Int J Pharm Bio Sci 2013 Jan; 4(1): (B) 59 - 69

Research Article **Microbiology**

International Journal of Pharma and Bio Sciences

ISSN 0975-6299

EVALUATION OF *IN VITRO* CONDITIONS INFLUENCING PHOSPHATIDYLINOSITOL-SPECIFIC PHOSPHOLIPASE C PRODUCTION BY *STAPHYLOCOCCUS AUREUS* ATCC 9144

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ABSTRACT

The aim of this study was to evaluate the growth conditions affecting production of hosphatidylinositol-specific phospholipase-C (PIPLC) by *Staphylococcus aureus* ATCC 9144. A simple colorimetric method for direct estimation of phospholipids was adapted for rapid assaying of PIPLC. First phase of the study involved preliminary screening of nutritional and physical parameters. In the second phase, Plackett-Burman statistical design was employed to identify the most critical factors. Of the eleven variables studied, peptone, sodium carbonate (buffer), and rotational speed exhibited significant positive influence while glucose showed a negative influence on PIPLC activity. Thus, low C/N ratio, aeration, and buffering agent were identified as important factors controlling production of PIPLC. These results contribute to the existing knowledge on PIPLC regulation in *Staph. aureus* and would help optimize production of this enzyme for its successful application in biochemical research and industry.

KEY WORDS: **Phosphatidylinositol-specific phospholipase C *Staphylococcus aureus* Enzyme production Plackett-Burman design Colorimetric assay**

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INTRODUCTION

Staphylococcus aureus is an important opportunistic pathogen known to cause food poisoning, nosocomial infections and life threatening infections such as septicemia, endocarditis and toxic shock syndrome[1]. It produces a number of toxins and enzymes that contribute to its pathogenesis and phosphatidylinositol-specific phospholipase C (PIPLC; E.C.4.6.1.13) is one of them. PIPLC, an important class of phospholipases, is produced by many bacterial species and eukaryotes including mammals. The enzyme hydrolyses phosphatidylinositol (PI) to yield diacylglycerol and inositol phosphate. In bacteria such as *Bacillus anthracis, Staphylococcus aureus, Listeria monocytogenes* and others , PIPLC is shown to contribute to their virulence[2-6]. In eukaryotes, it plays a role in transmission of signals[7]. Further, PIPLC can hydrolyse the glycosylphosphatidylinositol (GPI)-anchor of eukaryotic membrane proteins[8,9]. This unique property has made PIPLC an important research tool in studies involving the functionally diverse and important GPI-anchored proteins[10]. Bacterial PIPLCs also serve as convenient models to study mammalian PIPLC[11,12]. In addition, bacterial PIPLC has gained commercial importance because of its promising role in degumming of oil and in the production of diacylglycerol-rich cooking oil[13,14]. Successful application of PIPLC in membrane research and oil industry, therefore, demands its production in high yields. Many studies involving *Staph. aureus* PIPLC as a tool to study GPI proteins have been reported[15,16]. However, only few attempts have been made towards characterization of *Staph. aureus* PIPLC in terms of its requirements for optimal expression in culture. In light of the potential use of PIPLC in research and industry, and its role in pathogenesis, the main purpose of the study was evaluation of growth conditions that affect the expression of this enzyme in *Staph. aureus*. Previous study in our laboratory demonstrated that *Staph. aureus* ATCC 9144 produces PIPLC and releases GPI-anchored proteins from erythrocyte membranes. Hence this strain was used for further studies. After a preliminary screening of medium components and growth conditions, important variables were screened by the Plackett-Burman experimental design. This design is capable of rapidly identifying the significant factors which have strong influence on the response from a multi-parameters system. It is a two-level fractional factorial design for examining 'N' parameters in 'N + 1' number of experiments[17]. Further, in this study, PIPLC activity was estimated by a simple colorimetric assay which is an adaptation of the method for direct estimation of phospholipids reported by Raheja *et al* [18].

MATERIALS AND METHODS

Phosphatidylinositol (PI) was obtained from Sigma-Aldrich, USA. All media components were from Himedia (India). Other chemicals and reagents used were of AR grade.

(i) Organism and culture conditions

Bacterial strain *Staphylococcus aureus* ATCC 9144 procured from National chemical Laboratory, Pune, India, was stored at 4ºC. The strain was revived periodically by subculturing at 37ºC every 2–3 months on nutrient agar slant. The nutrient medium used for inoculum preparation was also used as the basal enzyme-producing medium. The medium[19] contained (g l^{-1}): peptone, 20 ; beef extract, 30; glucose, 2.0; NaCl, 2.0; Na_2HPO4, 0.4; and Na_2CO_3, 2.5; pH 7.0.

(ii) Inoculum preparation

A loopful of *Staph. aureus* culture from the slant was transferred to 10 ml nutrient medium and incubated at 37ºC for 24 h. A loopful from this tube was grown on blood agar plate at

37ºC for 24 h. Growth from blood agar was then transferred to 50 ml nutrient medium and incubated on a rotary shaker (150 rev min^{-1}) at 37ºC for 24 h. After incubation, using sterile saline, the absorbance of the culture was adjusted to 1.00 at 600 nm and this was used as seed culture. Of this seed culture, 1% (v/v) was used to inoculate 50 ml of the production medium under study. The inoculated flasks were incubated at 37ºC on the shaker (150 rev min^{-1}) for 24 h. The cell-free extract obtained by centrifugation at 12,000 g for 20 min was then assayed for PIPLC activity.

(iii) PIPLC assay

PIPLC activity was determined using phosphatidylinositol (PI) as the substrate. A 10 mmoles l^{-1} solution of PI was prepared in 0.05 % (v/v) Triton X-100 and subjected to sonication for three min. Briefly, the reaction mixture consisting of 10mM PI (200 µl), 0.1 mmoles l^{-1} Tris maleate buffer pH 6.0 (200 µl) and enzyme extract (500 µl), was incubated at 37ºC for one hour. The reaction was terminated by adding 2.5 ml of chloroform: methanol: chloroform: methanol: HCl (66:33:1) mixture, vortexed for a min and centrifuged at 10,000 g for 10 min. From the lower chloroform layer, 400 µl aliquot was withdrawn in calibrated test tubes and the amount of unhydrolysed phospholipid was estimated as described by Raheja *et al*[18], with modifications. . To the above 400 µl aliquot, 3600 µl chloroform and 200 µl chromogen[17] was added and vortexed for a minute. The plugged tubes were placed in a boiling water bath for 1.5 min and then cooled. The volume in each tube was made to 4.0 ml with chloroform and placed at room temperature for 30 min. The blue organic layer was pipetted and estimated at 710 nm. A standard plot was obtained with 0.1– 0.5 mg PI using 1 g l^{-1} of standard PI. This was used to determine the unhydrolysed PI from which the hydrolysed PI was calculated. One unit of enzyme activity was defined as the nanomoles of substrate (PI) hydrolysed per minute at 37ºC.

(iv) Determination of Cell Density

At the end of incubation period, known volumes of culture broth were centrifuged at 12,000 g for 20 min and the pellet obtained was washed twice with sterile saline. The cell mass thus obtained was again suspended in the same volume of sterile saline and cell density determined turbidimetrically at 600 nm.

(v) Screening of nutritional factors

Various carbon sources such as glucose, fructose, maltose, lactose, sucrose and starch (10 g l^{-1}); nitrogen sources namely peptone, yeast extract, beef extract (20 g l^{-1}) and BSA (15 g l^{-1}); metal ion compounds such as $CaCl_2$, $ZnCl_2$, and $MgSO_4$ (1.0 mmoles l^{-1}) were screened for enhanced PIPLC activity. Effect of NaCl was studied by its removal from the 'basal medium'. Effect of inorganic phosphate concentration was studied using Na_2HPO_4 at 0, 1.0 and 5.0 mmoles l^{-1}. One control was maintained without the addition of any putative inducer under study.

(vi) Screening of physical parameters

Physiological parameters such as pH (4–8 units) and incubation temperature (20ºC – 50ºC) were tested for maximum enzyme yield.

(vii) Plackett-Burman experimental design

Plackett-Burman design was employed to identify important medium components affecting PIPLC production. Total of 11 parameters (variable k = 11), (Table 1) were selected for the study with each variable being represented at two levels, high (+) and low (−), in 12 trials. The number of positive and negative signs per trial were (k + 1)/2 and (k – 2)/2, respectively. Each row represents a trial and each column represents an independent assigned variable. Each trial was run in duplicate and the average of enzyme activity

was taken as the response. The effect of each variable was determined by the following equation: $E_i = (\sum P_{i+} - \sum P_{i-}) / n$ Where, *Ei* is the effect of parameter *i* under study, P_{i+} and P_{i-} are responses (enzyme activity) of trials at which the parameter was at its high and low levels, respectively, and *n* is the number of trials at each level (n = 6).

Table 1

Plackett–Burman experimental design matrix along with the observed response for PIPLC production by Staph. aureus ATCC 9144.

Run	Variables											PIPLC Activity (U ml^{-1})
	X1	X2	X3	X4	X5	X6	X7	X8	X9	X10	X11	
1	+	-	+	+	+	-	-	+	-	+	-	31.699
2	-	-	+	+	+	-	+	-	+	-	+	22.599
3	-	-	-	-	-	-	-	-	-	-	-	20.227
4	-	+	-	+	+	+	+	-	+	-	-	21.954
5	+	+	-	-	-	-	-	+	+	+	+	26.819
6	+	+	+	-	-	+	+	-	+	-	-	22.584
7	+	-	+	+	-	+	-	-	-	+	+	49.508
8	-	+	+	-	-	-	+	+	+	+	-	21.218
9	-	+	-	-	-	+	+	+	+	-	+	8.995
10	-	+	+	-	+	+	-	+	-	-	+	10.587
11	+	-	-	+	+	+	-	+	-	+	-	47.901
12	+	-	-	+	+	-	+	-	-	+	+	46.264

X1:Peptone, X2:Glucose, X3:Beef extract, X4:Na_2HPO_4, X5:Na_2CO_3, X6: NaCl, X7:$MgSO_4 \cdot 7H2O$, X8:Tween-20, X9: initial pH, X10:rotational speed and X11:incubation temperature

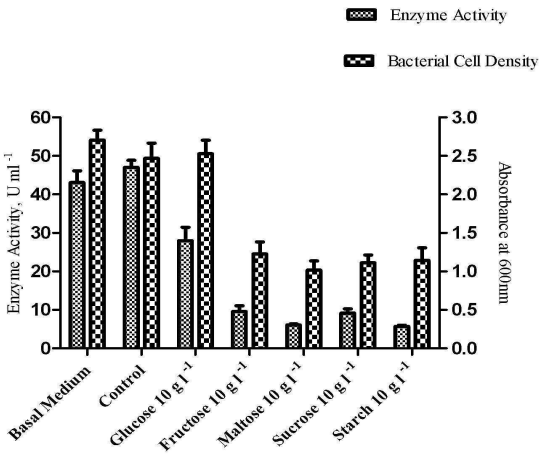
RESULTS

The effects of various factors on PIPLC production by *Staph.aureus* ATCC 9144 were studied.

(i) Screening of nutritional factors

Among the carbohydrates, only glucose (10 g l^{-1}) supported good bacterial growth; however, PIPLC activity was less than that produced in the Basal medium (Fig 1).

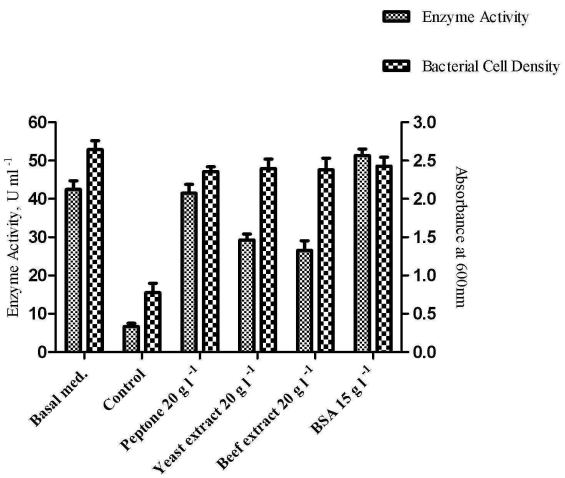
Figure 1
Effect of carbon source on PIPLC production by Staph. aureus ATCC 9144



**** Basal medium (g l^{-1}): peptone, 20 .0; beef extract, 3.0; glucose, 2.0; NaCl, 2.0; Na_2HPO4, 0.4; and Na_2CO_3, 2.5; pH 7.0. † Control without any carbon source. ‡ Values are a mean of three- replication***

Amongst the organic nitrogen sources, PIPLC production was maximum with BSA (15 g l^{-1}). Basal medium containing peptone and beef extract was found suitable for PIPLC production than peptone alone (Fig 2).

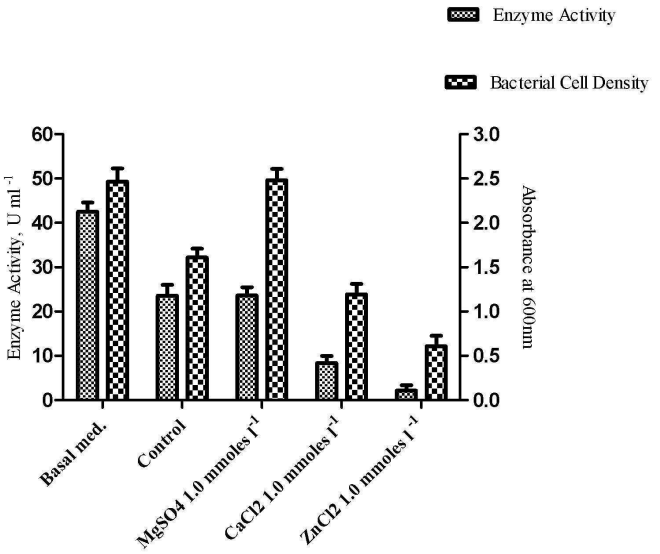
Figure 2
Effect of nitrogen source on PIPLC production by Staph. aureus ATCC 9144



**** Basal medium (g l^{-1}): peptone, 20 .0; beef extract, 3.0; glucose, 2.0; NaCl, 2.0; Na_2HPO4, 0.4; and Na_2CO3, 2.5; pH 7.0. † Control without any nitrogen source. ‡ Values are a mean of three- replication***

Further, when effect of inorganic phosphate was studied, it was evident that PIPLC elaboration was independent of the phosphate concentration in the medium (data not shown).. At 1.0 mmoles l^{-1} concentration, $ZnCl_2$ and $CaCl_2$ inhibited both growth and enzyme production (Fig 3). $MgSO_4$ promoted growth but not PIPLC production. Complete removal of NaCl from the Basal medium decreased the cell density and also PIPLC production.

Figure 3
Effect of metal ions on PIPLC production by Staph. aureusATCC 9144

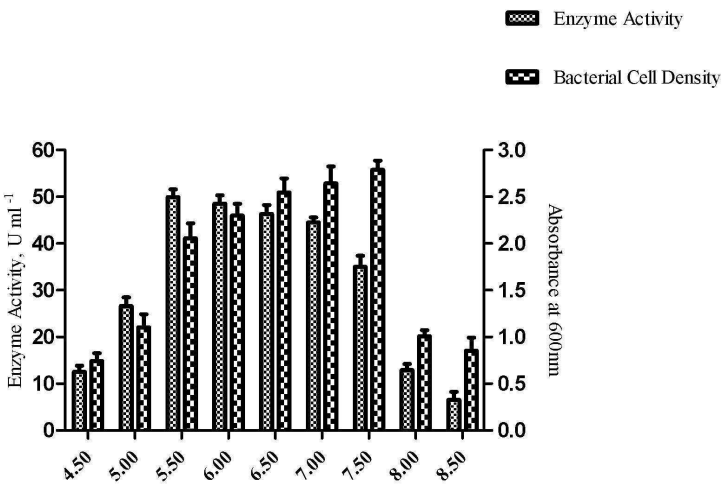


**** Basal medium (g l^{-1}): peptone, 20 .0; beef extract, 3.0; glucose, 2.0; NaCl, 2.0; Na_2HPO4, 0.4; and Na_2CO3, 2.5; pH 7.0. † Control without NaCl. ‡ Values are a mean of three- replication.***

(ii) Screening of physical parameters

Staph. aureus ATCC 9144 showed growth at a wide pH range from pH 5.0 to pH 8.0 with maximum growth at pH 7.0–7.5. However, PIPLC activity was found to increase with decreasing pH from 7.0 to 5.5 (Fig 4). A pH of 5.5 was found to be the optimum for PIPLC production.

Figure 4
Effect of Initial pH of medium on PIPLC production by Staph. aureus ATCC 9144



***Basal medium (g l^{-1}): peptone, 20 .0; beef extract, 3.0; glucose, 2.0; NaCl, 2.0; Na_2HPO4, 0.4; and Na_2CO_3, 2.5; † Values are a mean of three- replication.**

Optimum temperature for PIPLC production and cell growth was found to be 37ºC (data not shown). .

(iii) Plackett-Burman experimental design and analysis

Based on the above preliminary investigation eleven factors were further evaluated for identifying the most significant parameters influencing PIPLC production. The main effect of a factor was calculated as the difference between the average of measurements made at the high (+) and the low (-) levels of that factor. The significance of each variable was determined via a Student's t test (Table 2).

Table 2

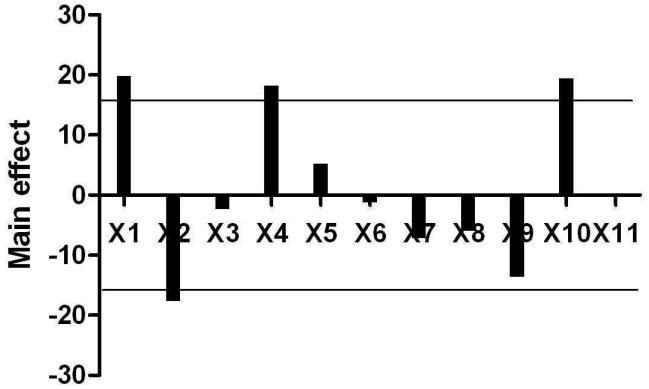
Statistical analysis of the Plackett-Burman design of each variable at different levels for PIPLC production by Staph. aureus ATCC 9144

Variable with Designate	Lower level (-)	Higher Level (+)	Main effect	SED	t exp	p value	Confidence level (%)
X1 Peptone (g l^{-1})	10	20	19.865	5.436	3.654	0.0044	99.56
X2 Glucose (g l^{-1})	2.0	10	-17.673	6.147	2.875	0.0165	98.35
X3 Beef extract (g l^{-1})	2.5	5.0	-2.327	8.275	0.281	0.784	21.6
X4 Na_2C0_3 (g l^{-1})	1.0	2.5	18.249	6.001	2.993	0.013	98.7
X5 Na_2HPO_4 (g l^{-1})	0.2	0.5	5.275	8.138	0.648	0.531	46.9
X6 NaCl (g l^{-1})	2.0	5.0	-1.216	8.299	0.146	0.886	11.4
X7 $MgSO_4$ (g l^{-1})	0.4	1.0	-7.18	7.991	0.899	0.389	61.1
X8 Tween-20 (g l^{-1})	2.0	4.0	-5.987	8.089	0.740	0.476	52.4
X9 pH (Units)	5.5	7.5	-13.669	7.094	1.927	0.082	91.8
X10 Rotational speed	100	200	19.410	5.598	3.467	0.006	99.4
X11 Temperature (ºC)	30	37	-0.135	8.308	0.016	0.987	1.3

The components were screened at confidence level of 95% based on their effects. The influence of the parameters on enzyme activity was graphically presented (Fig 5) to illustrate the relative magnitude and the statistical significance of the impact.

Figure 5

The influence of parameters on enzyme activity



X1:Peptone, X2:Glucose, X3:Beef extract, X4:Na2HPO4, X5:Na2CO3, X6: NaCl, X7:MgSO4·7H2O, X8:Tween-20, X9: initial pH, X10:rotational speed and X11:incubation temperature † Length of the column represents significance of the influence of studied factor on enzyme activity. ‡A reference line is drawn at a significant level of 0.05 and used to determine which factor was significant at 95% confidence level.

Positive sign of the effect of the tested parameter indicated greater enzyme activity at higher level of the parameter. However, if the sign was negative, PIPLC activity was greater at a low level of the

parameter. Peptone (X1), Na_2CO_3 (X4), and rotational speed (X10) showed significant positive influence on enzyme production whereas glucose (X2) showed a significant negative effect.

DISCUSSION

This is the first attempt to evaluate the *in vitro* conditions affecting PIPLC production by *Staph. aureus,* using the Plackett-Burman statistical design. For assay of bacterial PIPLC several direct and indirect methods have been proposed and developed. In direct assay methods using PI as the natural substrate, myo-inositol phosphates (IP) generated by enzyme action are quantitated[20]. This involves decomposition of IP by exhaustive oxidation in presence of concentrated acids prior to colorimetric determination. Alternatively, IP are determined by their radioactivity using [inositol-2-^{3}H] as the substrate[21]. The indirect assay methods employing chromogenic substrates such as 4-nitrophenyl1-phosphoinositol[22] are rapid but are not preferred for studying the kinetic parameters of the enzyme[10]. In this context, the present PIPLC assay demonstrates the suitability of a direct phospholipid estimation method for rapid assaying of PIPLC. The expression of extracellular enzymes in culture medium invariably depends on its composition. Carbon and nitrogen sources are the most important constituents affecting enzyme production. Preliminary investigation (Fig 1) and the negative effect of glucose obtained from Plackett-Burman experimental design imply diminished PIPLC production in presence of high glucose content in the medium. Like many other *Staph. aureus* virulence factors, PIPLC expression is regulated by the accessory gene regulator (*agr*) locus[19]. Further, it is reported that glucose inhibits *agr* expression and this inhibitory effect has been attributed to lowering of pH as a result of glucose metabolism[23]. A strong negative effect of glucose and the positive influence of peptone indicate a low C/N ratio enhancing PIPLC production.

Although BSA as organic nitrogen source resulted in maximum PIPLC activity (Fig 2), its addition in the medium increases the cost of enzyme production considerably. Hence, peptone as a cheaper alternative was used in the medium for further screening. Other organic nitrogen sources showed a decrease in PIPLC activity (Fig 2). However, there was no complete inhibition as reported for general C-type phospholipases[24]. Inhibition of these phospholipases by complex organic nitrogen sources is attributed to the presence of high amounts of organic phosphates which are ultimately converted to inorganic phosphates by phosphatases. Production of C-type phospholipases such as phosphatidylcholine-specific phospholipase C (PCPLC) is reported to be inhibited by high levels of inorganic phosphate in the medium[25]. However, PIPLC production was found to be independent of phosphate concentration in the medium (data not shown) (Fig S2; Supporting information) as reported earlier[26, 27]. This implies that some other factor is responsible for reduced PIPLC elaboration by *Staph. aureus* in presence of complex organic nitrogen sources such as yeast extract and beef extract. It also points to the fact that, unlike other C-type phospholipases, PIPLC does not play a role in phosphate- scavenging mechanism but functions as a virulence factor.

The optimum pH of 5.5 (Fig 3), is in accord with that reported by Marques *et al*[28]. However, the increase in PIPLC production was not five-fold as reported by them for the high yielding clinical isolates of *Staph. aureus.* This is also reflected by the Plackett-Burman experimental result where initial pH of the medium showed a statistically insignificant negative influence. Nonmaintained pH of the growth medium decreases *agr* expression[23]. The significant positive impact of Na_2CO_3

indicates the importance of buffered medium for the *agr* controlled PIPLC production. Although *Staph. aureus* is a facultative anaerobe, high agitation speed of 200 rev min^{-1} for effective aeration was another key factor found responsible for maximum PIPLC activity.

CONCLUSION

Plackett-Burman design was effectively employed for screening the important factors among a large number of variables. It allows one to obtain unbiased estimates of linear effects of all factors with maximum accuracy for a given number of observations[29]. The results indicate that nutritional factors (low C/N ratio), buffering agent and physical parameters such as pH and aeration influence expression of PIPLC by *Staph. aureus* ATCC 9144. The environment surrounding an infection site is crucial in determining bacterial proliferation and expression of extracellular proteins and toxins. Present report contributes to identification of environmental factors that may modulate the expression of PIPLC by an infecting *Staph. aureus* strain *in vivo* or in food stuffs. Further, emerging importance of microbial PIPLC in research and industry demands the enzyme to be produced in high yields. This report, which is the first of its kind, has major implication in optimization of conditions for PIPLC production from *Staph. aureus*.

ABBREVIATIONS

PIPLC : Phosphatidylinositol-specific phospholipase C
PI : Phosphatidylinositol
IP : myo-inositol phosphate

ACKNOWLEDGEMENT

This work was supported by the financial assistance from the University Grants Commission, India.

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