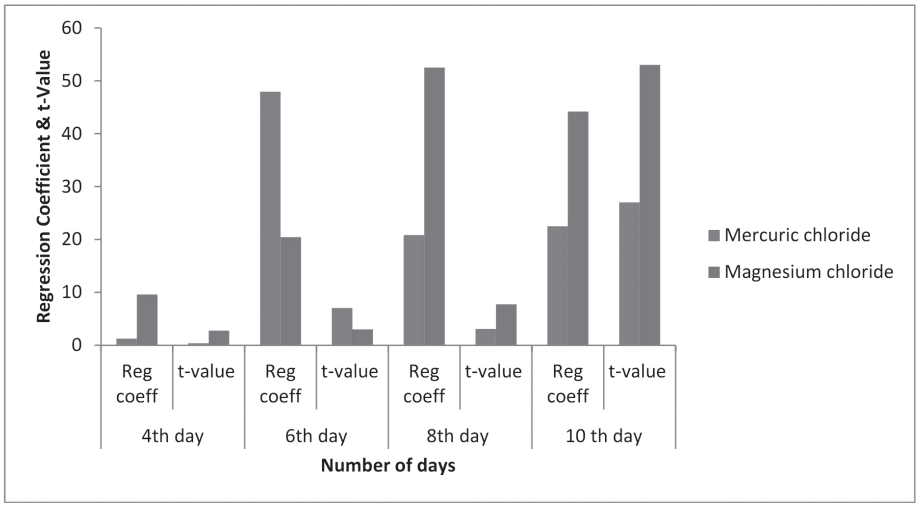

Fig 2: Variation in the Regression coefficients and t-Values of the Selected and Short Listed Micronutrients of Chloride forms for Vancomycin Production on 4th, 6th, 8th and 10th Day.

ranking 1st on more number of days as in case of lithium sulphate or significance of the nutrient during production stage rather than during growth stage like magnesium chloride ranking 1st on 8th and10th days.

Discussion

Presence of required nutrients in appropriate forms and at non-inhibitory concentrations is the main objective in developing a microbial culture medium (13). As micro-

organisms exhibit diverse nutritional requirements various nutrients can be screened by application of statistical methods like Plackett-Burman (1946) designs. A single dimensional approach is the most commonly used strategy in the laboratory but, as it is very laborious and time consuming, a statistical method is considered the best as it is rapid and reliable in short listing of nutrients. Therefore Plackett-Burman is considered best method as it (14) as it screens up to n-1 variables in just n number of experiments.

Trace elements are the micronutrients necessary for cell metabolism and act as cofactors of many enzymes of general cell metabolism, Secondary metabolism is also known to be affected by the presence or absence of trace elements as they may be responsible for activation of some of the biosynthetic pathways (15,16,17). Not many reports are available on influence of trace elements on *Streptomyces* except a few like that of Abbas (18, 19), on *S.coelicolor* (a model of the Streptomycetes) where different metals were studied mostly in chloride form and reported different effects. This study reported that mercury and cadmium are toxic where as copper nickel, and lead have inhibitory effect for actinorhodin production. Elements like manganese, zinc and cobalt showed complex effects and calcium and magnesium showed increase in cellular reactions. Both calcium and magnesium promoted growth and antibiotic production with magnesium having more positive effect. Similar trends were observed in the present study of vancomycin production.

Different trace elements in both sulphate and chloride forms were screened using Plackett-Burman design to find their influence on vancomycin production. Among the different forms of sulphate lithium sulphate was found to be significant. Since there are no reports of lithium as a significant trace element, the finding in the present study depicted the importance of lithium as a growth factor for *A.orientalis* thus, included in the optimized medium. Similarly effect

Table 1: Regression coefficients and t-values calculated from vancomycin yields of *A.orientalis* for different trace elements in sulphate form using Plackett-Burman design.

S. No	Ingredient	4th day		6th day		8th day		10th day	
		Reg coeff	t-value	Reg coeff	t-value	Reg coeff	t-value	Reg coeff	t-value
1	Intercept	210.00	35.97	607.50	66.80	1011.67	115.87	1184.17	137.93
2	Calcium sulphate	124.17	67.25	-115.00	39.99	-18.75	6.79	-28.75	10.59
3	Cupric sulphate	35.83	19.41	-46.67	16.23	-37.92	13.73	-37.92	13.97
4	Cesium sulphate	19.17	10.38	-26.67	9.27	-32.08	11.62	-2.08	0.77
5	Ferric sulphate	7.50	4.06	46.67	16.23	1.25	0.45	-14.58	5.37
6.	Ferrous sulphate	-50.00	27.08	3.33	1.16	45.42	16.45	-24.58	9.06
7	Lithium sulphate	110.00	59.58	156.67	54.48	184.58	66.85	138.75	51.11
8	Molybdenum sulphate	-50.00	27.08	-1.67	0.58	-17.92	6.49	-22.08	8.13
9	Magnesium sulphate	-33.33	18.05	-141.67	49.26	-101.25	36.67	-45.42	16.72
10	Manganous sulphate	7.50	4.06	-128.33	44.63	-47.92	17.75	-72.08	26.55
11	Nickel sulphate	-193.33	104.71	-215.00	74.76	-205.42	74.39	-111.25	40.98
12	Zinc sulphate	-119.17	64.54	-136.67	47.52	-82.08	29.73	-42.08	15.50

Probability- 0.00011: Highly significant

Table 2: Regression coefficients and t-values calculated from vancomycin yields of *A.orientalis* for different trace elements in chloride form using Plackett-Burman design.

S. No	Ingredient	4th day		6th day		8th day		10th day	
		Reg coeff	t-value	Reg coeff	t-value	Reg coeff	t-value	Reg coeff	t-value
1.	Intercept	505.00	45.78	842.50	39.12	1221.67	56.87	1220.00	462.96
2.	Calcium chloride	-2.08	0.60	-20.42	2.99	-17.50	2.57	-10.83	13.00
3.	Cesium chloride	-18.75	5.38	-21.25	3.12	-27.50	4.05	-10.83	13.00
4.	Cobalt chloride	1.25	6.36	-42.92	6.30	-12.50	1.84	10.83	13.00
5.	Cupric chloride	-7.08	2.03	-32.92	4.83	-59.67	8.71	-44.17	53.00
6.	Ferric chloride	24..58	7.04	77.92	11.44	-27.50	4.05	-44.17	53.00
7.	Cadmium chloride	17.92	5.14	77.08	11.32	-7.50	1.10	10.83	13.00
8.	Mercuric chloride	1.25	0.36	47.92	7.04	20.83	3.07	22.50	27.00
9.	Magnesium chloride	9.58	2.75	20.42	2.99	52.50	7.73	44.17	53.00
10.	Manganous chloride	-18.75	5.38	-32.92	4.83	5.83	0.86	10.83	13.00
11.	Nickel chloride	9.58	2.75	-0.42	0.06	25.83	3.80	10.83	13.00
12.	Zinc chloride	-43.75	12.54	-88.75	13.03	-164.17	24.17	-177.50	213.00

Probability -0.00012: Highly significant

of different chlorides were studied and found that mercuric chloride, cadmium chloride and nickel chloride appeared in list of significant nutrients but with lower ranks in order of ranking. Magnesium chloride ranked almost 1st and further selected for 2nd step screening. Chloride salt was significant as vancomycin molecule contains several chlorine atoms and hence a necessity for chlorine (20,21) and thus included in the optimized medium.

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Production of Pullulan using Jaggery as substrate by *Aureobasidium pullulans* MTCC 2195

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Abstract

Shake-flask fermentation, under batch cultivation, was investigated for the production of fungal exopolysaccharide, pullulan using jaggery (a traditional concentrated sugar cane juice) as a carbon substrate by *Aureobasidium pullulans* MTCC 2195. Change in the initial pH (from 3.0 to 7.0) of media containing jaggery was varied to study the effect of pH in the fermentation and maximum pullulan yield was obtained at a pH of 5.0. An increase in the initial concentrations (50, 75, 100 g/L) of jaggery in the media produced the maximum pullulan content as 21.6, 19.7 and 18.6 g per 100 g of jaggery, respectively, used. A sucrose based defined media were also used for comparison purposes. Fourier Transform InfraRed (FTIR) spectroscopic analysis was done to confirm the functional groups of synthesized pullulan and compared with that of commercial pullulan.

Keywords : Jaggery, Pullulan, pH, FTIR, *Aureobasidium pullulans* MTCC 2195

Introduction

The recent trends in cost-effective production of microbial polysaccharides, cheaper substrates are being used to meet the economic advantage of production. One of such is fungal Exopolysaccharide (EPS) called pullulan owing to its excellent properties, became potential compound in several high-value technological platforms like food, biomedical, pharmaceutical, film and packaging industries (1-4).

Pullulan, a water-soluble, neutral, linear, homo-polysaccharide mostly produced by a ubiquitous black, yeast-like fungus, *Aureobasidium pullulans*. Pullulan, regarded as GRAS (Generally Recognized As Safe, by USFDA), composed of maltotriose repeating units connected by α-1, 6 linkages (3 glucose moieties in maltotriose joined by α-1, 4 glycosidic bonds). However, the fermentative production of pullulan in submerged conditions on wide variety of agro-industrial residues and wastes as low-cost substrates, were attempted by many researchers (Table 1) (5-13). In earlier studies, *jaggery* (also known as *Panela* or *gur*), a traditional non-centrifuged, un-purified and concentrated sugar cane product, from cottage industries, contains 75-85% sucrose was used for production of pullulan by other strains of *Aureobasidium pullulans* (5, 13).

In this present study, a medium composed of *jaggery* as a carbon source for pullulan production by *Aureobasidium pullulans* MTCC 2195 was attempted. The specific aims were to vary the initial concentrations of *jaggery*, initial pH and to determine the kinetics of pullulan fermentation. Characterization of produced pullulan was also performed to confirm the results.

Materials and Methods

Materials Used: Jaggery was purchased from the local market and found to be mainly composed of sucrose. The composition of Standard Cultivation Medium (SCM) used in the shake-flask fermentation (in g/L) is sucrose, 50.0; yeast extract, 3.0; KH_2PO4, 5.0; KCl, 0.5; $MgSO_4 \cdot 7H_2O$, 0.2; NaCl, 1.0 and distilled water 1 L. All the chemicals were purchased from Qualigens Chemicals and the biochemicals are purchased from M/s. HIMEDIA chemicals Ltd. Standard pullulan was purchased from M/s. Kumar Organics Pvt. Ltd., Bengaluru, and was used in structural characterization.

Micro-organism and Inoculum Development: Micro-organism used in this study, *Aureobasidium pullulans* MTCC 2195, was obtained from Microbial Type Culture Collection and Gene Bank (MTCC), IMTECH, Chandigarh and maintained on potato dextrose agar (PDA) slants at 4^0C and subcultured prior to each experimental run. A loop-full of freshly grown cultures from PDA agar slants were transferred to a 250 ml conical flask containing 50 ml Standard cultivation media. Media pH (initially) was adjusted to 5.0 (after autoclaving at 121^0C, for 15 min.) and incubated at 30^0C for 48h on a rotary shaker at 150 rpm. This resulted suspension (at 5% v/v) was then used as inoculum for *jaggery* medium fermentations.

Shake flask fermentation: Shake flask fermentations were carried out with Standard Cultivation Medium components at initial sucrose concentrations (g/L) of 50, 75 and 100, individually. Further, the cultivation media (JCM) was made by replacing sucrose with jaggery (based on weight but not on % of sucrose content) for the same concentrations (g/L) of 50, 75 and 100. Both SCM and JCM in 100 ml aliquots were distributed in 500 ml Erlenmeyer flasks and autoclaved. These sterilized media were inoculated, 5% (v/v) aseptically and incubated for 172 hours at 30^0C and 150 rpm on a rotary shaker. Fermentation broth samples were collected, aseptically, at irregular intervals and determined the concentrations of dry cell biomass, pullulan and residual sugar.

Effect of initial pH on fermentation: In order to study the influence of pH on the shake flask fermentation for the pullulan production, the pH of *jaggery* in cultivation medium (after autoclaving) was adjusted to 3.0, 4.0, 5.0, 6.0 and 7.0, individually, using either 1N HCl or 1N NaOH and left uncontrolled during the fermentation. A 100 ml sterile media in 500 ml Erlenmeyer flasks by inoculating 5% (v/v) inoculum were incubated for 156 h at 30^0C and 150 rpm on a rotary shaker. The samples were withdrawn for every 12 hours and analysed for cell biomass, Pullulan and residual sugar contents.

Estimation of dry cell biomass and pullulan: At specific intervals of time, 2 ml of broth volume from each flask was centrifuged at 10,000 rpm for 20 min at ambient temperature to separate the cell biomass (pellet) from supernatant liquid. The collected cell biomass was washed twice with saline and distilled water and dried to constant weight in an oven at 90^0C. The dry cell biomass weight was expressed in g/L. The polysaccharide, pullulan, was precipitated (kept at 4^0C for 12 hours) using cell-free supernatant liquid by adding cold ethanol (in the ratio of 1:2 v/v). The precipitate obtained was filtered through a pre-weighed Whatman No.1 filter paper and dried to constant weight at 80^0C. The dry weight of pullulan and yield of pullulan were expressed in g/L and gram EPS per 100 g of sugar consumed.

Estimation of residual sugar concentration: The residual sugar content in the cell-free fermentation broth was measured by the Miller's method (14) using double beam ELICO SL-159 UV-Visible Spectrophotometer.

Characterization of pullulan by FTIR spectroscopy: The precipitated pullulan from each flask, at the end of fermentation was characterized to compare the quality of pullulan obtained from sucrose and *jaggery* media. The

structural characterization of pullulan was carried out using Fourier Transform InfraRed (FTIR) spectroscopy and IR spectra were recorded with Shimadzu IRTracer-100 Fourier Transform Infrared (FTIR) Spectrophotometer.

Results and Discussion

Effect of initial pH on fermentative production of pullulan in jaggery medium: Effect of environmental variables like pH of the cultivation medium enhanced the yields of pullulan formation by *A. pullulans* on wide variety of carbon substrates (5-13, 15-19). Changes in initial pH of fermentation medium affects the growth rate of *Aureobasidium pullulans* MTCC 2195 using coconut by-products, bakery waste, cassava and maize powders, cashew fruit juice, were reported by Thirumavalavan *et al*. (9, 10). In another study, Vijayendra et al. (5) varied the initial pH from 2 to 7 for the study of *A.pullulans* CFR-77 growth and reported that maximum pullulan production was achieved at an initial pH of 5.0. Here, we attempted the change in initial pH (3.0 to 7.0) effect on the kinetics of *A.pullulans* MTCC 2195 in jaggery medium. Final (at the end of 156 h) pullulan and biomass concentration (g/L) as a function of pH is shown in Fig. 1. Pullulan content released into the medium increases with pH up to 5.0 and then decreased. The maximum concentration (9.88 g/L) of pullulan exopolysaccharide was obtained at pH 5.0.

Study of jaggery as a carbon source on growth of A. pullulans and pullulan production: Preliminary checking of *Jaggery* in replace of sucrose to study the growth of *Aureobasidium pullulans* MTCC 2195 in the cultivation media with agar on petri plates was performed. Several workers have exercised the wide variety of carbon substrates, ranging from agro-industrial residues and wastes for the economic production of Pullulan and the results are summarized in Table 1. The earlier investigations (5, 13) on *jaggery* reveal that *jaggery* could be used as a good carbon source, because of its high sucrose%, for the growth of *Aureobasidium pullulans* and towards cost-effective production of pullulan. S.V.N. Vijayendra et al. (5) reported that the yield of pullulan produced by *A. pullulans* depends on initial sugar concentration in the cultivation medium. So, in the present study the increasing initial levels of *jaggery* concentrations, 50 g/L, 75 g/L and 100 g/L were employed to determine the maximum pullulan content using *A. pullulans* MTCC 2195.

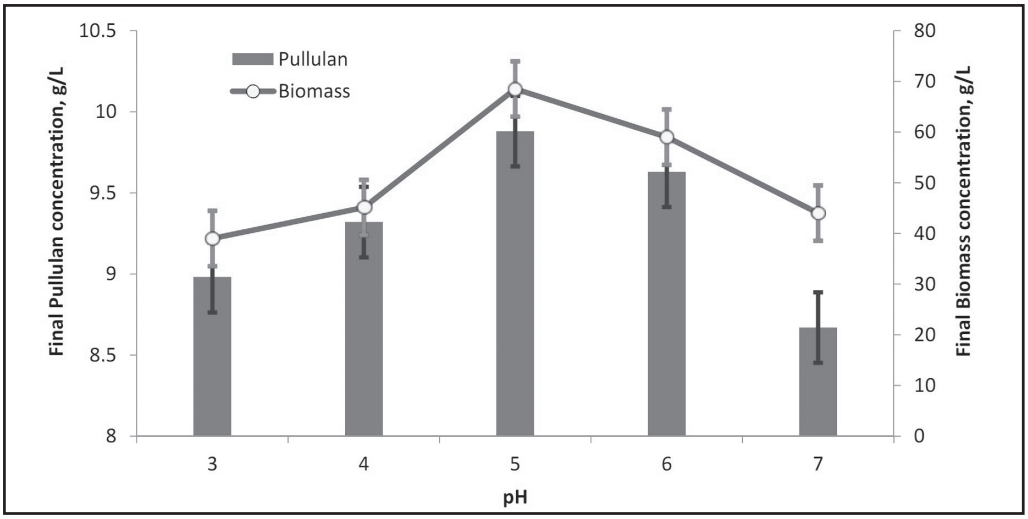

Fig. 1. Influence of pH on pullulan and biomass yield using jaggery as carbon source using *Aureobasidium pullulans* MTCC 2195 (error bars indicated).

Fig. 2 (a, b, c) shows the time course profiles of substrate, biomass and pullulan in shake-flask fermentations with increasing initial concentrations, 50, 75, 100 g/L of *jaggery* in the media, respectively. A bar diagram, Fig. 2 (d) indicates the pullulan concentration at specific time points in the above profiles. In all the plots, a considerable amount of sugar in the media was depleted in the early hours of fermentation and results a quick growth of *A.pullulans* and there was a concomitant increase in the pullulan production with decline in the residual sugar content in the media. The maximum pullulan concentrations obtained from *jaggery* medium containing 50, 75 and 100 g/L of sugar concentration were 10.8, 14.8 and 18.6 g/L, respectively.

Fig. 3 (a, b, c) shows the time course profiles of substrate, biomass and pullulan, in case of sucrose containing media with the similar initial sucrose concentrations. A bar diagram, Fig. 3 (d) indicates the pullulan concentration at specific time points in the above profiles. Comparative of *jaggery* and sucrose as carbon substrates for the production of pullulan in this study with literature, is listed in Table-2. Further, kinetic models fit the results of comprehensive biomass growth and pullulan formation and there by predicts kinetic parameters, in a good manner (9, 20, 21). Of these models, Logistic (L), Leudeking- Piret (LP), Logistic incorporated Leudeking-Piret (LILP), Modified Leudeking-Piret (MLP) and Logistic incorporated Modified Leudeking-Piret (LMLP) were tested to describe biomass, pullulan and sugar profiles in the batch cultivation of *Aureobasidium pullulans* (21).

Structural characterization of pullulan by FTIR spectroscopy: Structural characterization

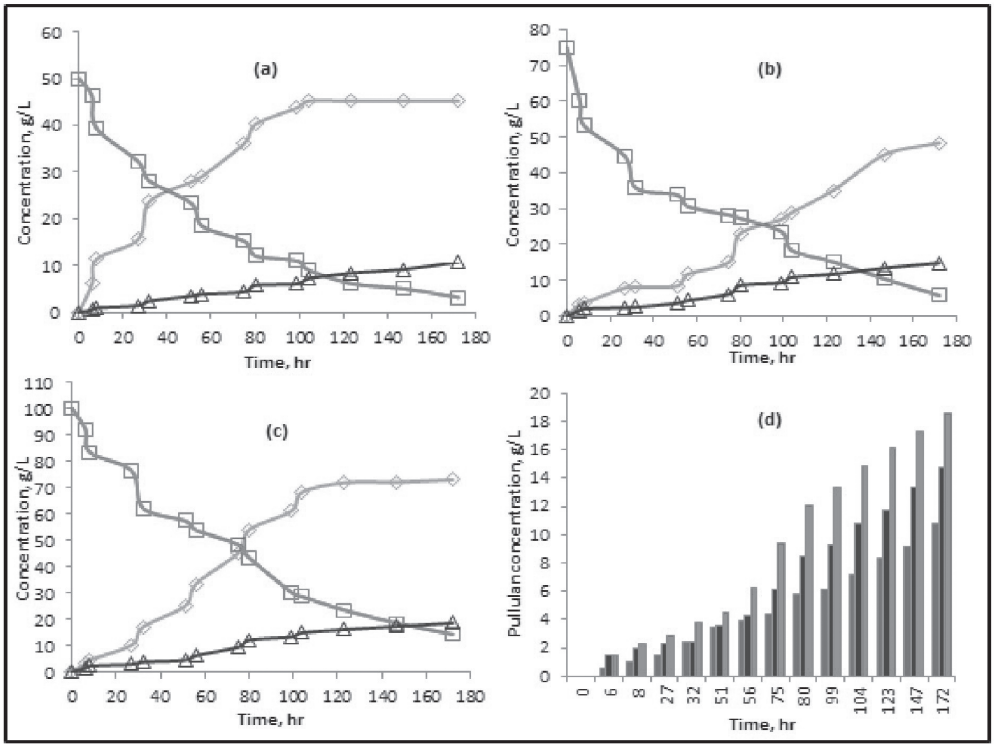

Fig. 2. Time course for batch fermentation of *A. pullulans* MTCC 2195 in (a) 50 g/L (b) 75 g/L (c) 100 g/L *jaggery* media: cell dry weight (Ê%), pullulan dry weight (Ä) and sucrose concentration (¡%); (d): variation of pullulan concentration with time in (a), (b),(c) profiles.

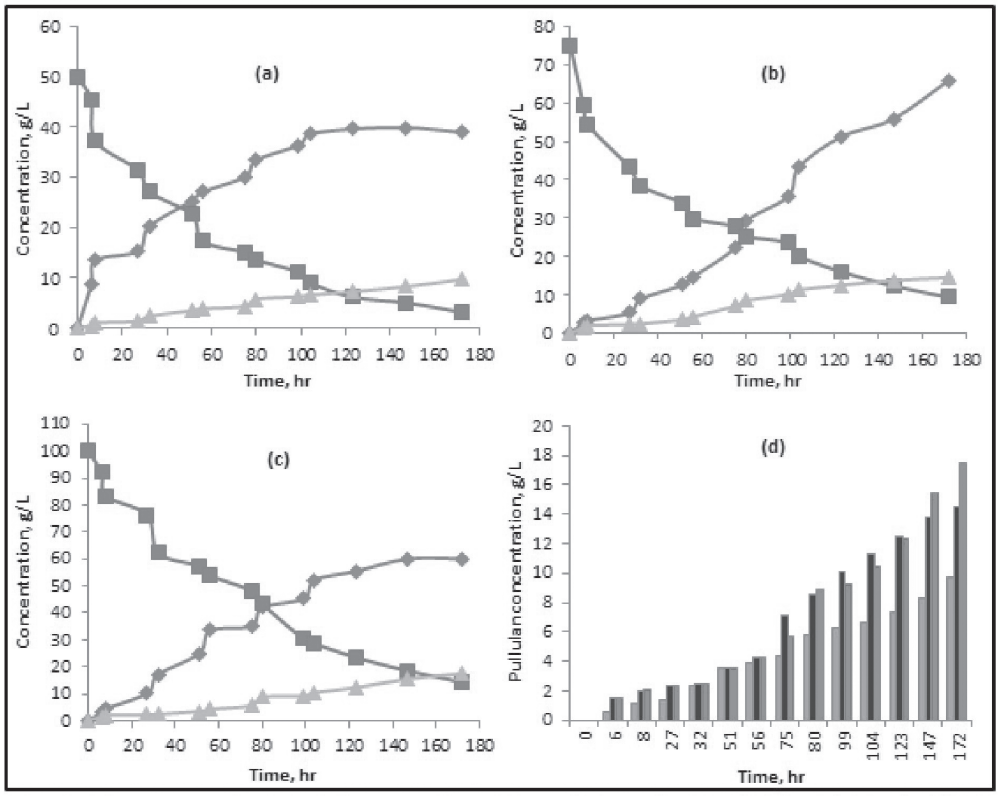

Fig. 3. Time course for batch fermentation of *A. pullulans* MTCC 2195 in (a) 50 g/L (b) 75 g/L (c) 100 g/L sucrose media: cell dry weight (Ê%), pullulan dry weight (Ä) and sucrose concentration (¡%); (d): variation of pullulan concentration with time in (a), (b),(c) profiles.

to examine the possible functional groups for commercial pullulan and synthesized pullulan from batch cultivation were done by Fourier Transform InfraRed Spectrophotometer and spectra were shown in figure 4. Top spectrum shows the absorption peaks of standard pullulan and bottom spectrum indicates that of synthesized pullulan from this study. A strong absorption peaks at ranges of 3300 cm^{-1} and 1640 cm^{-1} confirms repeating units of –OH and O-C-O, respectively. In the specific area (1500-650 cm^{-1}) is a characteristic of pullulan molecule as a whole. Another strong absorption at 1000 cm^{-1} characterizes the C-O group and peaks at 900 and 650 cm^{-1} proves the presence of α-1, 6- and α-D-glucopiranosid units, respectively. The similar spectra and frequencies were also observed in other works (6, 8, 22, 23).

Conclusion

The work attempted, here, first focuses the suitability of using *jaggery* as an effective and cheaper carbon substrate for the maximum production of exopolysaccharide, pullulan by *Aureobasidium pullulans* MTCC 2195. Then, the effect of initial *jaggery* concentration and pH in batch cultivation of polymer production was studied. The results obtained from *jaggery* media were compared to sucrose media and rates of *jaggery* utilization and pullulan production was high, when the initial sugar concentration was comparatively low. The FTIR analysis of synthesized pullulan was also performed to confirm the functional groups in it. All the above results indicate that *jaggery*, an agro-industrial residue, as alternative for carbon source for melanin-free pullulan production. Further, to

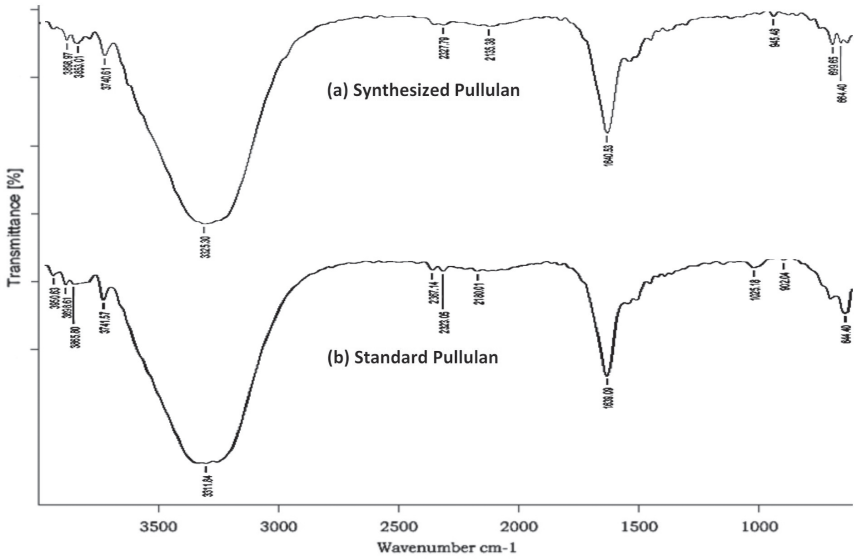

Fig. 4 (a). FTIR spectrum of pullulan synthesised in this work (b) FTIR spectrum of standard ullulan commercially purchased.

Table 1. Survey on *Aureobasidium pullulans* variants producing Pullulan using different agro-industrial wastes/ residues as substrates, fermentation conditions and yields

Organism	Type of Carbon Substrate pH	Fermentation conditions Time, hr		Pullulan content,g/L	Reference
A. pullulans CFR-77	*Jaggery*	5.0	72	51.9	5
A.pullulans HP-2001	Soybean pomace	5.7	96	7.5	6
A pullulans NRRL Y-6220	Soya Bean Oil	4.0	120	17.4	7
A pullulans NRRL Y-2311-1	Soya Bean Oil	3.5	96	26.24	
A.pullulans ATCC 42023	CSL+ sucrose	2.2	120	65.3	8
	clarified cane molasses	2.2	120	47.84	
	potato starchy waste	3.5	120	22.33	
	Hydrolysed sweet whey	2.3	120	12.4	
	hydrolysed rice straw	4.0	120	9.36	
A.pullulans MTCC 2195	Cashew juice	6.5	156	92.5	9
	Maize	6.5	96	71.0	
	Cassava	6.5	96	65.0	
	Bakery waste	6.5	96	27.0	
A.pullulans AP329	Sweet potato	5.5	96	29.43	11
A.pullulans MTCC 2195	Coconut water	7.0	144	38.3	10
	Coconut milk	7.0	144	58.0	
A. pullulans MTCC 2195	Jack fruit seed powder	7.0	168	22.49	12
A.pullulans RBF 4A3	Jaggery+ DOJSC+ CSL	-	72	66.25	13

CSL- Corn Steep Liquor, DOJSC- De-Oiled Jatropha Seed Cake

Table 2. Comparison of Pullulan production using Jaggery and Sucrose as carbon sources

Carbon substrate		Kinetic parameters					
Type	**Initial concentration, g/L**	**Pullulan content, g/L**	**Dry cell weight, g/L**	**Residual sugar, g/L**	**Yield, g Pullulan 100g sugar utilized**	**Pullulan/ productivity, g/(L.h)**	**Reference**
Jaggery	50	10.8	45.2	3.2	21.6	0.062791	This study
	75	14.8	48.35	5.7	19.7	0.086047	
	100	18.6	73.01	14.3	18.6	0.10814	
Sucrose	50	9.7	39.0	3.2	19.4	0.056395	
	75	14.5	65.9	9.4	19.3	0.084302	
	100	17.5	60.1	14.3	17.5	0.101744	
Jaggery	50	23.01	7.25	5.68	51.9	0.239688	5
	75	24.56	9.24	11.25	44.61	0.255833	
	100	25.03	11.25	39.17	41.16	0.260729	
Jaggery[a,]	180	66.25	—	—	36.81	0.92014	13

[a,] - combination of Jaggery, De-oiled Jatropha Seed Cake, Corn Steep Liquor

elucidate what affects the fate of pullulan formation under different fermentation conditions, the fermentation have to be studied in5L reactor.

Acknowledgements

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Study of Potential Plant Growth-Promoting Activities and Heavy Metal Tolerance of *Pseudomonas aeruginosa* HMR16 Isolated from Zawar, Udaipur, India

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Abstract

The use of plant growth-promoting rhizobacteria (PGPR) is steadily increasing in agriculture and offers an attractive way to replace chemical fertilizers, pesticides, and supplements. Genus *Pseudomonas* is widespread bacteria in agricultural soils and has many traits that make them well-matched as PGPR. *Pseudomonas aeruginosa* HMR16 isolated from heavy metal contaminated soil of Zawar, Udaipur was tested for direct plant growth promoting activities (IAA production, production of ammonia and phosphate solubilization) and indirect growth promoting activities (HCN production, siderophore production). Effect of heavy metals such as zinc, lead, chromium on growth of *Pseudomonas aeruginosa* HMR16 was also determined on nutrient agar and nutrient broth medium supplemented with respective heavy metals concentrations. *Pseudomonas aeruginosa* HMR16 was found to be positive for the production of plant growth-promoting hormone (IAA), ammonia, HCN, siderophores and solublize phosphate with solubilization efficiency of 115.38. Minimum inhibitory concentration (MIC) of zinc, lead, and chromium against *Pseudomonas aeruginosa* HMR16 strains were 16.0mM, 1.25 mM and 0.30mM respectively. *Pseudomonas aeruginosa* HMR16 showed various plant growth promoting activities and tolerance to heavy metals thus it can be used to improve plant growth in heavy metal contaminated soil.

Keywords: Zawar; PGPR, Heavy metals, *Pseudomonas,* IAA, Phosphate solubilization

Introduction

Heavy metal pollution in soils is the most serious environmental problem and has significant implications for most of the organisms. In plants, such effects of heavy metal contamination may include growth inhibition, structural damage, and a decline of physiological and biochemical activities. Metal-contaminated soils represent one of the most difficult challenges facing bioremediation. Phytoremediation assisted with the heavy metal tolerant bacteria offers more benefits than conventional technology in accumulating heavy metals from the soil as it is less expensive and safer for the environment. The soil bacteria that aggressively colonize the root zone and promote plant growth are generally termed as Plant Growth Promoting Rhizobacteria (PGPR). Fluorescent *Pseudomonads* are the dominant group of bacteria that preferably lives in close vicinity to the root or on root surface and play a crucial role in soil health and plant development (1). These bacteria have several mechanisms to survive in heavy metal contaminated soil and also influence plant growth directly or indirectly (2). Their tolerance to adverse environmental conditions, capacity to solubilize phosphate, hydrocyanic acid, indole acetic acid, siderophores and ability to effectively colonize roots is responsible for plant growth promotion (3).

The aim of this study was to determine the potential plant growth promoting activities of indigenous *Pseudomonas aeruginosa* HMR16 strain isolated from heavy metal contaminated site of Zawar, Udaipur and to determine the minimum inhibitory concentration of various heavy metals i.e. zinc, lead and chromium.

Materials and Methods

***Source and maintenance of bacteria*:** Heavy metal tolerant bacterial isolate *Pseudomonas aeruginosa* HMR16 (Accession No. KU174205) previously isolated from heavy metal contaminated sites of Zawar, Udaipur (4) on Nutrient agar medium supplemented with zinc sulphate heptahydrate was used in this study. Isolate *Pseudomonas aeruginosa* HMR16 was routinely grown for 24h at 37°C on Nutrient agar medium supplemented with 1 mM zinc sulphate heptahydrate concentration and stored at -20°C in glycerol.

In vitro screening of bacterial isolate for their plant growth promoting (PGP) activities

***Production of IAA*:** IAA production was quantitatively measured by the method as described by Gordon and Weber (5). Bacterial culture was grown for 48 h on LB broth and LB broth amended with 100 µg/mL tryptophan. Fully grown culture was centrifuged at 5000 rpm for 15 min. The supernatant (2 mL) was mixed with two drops of orthophosphoric acid and 4 mL of the Salkowski reagent (50 mL, 35% of perchloric acid, 1 mL 0.5 M $FeCl_3$ solution). Optical density was taken at 530 nm with the help of spectrophotometer. Concentration of IAA produced by culture was measured with the help of standard graph of IAA obtained in the range of 10-100 µg/ mL.

***Production of NH3*:** Bacterial isolate was tested for the production of ammonia as described by Cappuccino and Sherman (6). Overnight grown bacterial culture was inoculated in 10 mL peptone broth and incubated at 35±0.1 °C for 48 h in Incubator Shaker. After incubation 0.5 mL of Nessler's reagent was added.

Production of HCN: Bacterial isolate was screened for the production of hydrogen cyanide by adapting the method of Castric (7). Briefly, bacterial culture was streaked on nutrient agar medium containing 4.4 g per liter of glycine. A Whatman filter paper No. 1 soaked in 0.5% picric acid solution (in 2% sodium carbonate) was placed inside the lid of a plate. Plates were sealed with parafilm and incubated at 25 ± 2°C for 4 days.

***Production of Siderophore*:** Bacterial isolate were assayed for siderophores production on the Chrome azurol S agar medium described by Schwyn and Neilands (8). Chrome azurol S agar plates were prepared and divided into equal sectors and spot inoculated with test organism (10µL) and incubated at 25±2ºC for 48-72 h.

Detection of phosphate solubilizing activity: Phosphate solubilizing activity was tested on the modified Sperber's medium by spot assay. A total of 10µL bacterial culture was spotted on the medium. Petriplates were incubated at 37 ºC for 24 h. The potential to solubilize insoluble phosphates on the modified Sperber's medium was determined by measuring clear zone around the colony. The colony and zone diameter was measured. The solubilization efficiency (SE) is calculated by formula given below.

$$SE = \frac{\text{solubilization diameter (s)}}{\text{growth diameter (g)}} X100$$

Determination of MIC (Minimum Inhibitory Concentration) *:* Minimum inhibitory concentrations (MICs) of the metals were determined by the plate-dilution and broth method. The metal salts used were zinc sulphate [$ZnSO_4.7H_2O$], lead nitrate [$Pb(NO_3)_2$] and potassium dichromate [$K_2Cr_2O_7$]. Heavy metal tolerant isolate *Pseudomonas aeruginosa* HMR16 was inoculated aseptically on Nutrient agar plates, supplemented with different concentrations (mM) of the following heavy metals: Zn (0.0- 16), Pb (0.0-1.5) and Cr (0.0-

0.35). The plates were incubated at 37 ºC for 48 h. The concentration of metal where there was no growth observed is considered as the MIC of the metal against the strain tested. The broth method was accomplished using tubes of nutrient broth spiked with different concentrations of filter-sterilized divalent metal ions. Bacterial culture was pre-incubated in 100 mL metal-deficient nutrient broth to a mid-log phase and 0.1 mL sample was transferred into 10 mL nutrient broth containing metals in test tubes. Positive controls consisted of metal-deficient medium inoculated with bacterial cultures. The tubes were incubated on rotary shake incubator at 37°C for 48 h. The concentration of heavy metals at which no turbidity was observed by spectrophotometer at 620nm was considered as the MIC of bacterial isolates against heavy metals.

Results and Discussion

Pseudomonas is a well-known beneficial plant growth promoting bacteria. Under unfavourable conditions, rhizospheric bacteria may enhance the plant growth by optimizing the supply of nutrients, stimulating plant growth by the synthesis of phytohormones IAA, solubilization of inorganic phosphorus and inhibiting the activity of pathogens (9). Previously characterized *Pseudomonad* strains namely *Pseudomonas aeruginosa* HMR16 was screened for their plant growth promoting activity such as IAA, ammonia, HCN, siderophore, production and P-solubilization. Effect of heavy metals such as zinc, lead, chromium on growth of *P. aeruginosa* HMR16 was also studied. Among PGP activites, the phytohormone auxin (IAA) release by PGPR has various direct effects on plant growth under stress conditions (10). IAA is one of the most important phytohormone and function as important signal molecule in the regulation of plant development. IAA is a common product of L-tryptophan metabolism by various PGPR strains. Our results suggest that *Pseudomonas aeruginosa* HMR16 strain produce IAA. For estimation of IAA production, development of pink color in the tube after addition of orthophosphoric acid and Salkowski reagent to supernatant of *Pseudomonas* culture in LB broth supplemented with and without L-tryptophan confirmed that *Pseudomonas aeruginosa* HMR16 strain produce indole acetic acid (Fig 1a).Colorimetric estimation of IAA by *P. aeruginosa* HMR16 showed 1.50 μg/mL and 6.832 μg/mL IAA production in absence and presence of L-tryptophan respectively. These results were found to be much better than that of Karnwal (11) where *P.aeruginosa* AK2 produced 0.8 μg/mL and 3.9 μg/mL IAA in absence and presence of 100 μg/mL L-tryptophan respectively. Our findings of IAA in *Pseudomonas* isolate is in agreement with other worker where in the presence of 100 μg/mL L-tryptophan, *Pseudomonas* spp. produced IAA in the range of 6.00-7.93 μg/mL (12). Ammonia production by rhizobacteria also plays an important role in biocontrol activity of PGPRs. For estimation of ammonia, the development of faint yellow to dark brown color after addition of Nessler's reagent to overnight grown bacterial culture in peptone broth indicated the production of ammonia by *P. aeruginosa* HMR16 (Fig 1b). These results are in close agreement with those of Chacko et al., (13) who isolated the *Pseudomonas putida* from the rhizosphere. This bacterium shows different PGPR characteristics and was also found positive for the production of ammonia. HCN production by rhizobacteria has been postulated to play an important role in the biological control of pathogens (14). Genus *Pseudomonas* is one of the leading bacteria which inhibit growth of pathogenic fungus in agriculture fields. For estimation of HCN production, the changes in color of the filter paper from yellow (0.5% picric acid in 1% Na_2CO_3) to brown after 24 h incubation showed that *P. aeruginosa* HMR16 strain produce HCN (Fig 1c) which acts as an inducer of plant resistance. Lanteigne *et al.,* (15) isolated HCN producing *Pseudomonas* and observed biological control activity of *Pseudomonas.* Bakker and Schippers (16) also observed that nearly 50% of the pseudomonads from potato and wheat rhizospheres produce HCN which has a primary mechanism in suppression of root fungal pathogens. Siderophores are known to chelates

with iron and other metals and contribute to disease resistance by limiting the supply of essential trace minerals in natural habitats. Siderophore producing PGPR help to prevent plants from becoming chlorotic due to the availability of Fe to plants when they were grown in heavy metal polluted soil (3, 17). For estimation of siderophore production, formation of yellow-orange halo around the colony on CAS agar medium showed that *P. aeruginosa* HMR16 produce siderophore (Fig 1d). These findings are in close corroboration with those of Islam et al. (18) which shows that the highest orange halo zone was produced by *P. aeruginosa* bacterial isolate. Phosphate solubilising microorganisms solubilize insoluble phosphates mainly by secreting acids (19) which can be observed as a solubilization zone on the medium. Efficiency of phosphate solubilization by *P. aeruginosa* HMR16 was determined. The *Pseudomonas aeruginosa* HMR16 was found to be potent phosphate solubilizers showing clear halo zone around their colonies. The zone size was gradually increased when incubation was extended beyond 24 h. These results are similar to Kumar et al., (20) who isolated phosphate solubilising *Pseudomonas* sp and showed highest phosphate solubilization zone (20 mm) in PVK agar. The Solubilization efficiency of 115.38 and maximum zone size of 1.5 cm was recorded after incubation period of 24 h (Fig 1e). Reena et al., (21) found the phosphate-solubilizing capacity of *P. aeruginosa* as 126.11 on 6^{th} day which is comparatively low than that of our present study. Genus *Pseudomonas* is well-studied and is of great interest because of their high resistance to heavy metals and other toxic substances. In the present study efforts were made to determine the minimum inhibitory concentration (MIC) of zinc, lead, chromium for this bacterial isolate. The present results showed *P. aeruginosa* HMR16 to be tolerant to all the three heavy metals tested. Fairly high tolerance was observed towards elevated concentration of zinc followed by lead and chromium. On determining MIC by agar dilution method, the well defined colonies were observed after 48 h of incubation in the medium up to 14mM concentration of zinc, 1mM concentration of lead and 0.25 mM concentration of chromium. *Pseudomonas aeruginosa* HMR16 didn't show any growth on high concentration of zinc, lead and chromium i.e.16mM, 1.25mM and 0.30mM respectively (Table 1). On determining MIC by broth method, the bacterial culture was grown in nutrient broth supplemented with different concentrations of divalent metal ions. Growth in terms of turbidity was observed at all concentrations up to 10 mM concentration of $ZnSO_4.7H_2O$, 0.75 mM concentration of $Pb(NO_3)_2$ and 0.2mM concentration of $K_2Cr_2O_7$. However, growth was inhibited at 11 mM, 1.0 mM and 0.25mM concentration of zinc, lead and chromium respectively (Fig. 2, 3 and 4). This indicates that zinc is less toxic than lead and chromium to the *P. aeruginosa* HMR16. In the present study, *Pseudomonas aeruginosa* HMR16 showed high MIC of 16mM for zinc ions. This range of MIC is quite higher to *Pseudomonas aeruginosa* isolated from polluted sites in Assiut city, Egypt for which MIC of 9.2mM was observed (22). A maximum MTC of 5 mM for Zn was observed for *Pseudomonas* (23) which is low as compared to the MIC of Zn for *Pseudomonas aeruginosa* HMR16 in our study. *Pseudomonas aeruginosa* HMR16 shows MIC of 1mM for lead ions which is comparable with that of *Pseudomonas aeruginosa* AD4 isolated by Durve et al., (24). Sheng et al., (25) isolated *Pseudomonas* sp. from heavy metal contaminated site and showed MIC for zinc and lead as 0.34mM and 0.60mM respectively. These MIC values for Zn and Pb are low as compared to the MIC of Zn and Pb for *Pseudomonas aeruginosa* HMR16 in the present study. *Pseudomonas aeruginosa* HMR16 shows MIC of 0.25 mM for Cr ions. Our results are comparable with previous study by Singh et al., (26). It was observed in this study that high MIC value for heavy metals was obtained on agar medium as compared to broth medium. Hassen et al., (22) also tested the levels of tolerance of environmental bacteria to the different divalent metal ions including Zn^{2+} in nutrient broth and reported that the test in liquid media was sensitive

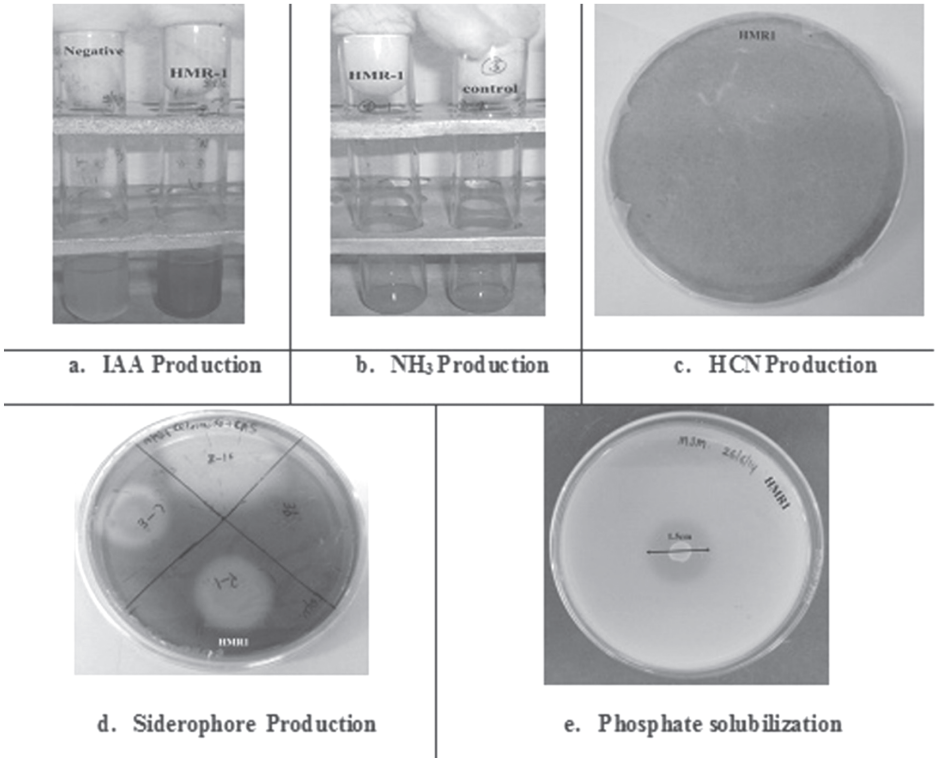

Fig. 1. Various Plant growth promoting activity of *Pseudomonas aeruginosa* HMR16

Table 1. Heavy metal tolerance of *Pseudomonas aeruginosa* HMR16.

S.No	Zinc ions concentration (mM)	*P. aeruginosa* HMR16	Lead ions concentration (mM)	*P. aeruginosa* concentration HMR16	Chromium ions (mM)	*P. aeruginosa* HMR16
1.	0	+++	0	+++	0	+++
2.	2.0	++	0.25	++	0.10	++
3.	4.0	+	0.5	+	0.15	+
4.	6.0	+	0.75	+	0.20	+
5.	8.0	+	1.0	+	0.25	+
6.	10.0	+	1.25	-	0.30	-
7.	12.0	+	1.5	-	0.35	-
8.	14.0	+				
9.	16.0	-				
+++ luxurious growth, ++ good growth, + less growth, - No growth						

Study of Potential Plant Growth-Promoting Activities

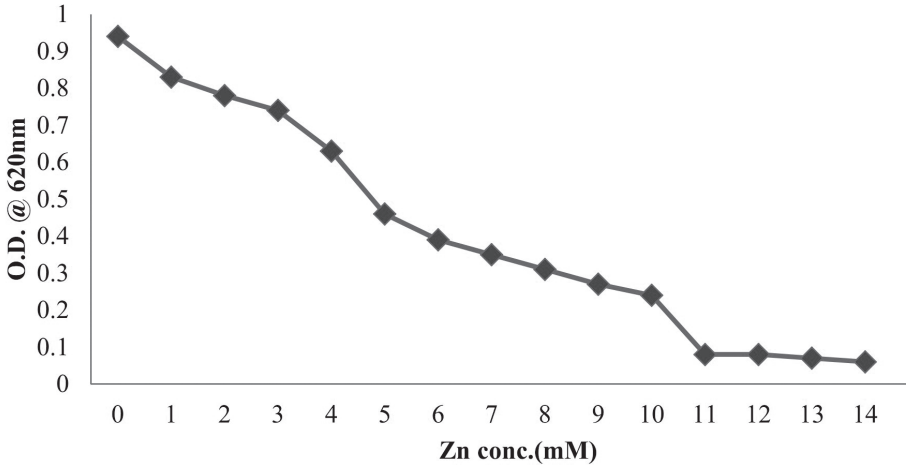

Fig. 2. Minimum inhibitory concentration of zinc for the strain *Pseudomonas aeruginosa* HMR16

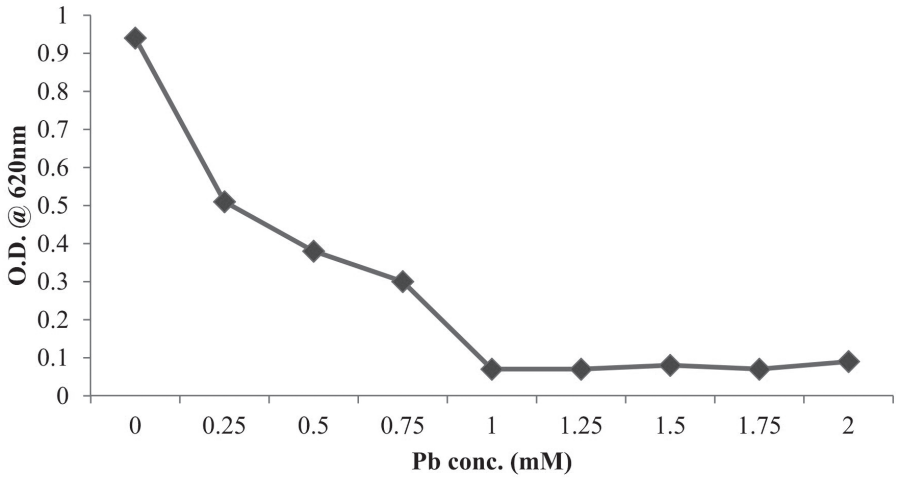

Fig. 3. Minimum inhibitory concentration of lead for the strain *Pseudomonas aeruginosa* HMR16

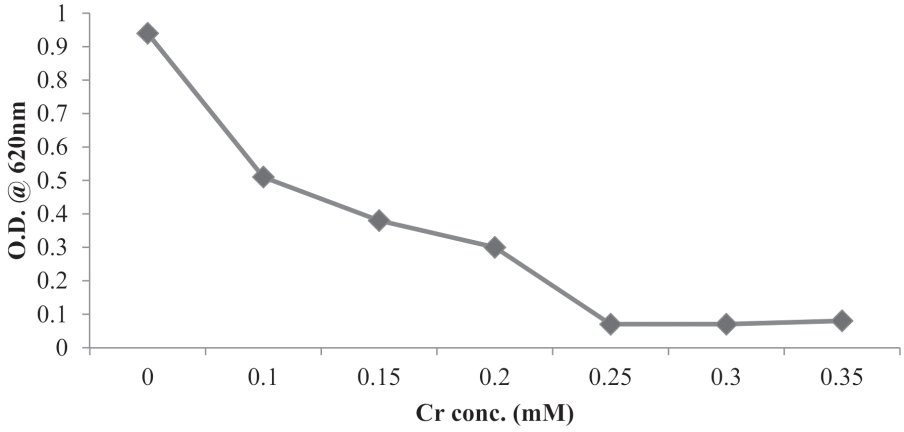

Fig. 4. Minimum inhibitory concentration of chromium for the strain *Pseudomonas aeruginosa* HMR16

at concentrations 10 to 1000 times lower than those obtained in solid media.

Conclusions

This study showed *Pseudomonas aeruginosa* HMR16 with multiple PGP activities IAA, HCN, siderophore, ammonia production and P-solubilization and tolerance to various heavy metals. Thus metal tolerant *Pseudomonas aeruginosa* HMR16 can be further explored for its application in promoting plant growth in heavy metal contaminated soil.

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Evaluating the Antiviral Activities of Some Bacteria Against Bovine Viral Diarrhea Virus (BVDV)

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Abstract

Viral-induced infectious diseases represent one of the major health threats and their control remains an unachieved goal. Present research aimed is to find antiviral substance derived from bacteria against Bovine Viral Diarrhea Virus (BVDV) which is used as a model in culture for HCV. Thirteen bacterial isolates were selected, evaluated and ability to produce antiviral activity against BVDV using Madin Darby Bovine Kidney cells (MDBK) as cell culture system. Among these isolates, eight were able to inhibit the activity of BVDV. These isolates were 2, 4, 7, 8, 10, 11, 12 and 13 appear to be highly inhibitive effects against BVDV virus type and among these, five isolates (2, 4, 7, 8, 10) were isolated and identified as *Bacillus cereus strain LJ01*, *Bacillus cereus strain FT9, Pseudomonas putida strain sda2*, *Bacillus thuringiensis Bt407, Xanthomasfuscans* based on 16S rDNA analysis from highly saline sea water. *Pseudomonas oleovorans*, *Pseudomonas* sp. and *Pseudomonas fluorescence* appears to be highly inhibitive against BVDV virus type. *Pseudomonas oleovorans*was selected to be subject to gradient extraction using ethyl acetate. Extract fractionation is done using chloroform 90%/10% methanol with increasing percentage of methanol till 100% by silica gel column chromatography. The antiviral fraction was subjected to GC/Mass for identification through matching compounds on database. The fraction containing compounds identified as saturated fatty acid esters called tetradecanoic acid and hexadecanoic acid methyl esters. Finally it can concluded that saturated fatty acids are considered to be an important antiviral against different viruses and it is further supported by 80% inhibition in BVDV plaque reduction assay.

Key Words - Antiviral, bacterial antiviral activity, bovine viral diarrhea virus, Madin Darby Bovine Kidney cells, plaque reduction assay.

Introduction

Viral infections are the causative agent of many human and animal diseases that have massive economic effects. Recent year's development of novel drugs candidates in accordance with the rise of new viral types and because of decreased availability of antiviral measures, in addition to the occurrence of new virus types and drug-resistance viral strains and have led scientists to research development on novel drug candidates (1).

The past years have seen extensive progress in the understanding of the biochemical mechanisms that involve the replication of different members of the Flaviviridae virus family such as the Flaviviruses kunjin west Nile, the Pestivirus Bovine viral diarrhea virus (BVDV), and hepatitis C virus (HCV) (2). The characterization

of the HCV molecular determinants in the replication process is particularly important because HCV is the major causative agent of liver disease in man (3). HCV and BVDV showed high degree of homology in terms of their genomic organization, strategies in protein expression, genome replication, and viral envelopment (2). Since studies on HCV are short listed because of the low rate of viral replication and the lack of efficient infectious cell culture systems, BVDV is applied as a model system for the life cycle of HCV to study the activity of antiviral drugs during an infection (4, 5).

The conventional approach to the control of viral diseases is to develop effective vaccines against the infectious viruses; however this is not always feasible. Because of their differences in the mode of replication, viruses present a larger therapeutic challenge than do bacteria (6). Also, the genetic flexibility of viruses causes new strains to emerge and the well-known example is bovine virus diarrhea virus (BVDV), the causative agent of BVDV. As of today, there are no vaccines to treat BVDV virus.

The use of biological methods to eliminate viral disease is required not only due to its wide availability but also to avoid the excessive use of chemotherapy, which can cause different side effects and immunological disorders and to avoid this side effect there is a need to produce novel drugs. Peptides have a wide variety of important biological activities including antifungal, antibacterial, antiparasitic, antiviral and antitumoural activities (7,8). Lipopeptides, produced by various strains of *Bacillus subtilis* are either cyclopeptides such as iturin or macrolactones such as surfactin and plipastatin (9). In general, lipopeptides exhibit significant inhibitory activity against phytopathogenic fungi, bacteria, virus (10, 11, 12). Compared with chemical agents, lipo-peptides are safer for the environment because they are produced from *Bacillus subtilis*. Therefore, they are likely to be widely used in medicine, agriculture and the food industry (13). In the course of screening for an extracellular polysaccharide producing bacteria from marine environment, *Pseudomonas* WAK-1 strain was found to produce extracellular glycosaminoglycan and sulfated polysaccharide (14,15).

Antiviral activities were found to be associated with lipid fractions of human milk samples due to the presence of unsaturated fatty acids and monoglycerides (16). On the other hand, Two compounds 2-hydroxymyristic acid (2-hydroxytetradecanoic acid) and 2-hydroxypalmitic acid (2-hydroxyhexadecanoic acid) were effective for inhibition of varicella-zoster virus (human herpesvirus 3, VZV) (17). Later, it was found that Lipholic extracts from different edible plants were considered to be antiviraly active againts herpes simplex virus (HSV-1) and parainfluenza (PI-3) detection of compounds by GC/Mass showed the presence of palmitic, myristic and linoleic and linolenic acids (18).

In this study the antiviral activities of different bacterial isolates was evaluated to subject some for further studies involving identification of the most active compounds responsible for the inhibition. *Pseudomonas oleovorans* was selected for studying the extract GC/MS scanning and evaluate its antiviral activity during extraction procedures.

Materials and Methods

Chemicals: Casamino acids (Difco), bacteriological peptone (LabM), yeast extract powder (LabM), beef extract powder (Sigma), sea plaque agarose (Lonza),agar agarbact (for bacteriology), methylene blue (Sigma), KCl, $MgSO_4.7H_2O$, $CaCl_2$ (anhydrous), NaCl. Unless otherwise mentioned, all chemicals used in this study were purchased from El-Gomhoria Company, Cairo, Egypt.

***Virus and cell culture*:** Madin Darby Bovine Kidney cells (MDBK) were used for propagation of Bovine Viral Diarrhea Virus (BVDV) for the plaque assay. Cells were grown at 37°C in 25ml culture flask containing DMEM composed of Minimum Essential Medium,10% fetal bovine serum, 1% penicillin-streptomyces and 0.1%

fungizone (amphotericinB). Cells were incubated 3days at 37°C in 5% CO_2incubator. After incubation cells were seeded into six or 12-well cell culture plates and allowed to grow to confluence. The cells were washed in phosphate buffered saline (PBS) then subjected to plaque assay.

***Maintains of bacteria*:** A total of 13 bacterial isolates, 10 of them (1-10) were isolated from highly saline sea water in Egypt, isolate number 11 (*Pseudomonas oleovorans)* was kindly obtained from the Centre of Culture of Microbiological Food Irradiation Department, National Centre for Radiation Research and Technology, Nasr City, Egypt. In addition, isolate number 12 (*Pseudomonas sp.)* was kindly obtained from the culture collection Chemistry of Natural and Microbial Products Department of National Research Centre. Also, isolate number 13 (*Pseudomonas fluorescence)* was kindly obtained from Navy American Medical Research Unit III, Cairo, Egypt. Bacterial isolates were maintained on slants nutrient agar slants containing (g/l): peptone; 5, beef extract; 3,NaCl 5 and agar 15. The pH was adjusted at 7. The cultures were incubated at 37°C for 24 hours (19). Then stored at 4°C and sub-cultured monthly. For long term storage, bacterial isolates were grown into nutrient broth medium at 30°C then centrifuged at 10,000 r.p.m. for 10 min. The pellets were washed twice with sterile saline solution (0.85%) then re-suspended in Protect Vials (Technical Service Consultants Ltd., UK) containing 10% sterile glycerol and stored at -20°C.

***Enrichment medium for bacterial growth** (20):* Bacteria were cultivated on nutritive medium containing (g/l): Glucose; 10, peptone; 10, beef extract; 1.5 and yeast extract; 3, pH was adjusted at 7.

***Production medium** (21):* Bacterial inoculums were inoculated on 50ml CYG (Casamino acids Yeat extract Glucose) broth medium in a 250 ml-Erlenmeyer flask.CYG broth medium composed from: (Casamino acids, 5 g; yeast extract, 0.5 g; glucose, 1 g; NaCl, 6.8 g; KCl, 0.4 g; $MgSO_4.7H_2O$, 0.2 g; $CaCl_2$ (anhydrous), 0.2 g; 1,000 ml of distilled water; pH 7.2). To prepare samples for plaque reduction assay flasks containing 50ml of CYG production medium were inoculated with (2ml) prepared inoculum from enrichment medium the flasks were agitated on rotator shaker at 30°C for 48 hours at 150 r.p.m. (22,23).

***Plaque Reduction Assay** (24):* The contents of each flask were centrifuged at 4000 r.p.m. at 4°C for 10 min. 0.2 ml of each bacterial culture suspension was filtered through 0.22µm membrane filter and mixed with equal volume of BVDV suspension containing approximately $59\times10^{-4.6}$PFU/0.2 ml. The mixtures were incubated at 37°C on shaking for 90 min. Mixtures were added on cells were grown on 6 well-plate and incubated for 90 min at 37°C allowing the mixture to adsorb on cells. In case of agarose overlay, the overlay was added in drops to cells. Plates were incubated for 3 days at 37°C in 5% CO_2. Then, the cells were fixed with 10% formalin and stained with 0.3% methylene blue. Plaques were counted and the percentage inhibition of plaque formation was estimated by comparing mean plaque numbers in tested wells with the mean plaque number in the control wells containing no tested compound.

***Determination of proteins** (25):* Sample solution containing 20µg of protein in a volume up to 0.1 ml was pipette into a test tube. The volume in the test tube was adjusted to 0.1 ml with distilled water. One milliliter of protein reagent was added to the test tube and the contents were mixed by vortex. Absorbance at 595 nm was measured after 2 min and before 1 hour in 1ml cuvette against a reagent blank prepared from 0.1ml of the distilled water and 1ml of protein reagent. A known volume of bovine serum albumin solution with a known concentration of protein was plotted against the corresponding absorbance. The resulting standard curve was used to determine the concentration of protein in the unknown samples.

Determination of carbohydrates *(26):* The basic principle of this method is that carbohydrates becoming dehydrated during the reaction with concentrated sulfuric acid resulted in furfural derivatives. The followed reaction between furfural derivatives and phenol leads to the development of detectible color. The standard procedure of this method is started with the addition of a 1 ml aliquot of a carbohydrate solution to 1 ml of 5% aqueous solution of phenol in a test tube and mixed together. Subsequently, the addition of 5 ml of concentrated sulfuric acid is performed rapidly to the mixture. The test tubes were allowed to stand for 10 min., and then they are vortex for 30 seconds and placed for 20 min. in a water bath at room temperature for color development. Then, light absorption at 490 nm is recorded on a spectrophotometer. Reference solutions are prepared in identical manner as above, except that the distilled water is used in replacement of 1 ml aliquot of carbohydrate.

PCR 16S rDNA and electrophoresis *(27):* Genomic DNA of the bacterial isolates (isolates number 2,4,7,8 and 10) were extracted using GeneJET Genomic DNA purification kit (Thermo scientific # k0721). The 16S rRNA fragments were partially amplified by polymerase chain reaction (PCR). A region of approximately 500 bp from the 16S rRNA gene was amplified using the universal primers: forward primer 5'-AGGAGGTGATCCAACCGCA-3' (location 1522-1540) and the reverse primer 5'- AACTGGA GGAAGGTGGGGAT-3' (location 1170-1189). Two μl of the purified were amplified in a 25 μl reaction mixture by using PCR-EZ D-PCR Master Mix PCR Master Mix (BIO BASIC INC.). The PCR was performed by using Veriti 96-Well Thermal Cycler (Applied Biosystems Catalog Number 4479071). The PCR program consisted of one cycle of DNA denaturation at 95°C (5 min.), 40 cycles of 95°C (45 sec.), 58°C (45 sec.) and 72°C (1min.), plus one additional cycle of a final chain elongation at 72°C (10 min). The PCR products were visualized using agarose gel electrophoresis and compared with 100 pb DNA Ladder (100bp – 3000 bp) (Thermo Scientific). The PCR product was purified using QIAquick PCR purification kit, then subjected to cycle sequencing purification using Centri-sep kit according to the manufacture instructions.

DNA sequencing *(27):* PCR products were purified by Qiagen extraction kit according to the manufacturer instructions before applying to DNA sequencer. Sequencing was performed by automated florescent dye terminator sequencing method originally developed by using (DYEnamic ET Terminator Cycle Sequencing Kit, Amersham Pharmacia Biotech. ABI 3130) at faculty of agriculture Cairo university research area, Egypt.

Extraction of bioactive compounds (28): The most potent bacteria *Pseudomonas oleovorans* was inoculated into four liters of CYG production medium incubated for 24hrs at 30°C under shaking conditions and centrifuged at 4000 rpm. at 4°C for 10 min . The filtrates were extracted three times by ethyl acetate. The extracts were evaporated until they were completely dry using a rotary evaporator with heated waterbath up to 40°C. The crude extract was weighed and tested for antiviral activity.

Cytotoxicity assays (29): Different concentrations of the extract sample were placed in contact with MDBK cells monolayer cultured 24hours then incubated at 30°C for 3days. After incubation period the viable cells were evaluated as non-toxic at the present concentration comparing with non-treated cells.

Antiviral activity of Pseudomonas oleovorans extract: Madin Darby Bovine Kidney (MDBK) cells were inoculated with a mixture of equal volumes of BVDV and a nontoxic concentration after incubation of the mixture for 2hrs at shaker incubator 37°C. The mixture was inoculated into cells and incubated for 2hrs at 37°C in 5% CO_2. After incubation the previously mentioned steps for plaque reduction assay were performed except staining using (10%) crystal violet.

Chromatographic fractionation: The ethyl acetate extract was subjected to further extraction by using equal volumes of methanol

and n-hexane. The extracted n-hexane part was subjected to GC/Mass for identification through matching compounds on database. The methanol dried extract was chromatographed on silica gel 60 column with step gradient elution using chloroform 90%/10% methanol with increasing percentage of methanol till 100% (28). Fractions (25 ml) were collected while the fractionation processes were monitoring by TLC analysis. The plates were examined under UV (short, long wave length)and *P*-anisaldehyde/ H2SO4 spray reagent. Methanol fraction containing desired antiviral substance was also subjected to plaque reduction assay and GC/ Mass for identification through matching compounds on database.

***Gas Chromatography/ Mass Spectrometry analyses*:** Agilent 6890 gas chromatograph equipped with an agilent mass spectrometric detector with a direct capillary interface and fused silica capillary column PAS-ms (30m×0.25mm I.D.×0.25um film thickness). Samples were injected under the following conditions:

Helium was used as carrier gas at approximately 1ml/min.pulsedsplitless mode. The solvent delay was 3 min. and the injection size was 1.0 µl. the mass spectrophotometric detector was operated in electron impact ionization mode an ioning energy of 70e.v. scanning from m/z 50 to 550. The ion source temperature was 250°C and the quadruple temperature was 150°C. The electron multiplier voltage (EM voltage) was maintained 1550v above auto tune. The instrument was manually tuned using perfluorotributyl amine (PFTBA). The GC temperature program was started at 60°C then elevated to 280°C at rate of 8°C/ min. and 15 min. holding at 280°C the injector temperature was set at 280°C. Wiley mass spectral data base was used in the identification of the separated peaks.

Results and Discussion

***Antiviral screening of the isolated bacteria*:** In this study *in vitro* assays were established and employed to screen thirteen bacterial extracts for their antiviral activity against BVDV. Five marine isolates, number 2, 4, 7, 8 and 10 represents a resource for antiviral drugs. These bacteria exhibited 100% antiviral activities. Also, isolate number 11 and isolate number 12 both show 100% antiviral activities while isolate number 13 showed 97% antiviral activity. Isolates number 5, 6 and 9 showing a plaque reduction 42%, 44% and 39% antiviral activities respectively. On the other hand, isolates number 1 and 3 both showed no antiviral activities against BVDV (results are shown in fig. 1 and 2). These results explain that each bacterial isolates leads to different antiviral substance that may differ in characteristics and mechanism than the other one. So that Future studies can be focused on the isolation of the active compounds contained in this culture filtrates.It is also possible that any of the observed antiviral effects resulted from interaction between compounds found within the same extract. On the other hand, fractionation and isolation could have leaded to the result of opposite effect of the elimination of any antiviral potential (1).

Genotypic characterization of the most promising unknown bacterial isolates showing the maximum antiviral activity against BVDV: Bacterial isolates number (2), (4), (7), (8) and(10) showing the maximum antiviral activity against BVDV was identified by16S rDNA. The 16S rDNA region was amplified by polymerase chain reaction (PCR) (about 500 bp) using universal primers. The molecular sizes of amplified regions were confirmed using agarose gel electrophoresis as shown in fig.3. According to sequencing similarities and multiple alignment, the isolate number (2) was found to be closely related to *Bacillus cereus strain LJ01* with 99% identity, isolate number(4) was found to be closely related to *Bacillus cereus strain FT9* with 96% identity, isolate number(7) was found to be closely related to *Pseudomonas putida strain Sda2*with 100% identity , isolate number 8 was found to be closely related to *Bacillus thuringiensisBt407* with 97% identity and finally isolate number (10) was found to be closely related to *Xanthomas fuscans* with 98% identity (Fig. 4).

***Antiviral activity of Pseudomonas oleovorans extract*:** Prior to plaque reduction assay, cytoxicity test was performed to determine the safe dose of the extract to be applied in test cell culture. The tested extract showed that concentrated samples (1mg/ml) were considered to be highly toxic to cells as it caused complete loss of cells monolayer. So the nontoxic concentration (0.02mg/ml) showing no physical signs of toxicity, e.g. partial loss of monolayer, rounding, shrinkage, or cell granulation is safe for use in plaque reduction assay to evaluate the extract antiviral activity against BVDV.

Pseudomonas oleovorans extract was evaluated for in-vitro antiviral activity against BVDV. The results in fig. 5 show that mixing the virus with a nontoxic concentration of *Ps. ol.* extract (0.02 mg/ml) prior to application on MDBK cell culture system causing reduction in the plaque formed by BVDV (81%). The antiviral activity of the fraction derived from the extract of *Ps. ol.* against BVDV (80%) increases the possibilities of drugs treatment which increasing the demand for new medicines (30). To our knowledge this is the first report on the antiviral activity against BVDV by *Ps. ol.* extract. On the other hand, when fatty acids were used as antiviral by as much as 20-fold concentration were required for maximum viral inactivation and considered highly active against the enveloped viruses (16). Today, over 40 compounds are commercially available in pharmacological markets including alternative antiviral medicines. The effectiveness of different antiviral extracts is characterized by their ability to occupy or denaturant the virus proteins that are present on the virus surface leading to the loss of the virus interaction with the host cells (30).

Fractionation and identification of the active compounds: The separation of the ethyl acetate crude extract leads to two layers methanol (90%) and n-hexane. The dried layer of n-hexane was subjected to GC/MS scanning leads to identification of about 20 compounds listed in table 1 when matching to database library (Fig. 6). GC/MS of the fraction containing the desired compounds was carried out resulting in identification of 18 compounds (Fig. 7 and Table 2). The compounds were identified by comparing the MS/MS fragmentation pattern with the library database. GC/MS analysis of the fraction showed the presence of tetradecanoic acid 12-methylester and hexadecanoic acid methyl ester which are the methyl esters of both tetradecanoic acid and hexadecanoicacid both acids were considered to have wide antiviral activities against different viruses. Tetradecanoic acid, also called myristic acid, is a common saturated fatty acid with the molecular formula $CH_3(CH_2)_{12}$ COOH. Palmitic acid, or hexadecanoic acid in IUPAC nomenclature, is the most common fatty acid (saturated) found in animals, plants and microorganisms. Its chemical formula is $CH_3(CH_2)_{14}COOH$. According to the available literature both them showed wide antiviral activities among a lot of viruses. The effect of fatty acids on virus particles was studied against enveloped viruses, i.e., vesicular stomatitis virus, herpes simplex virus and against a non-enveloped virus, poliovirus.Also, another two compounds considered the analogues of myristic acid called 2-hydroxytetradecanoic acid and 2-hydroxytetrapalmitic acid caused inhibition in the replication of *Varicella-zoster virus* (VZV, human herpes virus) (17).

Conclusion

Finally study concluded that seven bacterial isolates screened for their ability to produce antiviral compounds showed higher antiviral activity against BVDV. The unidentified isolates (2,4,7,8 and 10) were further identified using 16S rDNA. This study revealed the potentiality of the metabolites produced by *Pseudomonas oleovorans* against BVDV. The safe antiviral concentration of the total extract was 0.02 mg/ml. Bioassay guided fractionation of the total extract revealed that the activity was resided in the fraction contains myristic acid and palmitic acid methyl esters. The fatty acids methyl esters identified in this work could be used as new antiviral compounds from natural resources.

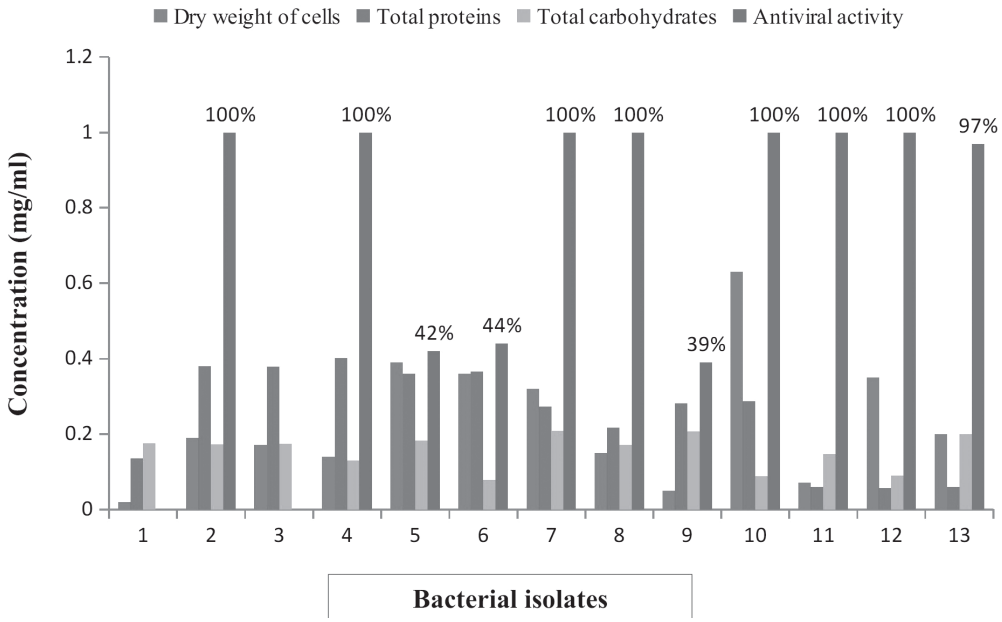

Fig. 1. Screening of bacterial antiviral activity against BVDV considering filtrate's both protein and carbohydrates contents. Bacterial cultures incubated 48 hours at 30°C on rotatory shaker at 150 r.p.m. Then 0.2 ml of each culture filtrate was evaluated for their antiviral activities against BVDV using plaque reduction assay. Both protein and carbohydrates contents were measured spectorphotometrically for each bacterial isolates. Also, cell dry weights were calculated for each isolate.

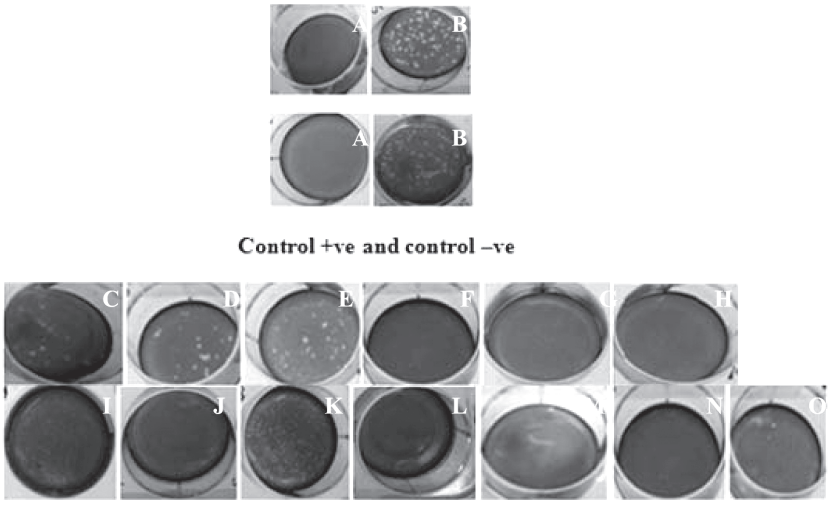

Fig. 2. Plaque reduction assay for bacteria appears in wells A, B, C, D, E, F, G, H, I, J, K, L, M, N and O for control +ve ,-ve, isolates number 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12 and 13 respectively .
Mixtures of 0.2ml of bacterial culture suspension and equal volume of BVDV suspension were added on cells were grown on 6 well-plate and incubated for 90 min at 37°C. Then, the cells were fixed with formalin and stained with methylene blue to reveal virus plaques on the cells.

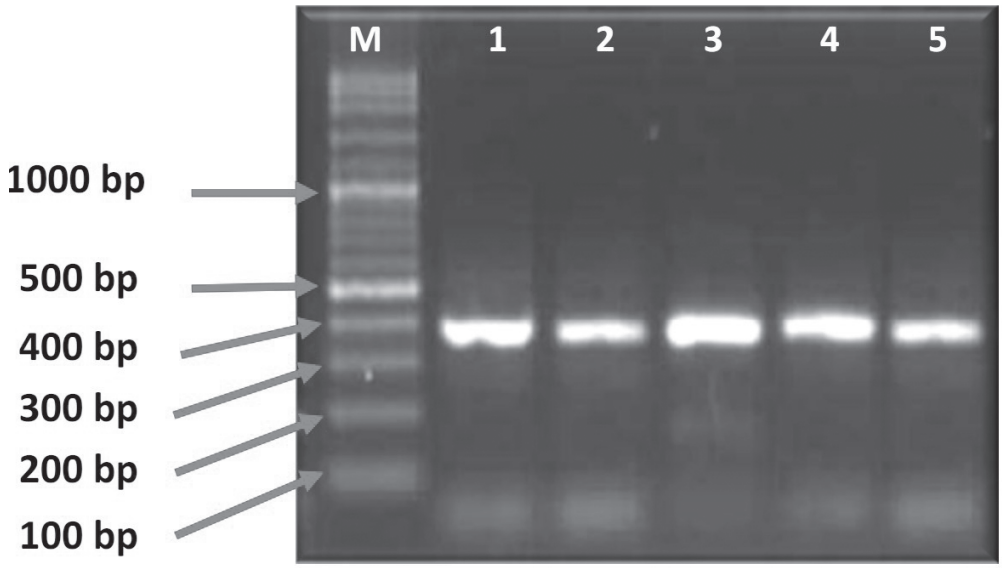

Fig. 3. Electrophoresis of PCR products for 16S rDNA of selected isolates.
Lane 1: M (molecular weight marker 100 bp DNA ladder); lanes 1, 2, 3, 4 and 5 are PCR products of isolates 2, 4, 7, 8 and 10 respectively.

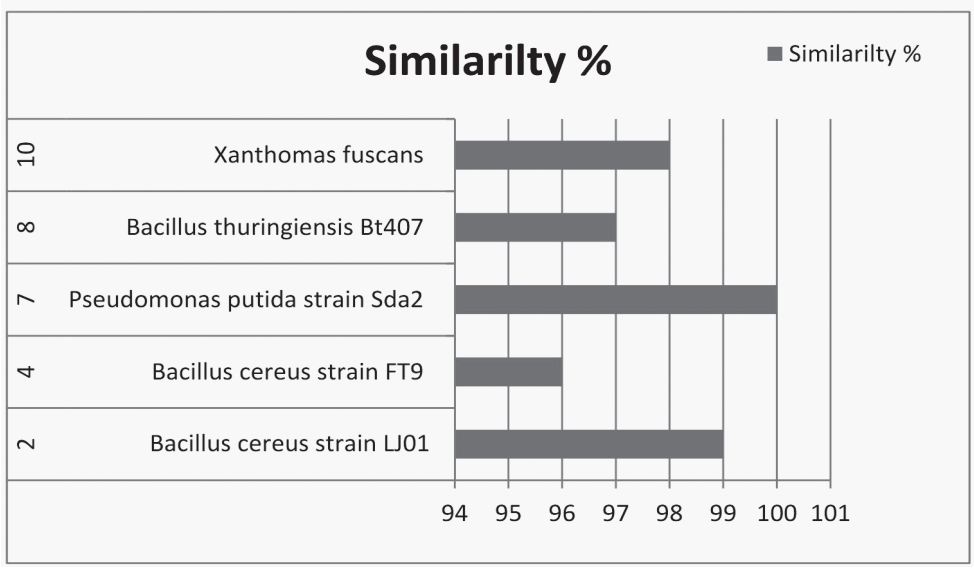

Fig. 4. Bacterial identification based on 16S rDNA sequencing alignment.

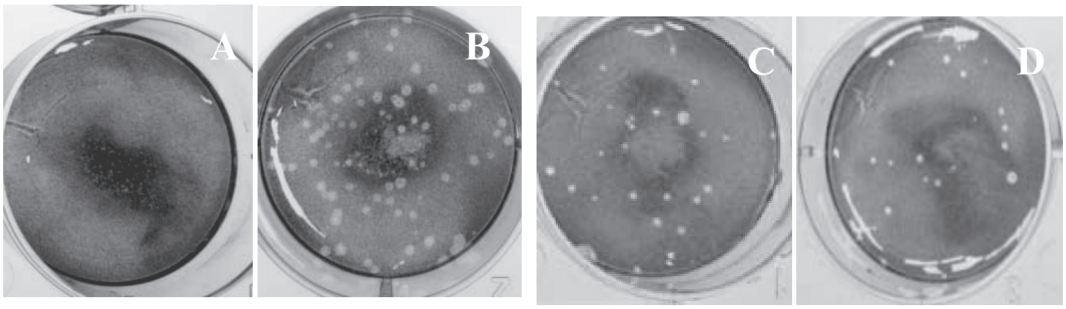

Fig. 5. Plaque reduction assay of Pseudomonas oleovorans extract.
Wells A, B, C and D represents control +ve , –ve, after extraction of ethyl acetate (81%) BVDV inhibition and after fractionation with chloroform: Methanol leading to inhibit BVDV with (80%).

Table 1. GC/MS analysis of n.hexane layer of Pseudomonas oleovorans extract.

No	Identified compound	R_t*	Molecular weight	Relative area %	Molecular formula
1	Propanoic acid 2-methyl hexyl ester	13.02	172.27	1.94	$C_{10}H_{20}O_2$
2	Butanoic acid, Butyl ester	13.40	144.21	2.96	$C_8H_{16}O_2$
3	Tetradecane	13.63	198.39	0.39	$C_{14}H_{30}$
4	1.Hexadecene	16.65	224.43	0.99	$C_{14}H_{30}$
5	1.Octadecene	19.45	252.48	0.29	$C_{18}H_{36}$
6	Hexadecanoic acid methyl ester	21.20	270.45	0.52	$C_{17}H_{34}O_2$
7	Eicosane	22.06	282.55	0.82	$C_{20}H_{42}$
8	Oleyl alcohol	22.29	268.48	0.31	$C_{18}H_{36}O$
9	9,12-Octadecadienoic acid	22.95	280.45	0.46	$C_{18}H_{32}O_2$
10	Hexatriacontane	23.25	509.97	0.15	$C_{36}H_{74}$
11	9-Eicosyne	23.71	278.52	0.32	$C_{20}H_{38}$
12	11-Dodecen-1-ol trifluoroacetate	23.95	280.33	0.24	$C_{14}H_{23}F_3O_2$
13	1,9-Tetradecadiene	24.20	194.36	0.28	$C_{14}H_{26}$
14	Docosane	24.39	310.60	1.31	$C_{22}H_{46}$
15	Tricosane	25.48	324.63	0.21	$C_{23}H_{48}$
16	Heneicosane	26.53	296.57	1.35	$C_{21}H_{44}$
17	Pentacosane	27.53	352.68	1.02	$C_{25}H_{52}$
18	2-Acetyl-N-methylaniline	28.71	149.19	1.05	$C_9H_{11}NO$
19	1,2-Benzendicarboxylicaciddinonyl ester	31.03	418.61	23.12	$C_{26}H_{42}O_4$

Retention time *

Table 2. GC/MS analysis of methanol fraction of Pseudomonas oleovorans extract.

No	Identified compound	R_t^*	Molecular weight	Relative area %	Molecular formula
1	2,4-Di-tret-butylphenol	13.518	206.32	0.90	$C_{14}H_{22}O$
2	Tetradecanoic acid 12-methylester	17.330	242.40	1.73	$C_{15}H_{30}O_2$
3	3-hydroxy-4-methoxybenzyl alcohol	17.993	154.16	19.45	$C_8H_{10}O_3$
4	Chloromesitylene	18.301	154.64	2.88	$C_9H_{11}Cl$
5	Hexadecanoic acid methyl ester	18.970	270.45	0.84	$C_{17}H_{34}O_2$
6	1,4-diaza-2,5-dioxo-3-isobutyl bicycle(4.3.0)nonane	19.210	210.00	19.49	$C_{11}H_{18}N_2O_2$
7	L-proline	19.616	115.13	4.22	$C_5H_9NO_2$
8	2,6-dimethyl-3,5 heptanedione	19.747	156.22	0.68	$C_9H_{16}O_2$
9	14 methylhexadecanoate	19.845	284.48	1.03	$C_{18}H_{36}O_2$
10	Phoratesulfoxide	19.947	260.31	3.96	$C_7H_{17}O_4PS_2$
11	N-Neopentyl-leucinol	20.736	329.48	0.99	$C_{18}H_{35}NO_4$
12	2,4,imidazolidinedione	22.039	100.08	2.20	$C_3H_4N_2O_2$
13	Benzyl (4□chlorophenyl) acetate	23.954	170.59	2.81	$C_8H_7ClO_2$
14	Cyclotetracosane	24.142	338.65	0.45	$C_{24}H_{50}$
15	3-benzyl-1,4-diaza-2,5,-dioxobicyclo(4.3.0)nonane	24.388	244.00	8.08	$C_{14}H_{16}N_2O_2$
16	Bis (2-ethylhexyl)phthalate	25.766	390.56	1.09	$C_{24}H_{38}O_4$
17	1-nonadecene	26.086	268.52	0.72	$C_{19}H_4O$
18	Tert-butyl ester of 3,4-dimethyl-5-(3,3-bis-methoxycarbonylmethyl-2-nitrobutyl	34.230	440.49	4.77	$C_{21}H_{32}N_2O_8$

Retention time *

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Genetic Architecture of Metric Traits in Bread Wheat (*Triticum aestivum L. EM. THELL)*

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Abstract

The objective of this study was to estimate gene effects and genetic variability for some quantitative traits of three bread wheat crosses (GW 395 × HI 1556, PHR 1012 ×GW 366 and GW 411 × VL920) by generation mean analysis. In most cases a digenic epistatic model was sufficient to explain variation in generation means. The additive-dominance model was adequate for number of effective tillers per plant, Grain yield per plant and days to 50 per cent heading. In some cases these models failed to explain variation in generation means, implying the presence of higher order interactions or interactions between linked loci. The traits viz., days to heading, days to maturity, plant height, grains per spike and 100-grain weight were controlled by both additive and non-additive type of gene actions. Whereas, the dominance together with epistatic gene effects was found to play major role in the expression of grain yield per spike. The adequacies of certain modes of inheritance as well as the importance and significance of gene effects and genetic components of variance for analyzed traits were dependent upon the particular crossing combination and experimental site.

Key words: bread wheat, gene effects, additive, non additive, dominance

Introduction

Wheat (*Triticum aestivum* L. em. Thell) is the most important cereal crop of the world. It is the main staple food of the rapidly increasing population of India that is why it occupies a prominent position in the cropping pattern of the country. Grain yield is a complex trait made up of the interaction between different yield components and environmental effects. Because of these complex interactions it is difficult to improve yield through breeding (especially in the early generations) if yield is the only factor recorded, suggesting that component traits should also be used as selection criteria for yield improvement. This is the reason why it is necessary to know the genetic architecture of yield components (6). Generation mean analyses provides information on the relative importance of average effects of the genes (additive effects), dominance deviations, and effects due to non allelic genic interactions, in determining genotypic values of the individuals and, consequently, mean genotypic values of families and generations (10). Generation mean analysis is a simple but useful technique for estimating gene effects for a polygenic trait, its greatest merit lying in the ability to estimate epistatic gene effects such as additive x additive (aa), dominance x dominance (dd) and additive x dominance (ad) effects (9). Besides gene effects, breeders would also like to know how much of the variation in a crop is genetic and to what extent this variation is heritable, because efficiency of selection mainly depends on additive genetic variance, influence of the environment and interaction between genotype and environment. The research reported in this

paper was carried out to provide information about gene effects and available genetic variability for the most important quantitative traits of wheat (*Triticum aestivum* L.) (2).

Material and Methods

The experimental materials used for the present study consisted of Six generations *viz.,* P_1, P_2, F_1, F_2, BC_1, and BC_2 generated from each of the three crosses namely, GW 395 × HI 1556, PHR 1012 ×GW 366 and GW 411 × VL920. Each of the crosses was grown in compact family block design with three replications. Each Net plot had one row for parents and F_1s, 2 rows for each of BC_1, and BC_2 and 4 rows of F_2 populations. Inter and intra row spacing was 22.5cm and 10 cm respectively with row length of 2.5m. The present investigation was carried out at the Centre of Excellence for Research on Wheat, SDAU, Vijapur during *rabi* crossing 2009-10, 2010- 11 and evaluation in 2011-12. Cultural practices recommended for the crop were followed uniformly. The data were recorded from 5 selected plants of parents and F_1, 40 F_2 plants and 20 BC_1 and BC_2 plants for following traits number of effective tillers per plant, days to 50 per cent heading, days to maturity, plant height, length of spike, spikelet per spike, grains per spike, grain yield per spike, grain yield per plant, 100-grain weight The data were analyzed by joint scaling test proposed by (1) to estimate genetic parameters by 3-parameter non-epistatic model (m, (d), and (h) and 6-parameter models assuming digenic epistatic interaction (m), (d), (h), (l), (j) and (l)

Results and Discussion

The cross GW 395 × HI 1556 showed significant differences for number of effective tillers per plant, days to heading, length of spike, spikelet per spike, grain yield per plant, 100-grain weight whereas cross PHR 1012 ×GW 366 also showed significant differences in generations for all the traits except days to maturity, spikelet per spike. All the traits except spikelet per spike and grain yield per plant showed significant differences in different generations. Only significant traits used for estimating other parameters.

A simple additive dominance model was adequate in GW 395 × HI 1556, PHR 1012 ×GW 366 for number of effective tillers per plant and grain yield per plant and GW 411 × VL920 for number of effective tillers per plant, days to heading therefore other traits shows inadequacy to the simple additive dominance model thereby indicating the involvement of more complex interactions or linkage Misra *et al*. (6) (Table 1). However, various gene effects were estimated on the basis of six parameter model.

Gene action studies (Table 1) revealed that an additive gene effect more pronounced for number of effective tillers per plant in all the three crosses and grain yield per plant in cross GW 395 × HI 1556, PHR 1012 ×GW 366. Additive gene effect was reported earlier also for effective tillers per plant (3) and grain yield per plant Sami *et al*. (7) and Zahid *et al*. (11). suggesting the potential for obtaining further improvement of these traits by selection practice of their progenies.

Among the digenic interactions, (Table 1) for days to heading revealed that significant additive (d), dominance (h) and additive × additive (i) played major role in the inheritance of this trait, however in crosses viz., GW 395 × HI 1556 and PHR 1012 ×GW 366, dominance × dominance (l) also observed, while additive × dominance was found to be significant in crosses GW 395 × HI 1556. The signs of (h) and (l) were indicating the presence of duplicate dominant epistasis in the expression of these traits, which would limit the range of variability in cross GW 411 × VL920 for days to maturity. . Since none of the signs of [h] were similar to the [l] type of epistasis, it was concluded that no complementary type of interaction was present in the genetic control of the studied traits. Plant height revealed that significant involvement of additive × additive (i) and dominance × dominance (l) gene effects in crosses PHR 1012 ×GW 366 and GW 411 × VL920 respectively. Importance of non-allelic

Table 1. The results of scaling test and gene effects for grain yield per plant and yield attributing traits.

	Days to 50 % heading	Days to maturity	No. of effective tillers /plant	Plant height	Length of spike,	Spikelet/ spike	Grains/ spike	Grain yield/ spike	Grain yield / plant	100-grain weight
GW 395 × HI 1556										
A	-0.73 ± 1.55		1.73 ± 0.94		2.58** ± 0.41	2.93** ± 0.70			5.12** ± 2.52	-0.68 ± 0.17**
B	-4.80** ± 1.54	NS	-0.33 ± 1.03	NS	1.32** ± 0.32	0.80 ± 0.65	NS	NS	4.83 ± 2.77	0.035 ± 0.094
C	-0.53 ± 2.61		0.46 ± 1.78		3.18** ± 0.62	3.07** ± 1.04			5.10 ± 4.27	-1.77 ± 0.25**
M	56.09 ± 0.45		13.57 ± 0.33		10.45 ± 0.13	17.52 ± 0.20			31.99 ± 0.76	5.01 ± 0.05
(d)	-3.40** ± 0.73		-2.17*± 0.31		-0.84** ± 0.21	-0.633 ± 0.40			-1.58** ± 0.74	-0.19* ± 0.08
(h)	1.77 ± 2.52		0.85 ± 0.52		-0.25 ± 0.68	-0.300 ± 1.17			0.04 ± 1.41	-0.68* ± 0.27
(i)	5.00* ± 2.33		-		-0.73 ± 0.65	-0.67 ± 1.12			-	-1.13** ± 0.27
(j)	-4.07* ± 1.98		-		-1.26* ± 0.48	-2.13**± 0.86			-	0.71** ± 0.18
(l)	-10.53** ± 3.93		-		4.65** ± 1.05	4.40* ± 1.91			-	0.49 ± 0.41
X^2(3)	10.87*		3.87		55.14**	20.31**			5.99	67.11**
HR 1012 ×GW 366										
A	-3.93** ± 1.45		-0.20 ± 0.94	-1.80 ± 2.53	2.66** ± 0.52	2.60** ± 0.84	15.13** ± 4.95	2.03** ± 0.25	4.60 ± 3.03	-0.39* ± 0.17
B	-5.60** ± 1.58	NS	1.06 ± 1.07	-0.53 ± 2.69	1.67** ± 0.45	0.67 ± 0.96	33.20** ± 4.32	0.48* ± 0.25	1.25 ± 2.83	-1.87** ± 0.26
C	-10.27** ± 2.36		-0.60 ± 2.17	-15.87** ± 4.29	5.55** ± 0.84	2.67 ± 1.58	59.40** ± 6.08	2.59** ± 0.42	7.27 ± 4.01	-3.05** ± 0.40
M	60.367 ± 0.40		8.86 ± 0.31	87.08 ± 0.73	9.47 ± 0.13	16.35 ± 0.23	35.00 ± 0.91	2.31 ± 0.08	22.25 ± 0.77	6.00 ± 0.09
(d)	-1.70* ± 0.80		1.77* ± 0.29	-0.93 ± 1.34	1.49** ± 0.23	1.13** ± 0.42	6.53* ± 2.59	0.17 ± 0.13	-2.26** ± 0.79	-0.51* ± 0.14
(h)	-3.67 ± 2.43		0.19 ± 0.57	-8.03 ± 4.26	0.90 ± 0.77	-1.17 ± 1.94	7.50 ± 6.79	0.41 ± 0.42	-0.44 ± 1.42	0.15 ± 0.47
(i)	-0.733 ± 2.27		-	-13.53** ± 3.96	1.22 ± 0.69	-0.60 ± 1.24	11.07 ± 6.34	0.07 ± 0.39	-	-0.78 ± 0.46
(j)	-1.67 ± 1.91		-	1.27 ± 3.21	-0.99 ± 0.54	-1.93* ± 0.96	18.07** ± 5.99	-1.56* ± 0.29	-	-1.48** ± 0.30
(l)	-8.80* ± 3.99		-	11.20 ± 6.86	3.11* ± 1.25	3.87 ± 2.29	37.27**±12.01	2.43** ± 0.65	-	-1.49* ± 0.69
X^2(3)	23.34**		1.39	17.18**	48.41**	10.17*	105.26**	75.19**	4.19	96.32**
GW 411 × VL 920										
A	1.47 ± 2.05	7.00 ± 3.51	1.60 ± 2.40	-5.93 ± 3.20	0.86 ± 0.52		15.13** ± 4.95	-0.08 ± 0.21		-0.06 ± 0.21
B	0.13 ± 2.38	11.13** ± 3.90	2.26 ± 2.28	-9.40** ± 2.85	-1.61** ± 0.43	NS	33.20** ± 4.32	0.08 ± 0.18	NS	-0.12 ± 0.19
C	0.53 ± 3.50	9.60 ± 5.77	1.13 ± 4.11	-13.00* ± 5.05	-0.32 ± 0.78		59.40** ± 6.08	0.77* ± 0.30		-0.11 ± 0.33
M	67.30 ± 0.61	106.60 ± 0.82	15.52 ± 0.69	99.67 ± 0.92	11.34 ± 0.17		35.00 ± 0.91	1.60 ± 0.05		2.90 ± 0.03
(d)	4.15** ± 0.60	-6.33** ± 1.80	-5.17* ± 0.67	1.23 ± 1.49	-0.59 ± 0.29		6.53* ± 2.59	0.20 ± 0.10		0.06 ± 0.03
(h)	-2.44* ± 1.20	-15.20* ± 5.43	1.39 ± 1.27	4.97 ± 5.01	1.14 ± 0.92		7.50 ± 6.79	1.10** ± 0.30		1.56** ± 0.08
(i)	-	-8.53 ± 4.89	-	2.33 ± 4.72	0.43 ± 0.90		11.07 ± 6.33	0.77** ± 0.27		-
(j)	-	4.13 ± 4.45	-	-3.47 ± 3.97	-2.47** ± 0.63		18.07** ± 5.98	0.16 ± 0.23		-
(l)	-	26.67** ± 9.22	-	-17.67* ± 7.80	-1.17 ± 1.43		37.27* ± 12.01	-0.77 ± 0.50		-
X^2(3)	0.533	9.82*	1.28	13.93**	18.75**		105.26**	10.23*		0.42

*, ** Significant at 5 % and 1% level respectively, NS indicate the non significant variation in generation

gene interactions for plant height was reported by Kaur and Singh (5). Estimation of gene effects for length of spike revealed that additive (d) and dominance × dominance (1) gene effects were significant Zahid *et al*., (2011) reported non additive gene action for expression of spike length. Spikelets per spike was under the main as well as interaction effects i.e. additive (d), dominance (h), additive × additive (i) additive × dominance (j) and dominance × dominance (1) interactions are significant in crosses GW 395 × HI 1556, PHR 1012 ×GW 366 Zahid *et al*. (11) also reported involvement of non additive gene action governed inheritance of spikelets per spike.

Additive × additive (i) additive × dominance (j) and dominance × dominance (1), type of gene action was significant in cross PHR 1012 ×GW 366 and GW 411 × VL920. Whereas in cross PHR 1012 × GW 366 the digenic interaction (j) and (l) were important for grain yield per spike, Sharma *et al.* (8) found that additive, dominance and epistatic effects. In all the crosses 100 grain weight was under the main as well as interaction effects i.e. additive (d), dominance (h), additive × additive (i) additive × dominance (j) and dominance × dominance (1) Kapoor *et al.* (3) also showed that all the three types of gene action additive, dominance and epistasis were involved for the inheritance of this trait.

Conclusions

Results obtained here revealed the importance of epistatic types of gene effects in the inheritance of all traits studied, and cannot be ignored when establish a new breeding program to improve wheat populations for economic traits. The inheritance of all traits studied controlled by additive and non-additive genetic effects, with greater values of dominance gene effect than the additive one in most cases. Farag (2009) reported that among the epistatic components, the dominance × dominance was greater in magnitudes than additive × additive and additive × dominance in most studied traits. When additive effects are larger than the non-additive, it clearly indicate that selection in early segregating generations would be effective, while, if the non-additive portion larger than additive, the improvement of the characters needs intensive selection through later generation. In order to get transgressive segregants it is necessary to break undesirable linkages and simultaneously exploit additive and non - additive gene effects. Biparental mating among potentially desirable plants may resorted to in early segregating generations for improvement for these traits.

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Phytochemistry and pharmacology of traditionally used medicinal plant *Cordia dichotoma* Linn (Boraginaceae)

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Abstract

Cordia dichotoma is a tall tree which grows in Sri Lanka, India and other warmer countries. Its medicinal properties are known since long time and it is traditionally used to cure several ailments. Its fruits are used as expectorant, astringent, coolant, emollient, purgative and anthelmintic. Anti-inflammatory, analgesic, hepatoprotective and several other pharmacological activities have also been reported from the plant. Aim of the current review was focused on the assessment of its present medicinal uses, phytochemistry and pharmacology in order to reveal its complete pharmacological and therapeutic potentials. Literature survey performed on electronic sources, scientific journals as well as books showed that this plant is of an enormous value because of its various potent pharmacological actions shown by it and several pharmacologically active principles like apigenin, arabinoglucan, quercetin which have been isolated from it. It will be certainly valuable to explore it for further research to be carried out on this medicinal plant.

Keywords - *Cordia dichotoma*, Pharmacology, Phytochemistry, Traditional uses

Introduction

Cordia dichotoma belonging to family Boraginaceae is a 3-4 m tall tree with 2-5 cm long petiole, 6-13×4-9 cm leaf blade ovate to elliptic, sparsely pubescent or glabrous, margin usually subundulate to undulate dentate which grows in Sri Lanka, India and other warmer countries (Fig. 1) (1). It is commonly known as Indian cherry in English and Lasora in Hindi. Its medicinal properties is known since long time and traditionally used to cure several ailments. Its fruits are used as expectorant, astringent, coolant, emollient, purgative and anthelmintic (2). Anti-inflammatory, analgesic, anticancer, hepatoprotective and several other pharmacological activities have also been reported from the plant (3, 4, 5). 'Instant Joshanda Granules', a polyherbal formulation, is extensively used by the people of India for the treatment of cough, sore throat, catarrh, common cold, respiratory distress, flu-like ailments and fevers of which *Cordia dichotoma* is the chief ingredient (6).

***Traditional uses*:** Leaf paste of *Cordia dichotoma* Forst is given to the animal with water for the treatment of diarrhea in Udaipur district of Rajasthan. Leaves and fruits of the plant *Cordia dichotoma* are given to the animals suffering from leucorrhoea (7). The fruit of the plant is used as purgative, diuretic, antihelmintic and is useful in dry cough, wound purification, mouth ulcer cure and jaundice cure and to increase male potency (8, 9). Leaves of the plant are used for headaches and ulcers including decoction for sore throat (10). The bark of the plant is used in the treatment of ulcerative colitis

and colic pain (8). 100 g powder of stem bark is taken with a glass of water to cure menstrual disorders by Korku tribe of Amravati district of Maharashtra, India (11). It is used as abortifacient in traditional or folkloric medicine (12). The pickled fruit is eaten as an appetizer in certain areas of Taiwan. The indigestible seeds and sticky pulp of the fruit can form a phytobezoar if ingested excessively (13).

Phytochemistry: The dry powdered seeds of *Cordia dichotoma* were found to contain glycosides, alkaloids, carbohydrates, tannins and saponins (14). Alkaloids, flavonoids, amino acids and proteins are present in the fruits of *Cordia dichotoma* (8, 9). Qualitative phytochemical tests, thin layer chromatography and TLC-bioautography of the ethanolic extract of leaves demonstrated the presence of common phytoconstituents phenols, tannins and glycosides as major active constituents (15, 16). The antinutritional factors such as phytic acid (0.2±0.1%), tannin (1.386±0.0152%) and oxalic acid (2.133±0.2081%) were found to be present in the powdered wild edible fruits of the plant. Saponin was absent (17). *Cordia dichotoma* Forst bark was identified as botanical source of *Shleshmataka* in Ayurvedic pharmacopoeia (18). Apigenin was isolated by column chromatography from methanolic fraction of crude methanolic extract of *C. dichotoma* bark (19). Arabinoglucan was isolated from the fruits of *Cordia dichotoma* Forst (20). The phytoconstituents arabinoglucan, D-glucose and L-arabinose were present in fruits; linolenic acid in seed; and quercetin in leaves (21). The structure and properties of the newly identified natural cellulose fabrics from the branches of the *Cordia dichotoma* indicated that they could be appropriate for blending and processing by biodegradable polymers to make green composites (22).

Pharmacological activities

Analgesic, antibacterial and cytotoxic activities: The crude ethanol extract of the leaves of *Cordia dichotoma* produced significant writhing inhibition in acetic acid induced writhing in mice at the oral dose of 500 mg/kg b wt ($p<0.001$) which was comparable to that of the standard drug diclofenac sodium at the dose of 25 mg/kg b wt. The extract showed significant zone of inhibition against both Gram negative and Gram positive bacteria *Streptococcus aureus, Streptococcus pyogenes, Vibrio cholerae, Streptococcus epidermis, Hafnia* and *Escherichia coli* in disc diffusion study which is comparable to that of Kanamycin (30 ìg/ml). The extract also showed potent toxicity against the brine shrimp *Artemia salina* (LC_{50}: 20 ìg/ml and LC_{90}: 180 ìg/ml) in brine shrimp lethality bioassay (3).

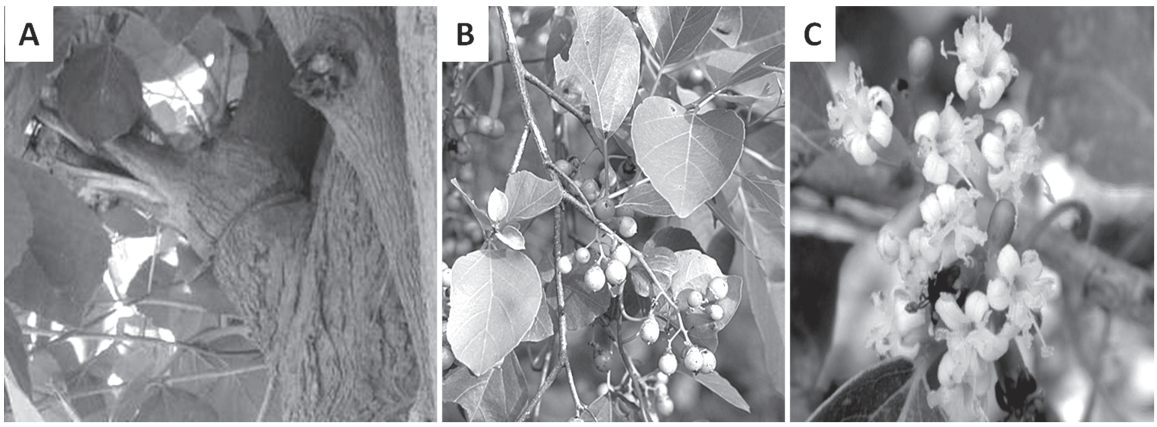

Fig. 1. Parts of *Cordia dichotoma*. (A) Whole plant; (B) twig with leaves and fruits; and (C) twig with flowers.

Anthelmintic activity: Five concentrations (10, 25, 50, 75 and 100 mg/ml) of ethanolic and aqueous extracts prepared from pulp obtained after separation of seeds from fruits of *Cordia dichotoma* Forst by Soxhlet extraction were studied for anthelmintic activity by using *Eudrilus euginiae* earthworms. Both ethanolic and aqueous extracts showed paralysis and death of worms in concentration dependent manner. Aqueous extract showed significant activity than ethanolic extract. The extracts also showed the presence of alkaloid, glycoside, saponin, flavonoid, triterpenoid, protein, amino acid and carbohydrate in preliminary phytochemical investigation (2).

Antibacterial activity: Antibacterial activity of alcoholic and aqueous extracts of thirty four medicinal plants including *Cordia dichotoma* were screened for potential antibacterial activity against six bacterial strains belonging to Enterobacteriaceae, viz., *Escherichia coli, Enterobacter aerogenes, Klebsiella pneumoniae, Salmonella typhimurium, Proteus mirabilis* and *Proteus vulgaris* by the agar well diffusion and disc diffusion methods. The ethanol extracts were more active than aqueous extracts for all the plants studied. Aqueous extract of *Cordia dichotoma* inhibited only to *Proteus mirabilis*, while ethanolic extract inhibited to *Klebsiella pneumonia* and *Proteus mirabilis* (23).

Antibacterial activity of *Cordia dichotoma* leaves extract studied by two different methods i.e. well diffusion and disc diffusion methods against the strain of *E. coli* revealed that it possesses antibacterial activity against *E. coli*. The activity of extract is due to the flavonoid active constituent. Phytochemical analysis showed positive result for tannin, protein and flavonoid (24).

Antibacterial activity of petroleum ether, chloroform, methanol and aqueous extracts of *Cordia dichotoma* Forst ripened fruits against urinary tract pathogens such as *Pseudomonas aeruginosa, Escherichia coli, Klebsiella pneumoniae, Proteus mirabilis, Proteus vulgaris* and *Staphylococcus aureus* using disc diffusion method was evaluated. Results of the study showed that the methanolic extract exhibited better antibacterial activity against the bacterial strains as compared to other extracts (25).

Antioxidant activity or free radical scavenging activit: The methanolic extract of seeds and leaves of *Cordia dichotoma* demonstrated positive antioxidant activity in a concentration dependent manner during investigation of their free radical scavenging potential using *in vitro* models viz., 1,1-diphenyl-2-picrylhydrazyl (DPPH) and hydrogen peroxide (H_2O_2) models. The IC_{50} values for the leaves were found to be 51.83 ìg/ml and 55.73 ìg/ml for DPPH and H_2O_2 models respectively. The IC_{50} values for seeds were found to be 57.22 ìg/ml and 48.28 ìg/ml for DPPH and H_2O_2 models respectively. In both the models, the methanolic extract of the leaves exhibited low IC_{50} values as compared to the seed's extracts. Thus, this activity was more pronounced in leaves as compared to seeds (26).

During nitroblue tetrazolium chloride (NBT) superoxide radical scavenging assay, maximum antioxidant activity of ethanol and acetone extracts of the fruits of the plant *Cordia dichotoma* was noticed to be 54.5 and 55.5 % respectively. The inhibitory potential expressed in IC_{50} values of acetone and ethanol extracts of *C. dichotoma* were found to be 131.0 and 149.0 ìg/ml respectively (27).

The activity of peroxidase (POX), superoxide dismutase (SOD) and catalase (CAT) was determined in the fruits. The SOD and CAT activities were increased in mature fruits than ripened fruits, whereas POX activity was found to be more in ripened fruits as compared to mature fruits (28).

C. dichotoma contains a considerable amount of phenols. The IC_{50} value was found to be 28 µg/ml for the methanolic extract and 36 for butanolic extract in DPPH scavenging assay. The study revealed that the bark has significant radical scavenging activity (18).

Antidiabetic activity: Marles and Farnsworth provided information on more than 1200 species of plants reported to have been used to treat diabetes and/or investigated for antidiabetic activity, with a detailed review of representative plants and some of great diversity of plant constituents with hypoglycemic activity (29). The aerial parts of *Cordia dichotoma* administered orally were devoid of normal antidiabetic activity.

The dose of 500 and 1000 mg/kg b. wt. of aqueous extract of the leaves administered orally to alloxan induced and normoglycemic Wistar rats showed a significant ($p<0.5$) decrease in blood glucose levels after 4, 8 and 24 h. The dose of 1000 mg/kg b. wt. of the extract in normoglycemic rats significantly ($p<0.05$) decreased the blood glucose levels at 8 and 24 h (30).

The methanolic extract of the fruits of *Cordia dichotoma* reduced the blood glucose level in glucose loaded animal and alloxan induced diabetic animal models when compared to diabetic control group and exerted significant hypoglycemic and antidiabetic activities compared to standard drug metformin. The extract also reduced the rate of body weight loss in normal and alloxan induced diabetic animals (31).

Antimicrobial activity: Alcohol is found to be a better solvent for extraction of antimicrobially active substances compared to water and hexane (32). Ethanolic extract of the leaves showed antimicrobial activity only against *S. aureus* (inhibition zone: 21-30 mm) and *S. dysenteriae* (inhibition zone: 10-20 mm) in the study antimicrobial activity against certain drug-resistant bacteria *Salmonella paratyphi*, *Staphylococcus aureus*, *Escherichia coli*, *Bacillus subtilis*, *Shigella dysenteriae* and a yeast *Candida albicans* of clinical origin. No correlation was observed between susceptibility of test strains with the extract and antibiotic resistance behavior of the microbial strains (15).

The acetone, chloroform, hexane, aqueous and ethanol extracts of the *C. dichotoma* leaves and the chloroform, aqueous and acetone extracts of the *C. dichotoma* stem bark did not show any antimicrobial activity against tested strains of bacteria and fungi determined by macrobroth dilution method on the concentration of 25 mg/ml to 0.10 mg/ml of the extracts. Only the hexane and ethanol extracts were active against only certain fungal strains (33).

The acetone and ethanol extracts of *Cordia dichotoma* fruits exhibited highest antimicrobial activity against *Staphylococcus epidermidis, Staphylococcus aureus, Escherichia coli, Candida albicans* and *Aspergillus niger*. Acetone extract showed maximum inhibitory zone of 19.1 mm whereas no inhibitory effect was observed for the aqueous extract (27).

Petroleum ether, benzene, chloroform, acetone, methanol and aqueous extracts of the plant were investigated for the antimicrobial efficacy by modified Kirby-Bauer disc diffusion method against the clinical isolates of oral cancer cases like the fungal pathogens *Aspergillus fumigates, Candida albicans* and bacterial pathogens *Escherichia coli, Staphylococcus aureus, Staphylococcus epidermidis, Klebsiella pneumonia, Pseudomonas aeruginosa, Proteus vulgaris, Proteus mirabilis*. Only *S. aureus* was inhibited significantly by the petroleum ether and acetone extracts (inhibition zone: 10 and 11.33 mm respectively; MIC: 250 and 125 ìg/ml respectively), while only *S. epidermidis* by aqueous extract (inhibition zone: 10.33 mm; MIC: 125 ìg/ml). Even, other isolates were not inhibited by the remaining extracts (34).

The methanolic and butanolic extracts of the bark showed remarkable zone of inhibition of bacterial growth and fungal growth comparable with that of standard drugs against the organisms tested *Escherichia coli*, *Pseudomonas aeruginosa, Staphylococcus pyogenes, Staphylococcus aureus*, *Aspergillus niger, Aspergillus clavatus*, and *Candida albicans*. The activity of the extracts increased linearly with increase in extract concentration (35).

Antiulcer activity: Ethyl acetate, butanol and butanone fractions of ethanolic fruit extract significantly decreased the volume of gastric secretion, free acidity, total acidity and ulcer index with respect to control during the study of anti-ulcer effect of fruit extracts (300 mg/kg b wt) in Wistar rats using pyloric ligation, aspirin and indomethacin induced ulcer models (36).

The aqueous extract of fresh ripened fruits was found to be more effective than alcoholic extract as compared to ranitidine in aspirin induced gastric ulcer model ($p<0.001$) as well as in pylorus ligation model in rats (37).

Anti-inflammatory activity: The ethanolic and aqueous extracts of *Cordia dichotoma* Forst seeds at a dose of 250 mg/kg and 500 mg/kg orally showed significant activity compared with the control and diclofenac sodium (10 mg/kg as standard) in dextran-induced paw edema and carrageenan-induced paw edema models in rats on different phases of acute inflammation (14).

Apigenin (5 mg/kg b wt, po) showed significant healing and reduction in inflammatory enzymes myeloperoxidase (MPO) (from 360±0.2 U/ml due to acetic acid induction to normal 222±22.5 U/ml due to treatment) and malondialdehyde (MDA) (from 9.98±1.5 nmol/ml to 2.11±1.5 nmol/ml) when screened for ulcerative colitis induced by intrarectal administration of 150 ìl, 5% acetic acid (pH 2.5) 3 cm from the anal margin. It was concluded that apigenin from *C. dichotoma* bark may be responsible for the treatment of ulcerative colitis (19).

The methanol fraction of methanolic extract of *C. dichotoma* bark showed effective treatment of ulcerative colitis. It showed good healing and lower pathological scores in treated animals. It significantly reduced MDA and MPO levels in tissue and blood. It showed antioxidant potential and contains a high level of phenolics (38).

Anticerebrovascular insufficiency activity: Long term cerebral hypoperfusion in rats resulted in propensity towards listlessness and anxiety (elevated plus maze test and open field paradigm) accompanied by deficits in memory and learning (Morri's water maze test) and tendency towards depression (Porsolt's swim test). Gliosis, cellular edema, astrocytosis and inflammatory changes were observed in forebrain. Treatment by *Cordia dichotoma* (250 mg/kg po for 28 days) alleviated these cognitive, behavioral and histopathological changes suggesting that *Cordia dichotoma* may be useful in cerebrovascular insufficiency conditions (39).

Binding property: The mucilage isolated from the aqueous filtrate of macerated seeds, whose pulp was previously removed, possesses binding property comparable to the starch. An increase in mucilage concentration led to decrease in friability and increase in disintegration time of the tablets (40).

Angiotensin converting enzyme inhibitory activity : The ethanolic extract of bark showed high ability to inhibit the angiotensin converting enzyme (41).

Diuretic activity : Petroleum ether, solvent ether and butanol fractions of alcoholic extract of the fruits 300 mg/kg b wt were tested for diuretic activity in rats for total urine volume, urine concentration of Na^+ and K^+ and showed increase in cation excretion and urine volume (42).

Hepatoprotective activity: The methanolic extract of the leaves (300 mg/kg) significantly reduced the alanine aminotransferase (ALT) ($p<0.001$), aspartate aminotransferase (AST) ($p<0.001$) and thiobarbituric acid reactive substances (TBARS) levels ($p<0.01$); and at 500 mg/kg extract dose significantly reduced the AST ($p<0.001$), ALT ($p<0.001$), TBARS ($p<0.01$) and lipid peroxide levels ($p<0.05$) in male Wistar rats with liver damage by carbon tetrachloride treatment (4).

Antifertility activity: The reversible nature of the developed phytopharmaceutical was studied by performing pharmacological analyses followed by chronic toxicity studies. The biochemical and

histological estimations detected the reversible contraceptive potential of the drug after withdrawal. The observations also suggested that the developed phytopharmaceutical had potential antifertility activity with safety aspects (43).

Wound healing activity: Petroleum ether, solvent ether, ethyl acetate, butanol and butanone successive fractions of the ethanolic extract of *Cordia dichotoma* fruits showed significant ($p<0.001$) wound healing activity on excision, incision and dead space wound models on Wistar albino rats (44).

Conclusion

Current review summarizes many important pharmacological studies, phytochemical investigations and isolated phytoconstituents of *Cordia dichotoma* which can be further assessed to find out lead molecules in the search of novel herbal drugs for the treatment of several diseases.

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NEWS ITEM

SCIENTIFIC NEWS

Wildlife Conservation of Tigers

It has been reported that the wildlife conservation of Tiger has resulted in increase of their population and it has gone up to 3,890, from the earlier 2010 estimated to be only 3,200, according to the World Wildlife Fund and the Global Tiger Forum. And it is being discussed during the 3rd Asia Ministerial Conference on Tiger Conservation being held at Delhi, which is being inaugurated by honorable Prime Minister Narendra Modiji. According to a 2014 National Survey, India's own tiger population has gone up significantly from 1706, as per its own national estimates, reported by the IUCN in 2010. The updated minimum figure, compiled from International Union for Conservation of Nature and Natural Resources (IUCN) data and the latest national tiger surveys, it can be attributed to multiple factors including increase in tiger populations in India, Russia, Nepal and Bhutan, improved surveys and enhanced protection.

Quantum Spin Liquid - a new state of matter has been detected

Recently, it has been successfully detected a mysterious new state of matter that causes electrons, thought to be nature's indivisible building blocks, to break into pieces. The state, known as a **quantum spin liquid**, was found in a two-dimensional material with a structure similar to grapheme, which was predicted 40 years ago, quantum spin liquids were thought to be hiding in certain magnetic materials, but had not been conclusively sighted in nature. "This is a new quantum state of matter, which has been predicted but hasn't been seen before," said Johannes Knolle from Cambridge University, one of the co-authors of the study published recently in the Nature Materials.

Zika Virus

Researchers from US, first time they determined the structure of the Zika virus, it reveals that the development of effective antiviral treatments and vaccines for the deadly disease. US Purdue University researchers has studied a strain of Zika virus isolated from a patient infected during the French Polynesia epidemic and determined the structure of the virus. The study, detailed online in the journal Science, found the structure to be very similar to that of other flavi viruses, which includes Dengue, West Nile, Yellow Fever, *Japanese encephalitis* and Tick-Borne Encephalitic viruses, with an RNA genome surrounded by a lipid, or fatty, membrane inside an icosahedral protein shell.

Anti-ageing pill that could see humans living a decade longer moves a step closer

An anti-ageing pill that could help us live up to a decade longer has moved a step closer with the discovery of a key component of the ageing process. Protein molecules - known as GSK-3 - are responsible for shortening our natural lives, according to a new study which suggests that life could be prolonged by effectively switching them off. Experiments with fruit flies - which also have the GSK-3 protein - found that the molecule could be inhibited by low-level lithium use, which extended their lives by 16 per cent. This has raised hopes that lithium - or more likely another, similar drug with fewer side-effects - could eventually be turned into a tablet to prolong human life. And perhaps more importantly, knowledge of the molecule could also unlock the secret to pushing back the onset of age-related diseases such as Alzheimer's, diabetes, cancer and Parkinson's, said Dr Jorge Ivan Castillo-Quan, lead author of the study.

The Max Planck Institute for Biology of Ageing and the European Molecular Biology Laboratory were also involved in the research, which is published in the journal Cell Reports.

Weasel temporarily shuts Large Hadron Collider

Spokesman Arnaud Marsollier says the world's largest atom smasher, the Large Hadron Collider at CERN outside of Geneva, has suspended operations because a weasel invaded a transformer that helps power the machine and set off an electrical outage on Thursday night.

Authorities say the incident was one of several small glitches that will delay plans to restart the $4.4 billion collider by a few days.Officials of the European Organization for Nuclear Research, known by its French acronym CERN, have been gearing up for new data from the 27-kilometer (17-mile) circuit that runs underground on the Swiss-French border.

El Nino dries up Asia as its stormy sister La Nina looms

Withering drought and sizzling temperatures from El Nino have caused food and water shortages and ravaged farming across Asia, and experts warn of a double-whammy of possible flooding from its sibling, La Nina.The current El Nino which began last year has been one of the strongest ever, leaving the Mekong River at its lowest level in decades, causing food-related unrest in the Philippines, and smothering vast regions in a months-long heat wave often topping 40 degrees Celsius (104 Fahrenheit).Economic losses in Southeast Asia could top $10 billion, IHS Global Insight told AFP.

The regional fever is expected to break by mid-year but fears are growing that an equally forceful La Nina will follow.That could bring heavy rain to an already flood-prone region, exacerbating agricultural damage and leaving crops vulnerable to disease and pests.

"The situation could become even worse if a La Nina event — which often follows an El Nino — strikes towards the end of this year," Stephen O'Brien, UN under-secretary-general for humanitarian affairs and relief, said this week. Much of Asia has been punished by a bone-dry heat wave marked by record-high temperatures, threatening the livelihoods of countless millions.Vietnam, one of the world's top rice exporters, has been particularly hard-hit by its worst drought in a century.In India, about 330 million people are at risk from water shortages and crop damage, the government said recently, and blazing temperatures have been blamed for scores of heat-stroke deaths and dead livestock.As a result, rice prices have risen this year but the UN's Food and Agriculture Organization and other experts say a major food crisis is not yet imminent as stocks remain ample.With La Nina looming, the situation has laid bare the region's lack of preparedness for the extreme weather swings expected to result from climate change, said Rajiv Biswas, Asia-Pacific Chief Economist at IHS Global Insight.

New reef system discovered in Amazon river

Scientists have discovered a new reef system at the mouth of the Amazon River, the largest river by discharge of water in the world. As large rivers empty into the world's oceans in areas known as plumes, they typically create gaps in the reef distribution along the tropical shelves — something that makes finding a reef in the Amazon plume an unexpected discovery. Scientists from University of Georgia in the U.S. and the Federal University of Rio de Janeiro in Brazil on an expedition to study the Amazon River plume looked for evidence of a reef system along the continental shelf. The Amazon plume is an area where freshwater from the river mixes with the salty Atlantic Ocean — affects a broad area of the tropical North Atlantic Ocean in terms of salinity, pH, light penetration and sedimentation, conditions that usually correlate to a major gap in Western Atlantic reefs."Our expedition into the Brazil Exclusive Economic Zone was primarily focused on sampling the mouth of the Amazon," said Patricia Yager, an associate professor at UGA. The team used multi-beam acoustic sampling of the ocean bottom to find the reef and then dredged up samples to confirm the discovery. We brought up the most amazing and colourful animals I had ever seen on an expedition," Ms. Yager said.The Amazon River plume and its effects on the global carbon budget converged with the discovery of the reef system

to provide scientists a wider view of the reef community. Microorganisms thriving in the dark waters beneath the river plume may provide the trophic connection between the river and the reef. "The paper is not just about the reef itself, but about how the reef community changes as you travel north along the shelf break, in response to how much light it gets seasonally by the movement of the plume," said Ms. Yager."In the far south, it gets more light exposure, so many of the animals are more typical reef corals and things that photosynthesise for food," she said. "But as you move north, many of those become less abundant, and the reef transitions to sponges and other reef builders that are likely growing on the food that the river plume delivers," Ms. Yager said.However, the reefs may already be threatened. "From ocean acidification and ocean warming to plans for offshore oil exploration right on top of these new discoveries, the whole system is at risk from human impacts," she said.

SCIENTIFIC FINDINGS

Identification of Potent Antibodies Against HIV - Scientists have developed a structure based vaccines that can jumpstart an effective immune response to HIV by rapidly offsetting antibodies against the virus, which so far have been unsuccessful. Previous studies have shown that the immune system can produce antibodies capable of "neutralizing" HIV, and stopping the AIDS-causing virus dead in its tracks. However, only less than a third of human bodies can produce "broadly neutralizing" antibodies in response to the HIV infection. Further, it also takes a year or more before the production gets into full swing. Scientists have isolated antibodies with a loop-like structure that binds tightly to HIV and disables it. Based on computer modelling, they re-engineered and optimized the antibodies' neutralizing capacity. The findings, reported in Proceedings of the National Academy of Sciences, revealed that the structure might aid in swiftly inducing the broadly neutralizing antibodies against HIV even in people who have not been exposed previously to the deadly virus as per lead researcher James Crowe Jr, professor at Vanderbilt University in the US.

New Genes Responsible for Stroke, Dementia Discovered - A team of US Scientists has found a new set of genes that may be responsible for stroke and dementia — the two most common and disabling neurological conditions. Researchers identified a new gene called FOXF2 that increases the risk of having a stroke due to small vessel disease in the brain. The small vessel disease not only causes stroke but is also a major contributor to the risk of dementia and also associated with gait problems and depression. The study may help researchers better understand, treat and prevent ischemic and hemorrhagic stroke and perhaps Alzheimer's disease and other dementias. "Our research has identified a gene affecting another type of ischemic stroke, due to small vessel disease and also suggests some genes may be associated with both ischemic and hemorrhagic stroke and may act through a novel pathway affecting pericytes — a type of cell in the wall of small arteries and capillaries," said Sudha Seshadri from Boston University Medical Center in the US. Who published their paper in the Journal Lancet Neurology.

How Zika Virus Causes Microcephaly - Researchers have now produced evidence of how Zika virus causes brain defects in babies. Several cases of microcephaly, a rare birth defect in which the brain fails to grow properly, continue to be reported in Brazil following the unprecedented epidemic of Zika virus, which was first detected in the country in May 2015. The researchers used the Zika virus infected neural cells and cultured them as neurospheres (a culture system composed of free-floating clusters of neural stem cells). While those cells that were not infected with Zika virus generated normal neurospheres, the Zika virus-infected neural cells generated neurospheres with abnormalities. At the end of six days, the virus killed most of the neurospheres, while hundreds of neurospheres grew in the control arm.To further investigate the

impact of Zika virus infection during neurogenesis, organoids were exposed to Zika virus and followed for 11 days. Ptaricia P. Garcez, the first author of a paper published today (April 11) in the Journal *Science* from the Federal University of Rio de Janeiro, Brazil, and others found that the virus reduced the growth of infected organoids by 40 per cent compared with brain organoids under control conditions.

Protein that may Reverse Deadly Heart Condition Identified - Scientists have identified a key protein that may potentially reverse cardiac fibrosis, an abnormal thickening of the heart valves which can lead to heart failure**.** CCN5, a matricellular protein, has been found to reverse established cardiac fibrosis in heart failure models, according to a study led by Roger J Hajjar, Professor at the Icahn School of Medicine at Mount Sinai in US, and Woo Jin Park, Professor at the Gwangju Institute of Science and Technology in South Korea. They induced extensive cardiac fibrosis in experimental animal models of heart failure, and then proceeded to transfer CCN5 to the hearts. Eight weeks later, the team examined the cellular and molecular effects. The results showed that CCN5 reversed cardiac fibrosis in the models. Researchers used trichrome staining and analysis of myofibroblast contents before and after CCN5 gene transfer to clearly show the reversal.

Indian-Led team of Doctors Develops Technology to Monitor Cancer Treatment - A team of Indian scientists from the prestigious Massachusetts Institute of Technology and Harvard Medical School have made an important breakthrough by developing a nanotechnology which will help monitor the effectiveness of cancer therapy within hours of treatment."We have developed a nanotechnology, which first delivers an anticancer drug specifically to the tumour, and if the tumour starts dying or regressing, it then starts lighting up the tumour in real time," said Shiladitya Sen Gupta, a principal investigator at Massachusetts Institute of Technology's (MIT) Brigham and Women's Hospital (BWH)."This way you can monitor whether a chemotherapy is working or not in real time, and switch the patients to the right drug early on. One doesn't need to wait for months while taking a toxic chemotherapy only to realize later and after side effects that the drug hasn't worked," Gupta, a co-corresponding author of the breakthrough research published online this week in 'The Proceedings of the National Academy of sciences'.

North Pole Completely Ice-Free During Summer- It's hard to imagine the North Pole not being covered with snow at any given time, but according scientists, there was a point millions of years ago when the Arctic was virtually ice-free during summers and the temperature of the ocean's surface ranged from 4 to 9 degrees Celsius (7.2 to 16.2 degrees Fahrenheit). Researchers from various scientific organizations, including the Alfred Wegener Institute Helmholtz Centre for Polar and Marine Research (AWI) in Germany, examined several sediment samples collected from the Lomonosov Ridge in the Arctic Ocean in order to shed light on the climate history of the region. By analyzing microfossils known as dinoflagellates that were included in the earth samples, the researchers were able to determine that the lower part of the sediment core was made up of deposits that were six to eight million years old. This meant that the samples are from the late Miocene period. The researchers also made use of "climate indicators," which allowed them to reconstruct the type of climate that existed in the central Arctic Ocean during the time. They discovered that six to 10 million years ago, the Artic and its surrounding ocean were not covered in ice during summertime. This contradicts earlier notion that the region had always been blanketed in dense sea ice all year round throughout its long history. The findings of the international climate study are featured in the journal Nature Communications.

NAVIC-India's very own GPS is ready with seventh navigation satellite launch : India's own navigational system, the set-up for which

was completed on Thursday with the launch of the seventh and final satellite, will be called NAVIC (Navigation with Indian Constellation), Prime Minister Narendra Modi announced after the launch.The seventh and final satellite of the Indian Regional Navigation Satellite System, the IRNSS 1G, was launched into a sub geosynchronous transfer orbit with a perigree (nearest point to earth) of 284 km and an apogee (farthest point to earth) of 20,657 km. The satellite was launched on board the Polar Satellite Launch Vehicle (PSLV), which took off from the Sriharikota launch pad at 12.50 p.m.With this launch, the IRNSS constellation of seven satellites is now complete. This will allow the Indian Space Research Organisation (ISRO) to focus on the process of designing front end chips which will receive the navigational signals sent out by the satellites. The system will be similar to the Global Positioning System (GPS) operated by the United States with 24 satellites and the Glonass, Galileo and BeiDou systems of Russia, Europe and China respectively.All satellites will undergo stabilisation testing and verification of their performance over the next few months before being pushed into use, according to ISRO officials.An area of 1,500 km from Indian boundaries will be covered under the navigational system. The Prime Minister invited other countries to make use of this system as well. “We have seven neighbours who rely on technology provided by other countries. They can use Indian services if they want,” he said in a video message addressed to ISRO engineers.With an accuracy of better than 20 m being claimed by ISRO, the navigation system will be offered as an open or Standard Positioning Service and a superior, coded military Restricted Service.

World’s tiniest thermometer using DNA created

Scientists have created the world’s tiniest thermometer that is 20,000 times smaller than a human hair, using DNA structures that can fold and unfold at specifically defined temperatures. The advance may significantly aid our understanding of natural and human designed nanotechnologies by enabling to measure temperature at the nanoscale, researchers said. Over 60 years ago, researchers discovered that the DNA molecules that encode our genetic information can unfold when heated.”In recent years, biochemists also discovered that biomolecules such as proteins or RNA (a molecule similar to DNA) are employed as nanothermometers in living organisms and report temperature variation by folding or unfolding,” said Alexis Vallee-Belisle, professor at University of Montreal in Canada.”Inspired by those natural nanothermometers, which are typically 20,000 times smaller than a human hair, we have created various DNA structures that can fold and unfold at specifically defined temperatures,” said Vallee-Belisle.One of the main advantages of using DNA to engineer molecular thermometers is that DNA chemistry is relatively simple and programmable.”DNA is made from four different monomer molecules called nucleotides - nucleotide A binds weakly to nucleotide T, whereas nucleotide C binds strongly to nucleotide G,” said David Gareau, from the University of Montreal.”Using these simple design rules we are able to create DNA structures that fold and unfold at a specifically desired temperature,” Gareau said.”By adding optical reporters to these DNA structures, we can therefore create 5 nm—wide thermometers that produce an easily detectable signal as a function of temperature,” said Arnaud Desrosiers, from the University of Montreal.These nanoscale thermometers open many exciting avenues in the emerging field of nanotechnology, and may even help us to better understand molecular biology. The study was published in the journal Nano Letters.

OPPORTUNITIES

The Rajiv Gandhi Centre for Biotechnology - (RGCB) RGCB has regular openings for post-doctoral training in select programs of Disease Biology and Plant Biotechnology. This programme is an ideal platform for young enthusiastic post-docs looking for comprehensive training as a prelude to applying

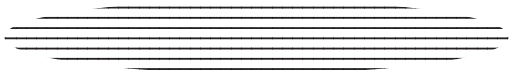
for faculty positions in India and abroad. Two systems of post-doctoral training are operational: 1. RGCB funded programme 2. Programmes funded by external funding agencies. Contact: http://rgcb.res.in/post-doctorial-training. Interested candidates may please contact the director@rgcb.res.in or mrpillai@rgcb.res.in

SERB - IUSSTF - Science and Engineering Research Board (SERB) in partnership with bi-national Indo-US Science and Technology Forum (IUSSTF) announces the SERB-Indo US Postdoctoral Fellowships for Indian Researchers as an initiative under the SERB Overseas Postdoctoral fellowship (SERB-OPDF). Completed Application should be submitted electronically to: E-mail : fellowship@ indousstf.org and fellows hipindous@gmail. com.Contact-http://iusstf.org/ story/53-95-SERB-Indo-US-Postdoctoral-Research-Fellowship-Program.html

DBT Research Associateship - Applications are invited from Indian nationals for DBT (DEPARTMENT OF BIOTECHNOLOGY) Research Associateship candidates will be selected based upon their performance in C.V and interview. Contact - http://biochem.iisc. ernet.in/dbtra.html

TWAS-DBT Postdoctoral Fellowship Programme - The Department of Biotechnology (DBT) of the Ministry of Science and Technology, India, and TWAS have established a fellowship programme for foreign scholars from developing countries who wish to pursue postdoctoral research in biotechnology. Deadline-31 August 2016. Contact-http://twas.org/opportunity/twas-dbt-postdoctoral-fellowship-programme, e-mails: fellowships@twas.org, dircics@gmail.com

CSIR-Nehru Science Postdoctoral Research Fellowship Scheme - CSIR-Nehru Science Postdoctoral Research Fellowship Scheme has been instituted to identify promising young researchers with innovative ideas and provide them with training and research opportunities in niche areas of basic science, engineering, medicine and agriculture. The scheme aims at facilitating their transition from mentored to independent research career. CSIR intends to offer one hundred (100) such fellowships every year to promising fresh PhD holders for working in CSIR laboratories with state-of-art R&D facilities. Contact -http://www.csir.res.in/external/ heads/aboutcsir/nehrufellowship.pdf



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