Global J. Environ. Sci. Manage., 1 (1): 63-70, Winter 2015
DOI: 10.7508/gjesm.2015.01.006

Optimizing the production of Polyphosphate from *Acinetobacter towneri*

J. Aravind; T. Saranya; P. Kanmani

Department of Biotechnology, Kumaraguru College of Technology, Coimbatore – 641049, India

Received 7 October 2014; revised 10 November 2014; accepted 1 December 2014; available online 1 December 2014

ABSTRACT: Inorganic polyphosphates (PolyP) are linear polymers of few to several hundred orthophosphate residues, linked by energy-rich phosphoanhydride bonds. Four isolates had been screened from soil sample. By MALDI-TOF analysis, they were identified as Bacillius cereus, Acinetobacter towneri, B. megaterium and B. cereus. The production of PolyP in four isolates was studied in phosphate uptake medium and sulfur deficient medium at pH 7. These organisms had shown significant production of PolyP after 22h of incubation. PolyP was extracted from the cells using alkaline lysis method. Among those isolates, Acinetobacter towneri was found to have high (24.57% w/w as P) accumulation of PolyP in sulfur deficient medium. The media optimization for sulfur deficiency was carried out using Response surface methodology (RSM). It was proven that increase in phosphate level in the presence of glucose, under sulfur limiting condition, enhanced the phosphate accumulation by Acinetobacter towneri and these condition can be simulated for the effective removal of phosphate from wastewater sources.

Keywords: *Polyphosphates, Biopolymer, MALDI-TOF, Sulfur deficient medium, Plackett-Burman, Neisser stain*

INTRODUCTION

Polyphosphate (polyP) appears to have always been an easy and rich source of energy from prehistoric times to today. It is one of the most widely distributed natural biopolymers, having been detected in many bacteria, fungi, yeasts, plants and animals (Dawes and Senior, 1973; Kulaev and Vagabov, 1983; Kulaev *et al.*, 1999). Polyphosphates are salts or esters of polymeric oxyanions formed from tetrahedral PO_4 (phosphate) structural units linked together by sharing oxygen atoms. They are linear, cyclic, cross-linked or branched polymers of orthophosphate residues linked by high-energy phospho-anhydride bonds equivalent to the bonds in ADP and ATP (Kulaev, 1975).

PolyP has been detected in abundance in all the living forms ranging from the prokaryotes to mammals, in the volcanic condensates, plants, and deep oceanic steam vents, indicating that it can be formed spontaneously by simple condensation of orthophosphoric acids under high temperature. They are present in the mammalian cells and sub- cellular organelles like lysosomes, mitochondria, mainly higher in nuclei. PolyP is more abundant in microbes than in plants and animals (Kornberg, 1995).

The microbial synthesis of intracellular polyP is primarily catalyzed by the enzyme polyphosphate kinase through the reversible transfer of the gamma phosphate of ATP to polyp (Geissdorfer *et al.*, 1998; Kornberg and Fraley, 2000). PolyP hydrolysis is mediated by exopolyphosphatases (PPX), endopolyphosphatases (Mullan *et al.*, 2002). Polyphosphate accumulating organisms (PAOs) are known as the microorganisms to absorb free phosphate in the environment and assimilate them as intracellular polyphosphate (poly-P) particles. This process was viewed as enhanced biological phosphorus removal (EBPR) in wastewater treatment systems (Bond *et al.*, 1999; Mino *et al.*, 1998; Oehmen *et al.*, 2007).

Many microorganisms (e.g., *Acinetobacter, Aerobacter, Bacillus, Pseudomonas, E.coli, Moraxella, Mycobacterium* and *Corynebacterium*) utilize phosphorus, which enters into the composition of several macromolecules in the cell (Streichan et al., 1990; Auling *et al.*, 1991). Some microorganisms have

*Corresponding Author Email: *dr.j.aravind@gmail.com*
Tel.: +91 422 266 9401; Fax: +91 422 266 9401

the ability to store phosphorus as polyphosphates in volutin granules. PolyP accumulation usually takes place under two conditions: PolyP overplus and PolyP luxury uptake. *Acinetobacter* spp. are able to accumulate more phosphate than is required for cell synthesis; the so-called luxury phosphate uptake. It is the process in which the organism produces PolyP when another nutrient rather than phosphate limits its growth (e.g: sulfur starvation), thereby permitting organism to accumulate excess phosphate reserves that would become highly valuable under future potential of phosphate starvation is called PolyP luxury uptake (Fuhs and Chen, 1975).

Although *Acinetobacter* sp. and *Burkholderia* sp. are found in low numbers, their capacity to accumulate poly-P intracellularly was the highest amongst all the isolates (Khoi and Diep, 2013). PolyP granules contain "acid-insoluble" polyP with long-chains and are present in the cytoplasm of various prokaryotes. In bacterial cells, there is also "acid-soluble" polyP with short-chains that can be found in various cell compartments (on the cell surface, in the perisplasm, and in the plasma membrane) (Kulaev, 1975).

Polyphosphates are capable of forming complexes with other polymers especially proteins, basic polypeptides and nucleic acids. This property is due to a simple interaction between cations and anions. Polyphosphate can be observed under bright-field or phase-contrast microscopy. Neisser's stain is used to observe these granules under bright-field microscope (Serafim *et al.*, 2002). Nuclear magnetic resonance (NMR) has been also recently used to detect polyphosphate granules in wastewater microorganisms (Florentz and Granger, 1983; Suresh *et al.*, 1984).

PolyP has some basic properties which determine its application in various fields of medicine, agriculture, industry, etc. PolyP can be used as an antibacterial agent in all processed meat, poultry, and fish products (Kulakovskaya *et al.*, 2012). It can also be used as a safe additive to meat processing as it enhances water binding, emulsification, colour retention, and antioxidant capacity. PolyP is used in wastewater treatment by sequestering iron, manganese, and alkaline earth metals such as calcium and magnesium. Calcium PolyP fiber is also used as a scaffold material for tendon tissue engineering in vitro (Sun and Zhao, 2002).

MATERIALS AND METHODS

Sampling and organism isolation

Soil sample were serially diluted and it was plated on Leeds *Acinetobacter* agar (LAM) and incubated at37 °C for 24 hours. Pink coloured colonies appearing on LAM agar plates indicate positive result for screening of *Acinetobacter* species (Jawad *et al.*, 1994).

Media

Phosphate uptake medium:

PAOs were cultivated at 30 °C for 22 h in the media contained (g/l): 5g CH_3COONa, 0.5g $MgSO_4.7H_2O$, 0.18g KNO_3, 0.25g KH_2PO_4, 0.5g peptone, 0.5g yeast extract and 0.5ml trace elements. The trace elements contained (g/l): 1.5g $FeCl_3.6H_2O$, 0.15g H_3BO_3, 0.03g $CuSO_4.5H_2O$, 0.18g KI, 0.12g $MnCl_2.4H_2O$, 0.06g $Na_2MoO_4.2H_2O$, 0.12g $CoCl_2.6H_2O$ and 10g EDTA. The pH of the medium was adjusted to 7.0 (Harold, 1966).

Sulfur deficient medium

PAOs accumulate larger amounts of PolyP in stationary phase sulfur deficient cultures or when inorganic PolyP is added to phosphate starved cells. The medium contained the following composition (g/l): 5g glucose, 0.5g KH_2PO_4, 1g NH_4Cl, 1g NaCl, 0.01g $MgCl_2$, 0.001g Na_2SO_4, 10g tris-(hydroxyl methyl-methylamine). The pH of the medium was adjusted to 7.0. The cultures were grown at 30 °C for 22 h (Harold, 1966).

Extraction of PolyP

After 22h of incubation, 4ml of cell suspension was harvested and centrifuged.The pellet was resuspended in 0.2N NaOH and kept in shaker at 160rpm for 20h. After 20h of lysis, the sample was again centrifuged. The supernatant was separated into two parts. One for direct orthophosphate assay and another was mixed with equal volume of 1N HCl and kept for hydrolysis in water bath at 100 °C for 10 min. Then it was cooled and orthophosphate assay was carried out. The difference in values of hydrolyzed and unhydrolyzed samples represents the presence of PolyP (Khoi and Diep, 2013).

Orthophosphate assay

To 0.2 ml of sample, 2.0 ml of molybdate reagent (15mM ammonium molybdate and 100 mM zinc acetate, pH to 5.0 w/HCl) was added followed by 0.5 ml of ascorbic acid reagent (10% ascorbic acid adjusted to pH 5.0 w/NaOH). The assays were incubated for 15 min at 30 °C and then

the absorbance was read at 850 nm. Concentrations were calculated from standard curves prepared by assaying KH_2PO_4 standards (Saheki *et al.*, 1985).

Medium optimization

Response Surface Methodology

The interactive studies on four significant factors A (Glucose), B (KH_2PO_4), C (Na_2SO_4) and D (NH_4Cl) on the response (Yield % of PolyP) were determined statistically using RSM (Box-Behnken design) Design Expert software (Trial Version 9.0.1).

A matrix consisting of 28 experiments with 4 replicates at the center point generated by the software was applied for maximizing the yield of PolyP. Production was carried out in 100 ml of sulfur deficient medium (pH 7.0), inoculated with *Acinetobacter towneri* and incubated at 37 °C for 22h in an orbital shaker. After incubation period, PolyP was extracted using alkaline lysis method.

The quadratic model was analyzed statistically. Analysis of Variance (ANOVA) table including F-test was used to judge the model's significance and regression factor. The second order polynomial equation was expressed in the form of contour and 3D plots.

RESULTS AND DISCUSSION

Isolation and Screening

Four different colonies were screened on LAM plates. By MALDI-TOF analysis, they were identified as *Bacillius cereus* 994000168 LBK, *Acinetobacter towneri* DSM 14962T HAM, *B. megaterium* DSM 32T DSM *and B. cereus* 4080 LBK.

The production of PolyP was analyzed in phosphate uptake medium and sulfur deficient medium and *Acinetobacter towneri* was able to produce 11.6 % (w/w) of polyphosphate under the phosphate uptake medium condition (Fig 1).

Acinetobacter towneri had higher accumulation of PolyP in both the media when compared to other organisms, and the production of PolyP was higher (24.57% as P) in sulfur deficient medium than in phosphate uptake medium (Figs. 1 and 2).

Response Surface Methodology

RSM deals with the interaction study of variables and helps in optimization of media components within a minimal number of experimental runs. Based on the results obtained from Plackett-Burmann (data not shown), four variables had been chosen for optimization via Box-Behnken design. The experimental

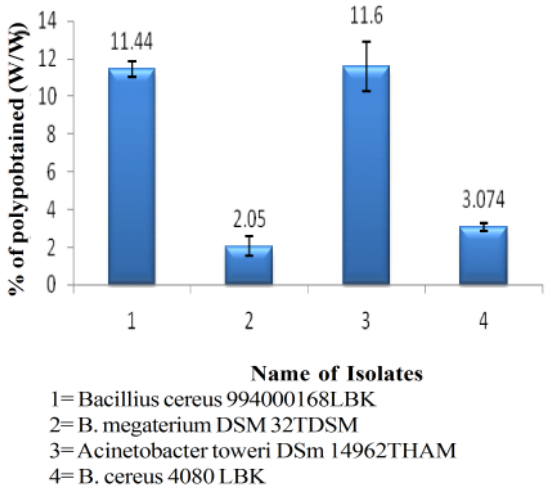

Fig. 1: Production of PolyP from different isolates in phosphate uptake medium

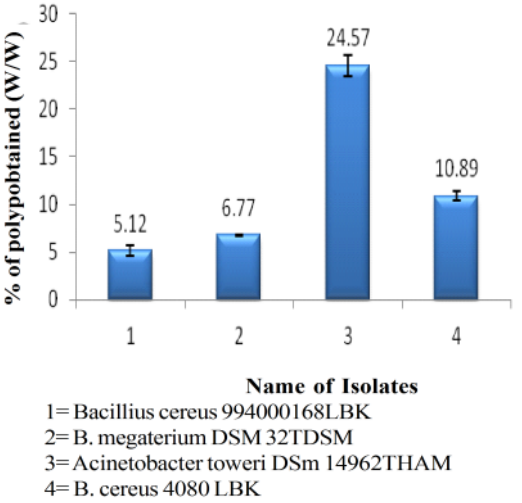

Fig. 2: Production of PolyP from different isolates in sulfur deficient medium

design setup is shown in Table 1.

Final equation in terms of coded factors

Yield = 25.45 + 0.89*A -1.40*B – 1.05*C – 0.91*D + 2.29*AB + 3.37*AC + 0.59*AD – 0.097*BC + 4.54*BD – 0.90*CD – 8.07*A^2 – 7.10*B^2 – 9.10*C^2 – 7.22*D^2 (1)

From equation (1), it has been shown that the combination of media components: glucose-NH_2PO_4, glucose-Na_2SO_4 and Na_2SO_4-NH_4Cl has positively influencing the productivity, while NH_2PO_4-Na_2SO_4 has negatively influenced the productivity of PolyP. Hence these condition can be effectively used by *Acinetobacter towneri* for removal of phosphate from phosphate containing effluent effectively

The ANOVA results and diagnostic case studies are given in Table 2. The actual vs. predicted values are depicted in Table 3.

The P value serves as a tool for checking the significance of each of the coefficients and its interaction with other factors. Low values of $P<0.05$ indicates the significant role of medium components on PolyP production which is further strengthened by their higher F-value obtained statistically. Also A^2, B^2, C^2 and D^2 are significant which also proves the impact of higher concentrations of these medium components interaction on polyphosphate production.

R^2 value gives a measure of how much variability in the observed response can be explained by the experimental

Table 1: Experimental design of Box-Behnken

Run	Factor 1 A: Glucose* (g)	Factor 2 B: KH_2PO_4# (g)	Factor 3 C: Na_2SO_4^ (mg)	Factor 4 D: NH_4Cl+ (g)	Response 1 Yield (%)
1	0.10	0.01	5.50	0.17	13.6
2	1.50	0.20	5.50	0.17	17.2
3	0.80	0.11	10.00	0.05	14.17
4	0.80	0.11	5.50	0.17	24.2
5	0.80	0.01	5.50	0.05	18.45
6	0.10	0.11	1.00	0.17	15.27
7	0.80	0.11	1.00	0.05	15.56
8	1.50	0.01	5.50	0.17	15.8
9	1.50	0.11	5.50	0.05	6.38
10	0.80	0.11	5.50	0.17	23.85
11	0.80	0.20	5.50	0.05	5.73
12	0.80	0.01	1.00	0.17	6.5
13	0.80	0.01	10.00	0.17	7.12
14	0.80	0.20	1.00	0.17	5.14
15	0.10	0.11	5.50	0.30	6.34
16	1.50	0.11	5.50	0.30	9.02
17	0.80	0.01	5.50	0.30	8.24
18	0.10	0.11	10.00	0.17	5.03
19	0.80	0.11	5.50	0.17	26.34
20	1.50	0.11	1.00	0.17	5.58
21	1.50	0.11	10.00	0.17	8.8
22	0.10	0.20	5.50	0.17	5.83
23	0.10	0.11	5.50	0.05	6.05
24	0.80	0.20	5.50	0.30	13.7
25	0.80	0.11	1.00	0.30	11.53
26	0.80	0.11	10.00	0.30	6.54
27	0.80	0.11	5.50	0.17	27.41
28	0.80	0.20	10.00	0.17	5.37

*Glucose high- 1.5g/l low-0.1g/l, #KH2PO4 high-0.2g/l low-0.1 g/l, ^Na2SO4 high-10mg/l low-1mg/l, +NH4Cl high-0.3g/l low-0.05g/l.

Table 2: ANOVA table for RSM quadratic model

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F
Model	1080.12	14	77.15	4.12	0.0075*
A-Glucose	9.47	1	9.47	0.51	0.4895
B- KH_2PO_4	23.35	1	23.35	1.25	0.2843
C- Na_2SO_4	13.13	1	13.13	0.70	0.4175
D- NH_4Cl	10.03	1	10.03	0.54	0.4772
AB	21.02	1	21.02	1.12	0.3086
AC	45.29	1	45.29	2.42	0.1438
AD	1.38	1	1.38	0.074	0.7902
BC	0.038	1	0.038	$2.031x10^{-3}$	0.9647
BD	82.63	1	82.63	4.41	0.0557
CD	3.24	1	3.24	0.17	0.6842
A^2	390.63	1	390.63	20.87	0.0005
B^2	302.14	1	302.14	16.14	0.0015
C^2	497.41	1	497.41	26.57	0.0002
D^2	312.55	1	312.55	16.70	0.0013
Residual	243.38	13	18.72	-	-
Lack of Fit	234.62	10	23.46	8.04	0.0565#
Pure Error	8.76	3	2.92	-	-
Cor Total	1323.49	27	-	-	-

R2 Value = 0.8161, * - Significant, #- not significant

parameters and their interactions. This model has R^2 value of 0.8161, which implies that it was better predictor up to 81.61%.

Adequate precision measures the signal to noise ratio. A ratio greater than 4 is desirable. In this model, the ratio of 7.096 indicate an adequate signal and this model can be used to navigate the design space.

The regression equation is expressed graphically in the form of contour plots which indicate the interaction among independent factors and their influence on yield of PolyP. The contour plots of all the combination of interactions were found to be elliptical. The contour plots were generated by varying the levels of two factors while keeping the third one constant (Figs. 3, 4, 5, 6, 7 and 8).

RSM design yielded a maximum yield of PolyP of 27.41% in trial no.27 and the values of factors were 0.80g glucose, 0.11g KH_2PO_4, 5.5 mg Na_2SO_4 and 0.17g NH_4Cl. This is a 1.1 fold increase in comparison with the pre-optimization maximum value in sulfur deficient medium.

CONCLUSION

PolyP, inorganic polymers are made up of phosphate residues linked by sharing oxygen atom. They were produced by *Acinetobacter* under luxuty phosphate uptake conditions. Based on the pre-optimization studies on sulfur deficient medium, it has been found that *Acinetobacter towneri* has higher production (24.57% w/w as P) of PolyP when compared to other organisms screened. Box-Behnken design applied successfully to obtain maximum productivity of PolyP under sulfur limiting conditions. Based on this design, the optimal values of factors, 0.80g glucose, 0.11g KH_2PO_4, 5.5g Na_2SO_4 and 0.17g NH_4Cl resulted in 1.1 fold increase in PolyP accumulation in *Acinetobacter towneri*. The model was statistically significant based on low P and high F values. It can be concluded that the model can be applied for production of PolyP from *Acinetobacter towneri* and can be simulated further for effective phosphates removal from effluents.

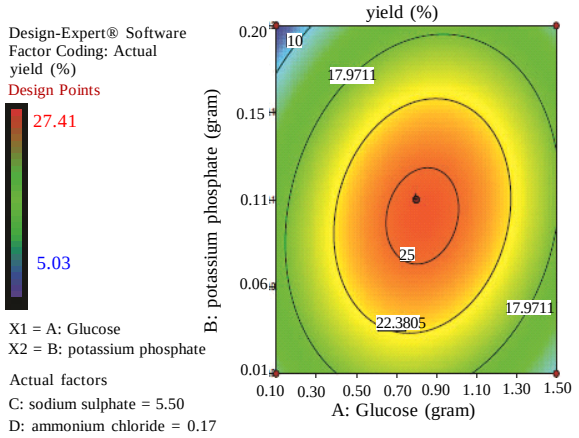

Fig 3: Contour plot – showing interaction of Glucose and KH_2PO_4 towards yield

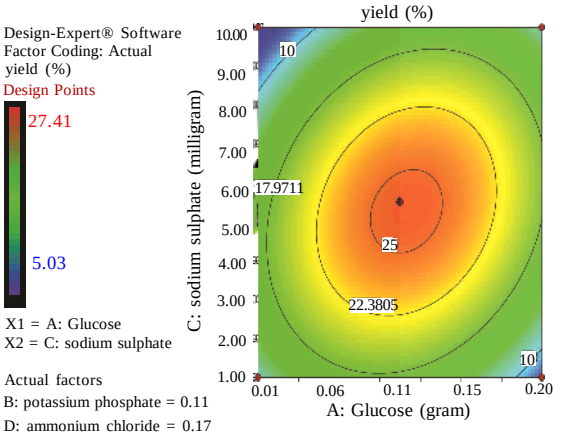

Fig 4: Contour plot-showing interaction of Glucose and Na_2SO_4 towards yield

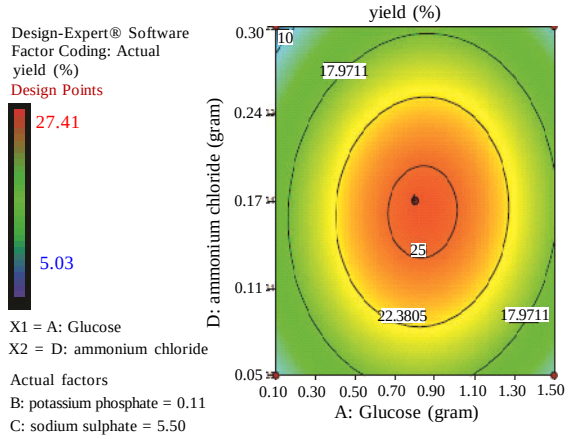

Fig 5: Contour plot – showing interaction of Glucose and NH_4Cl towards yield

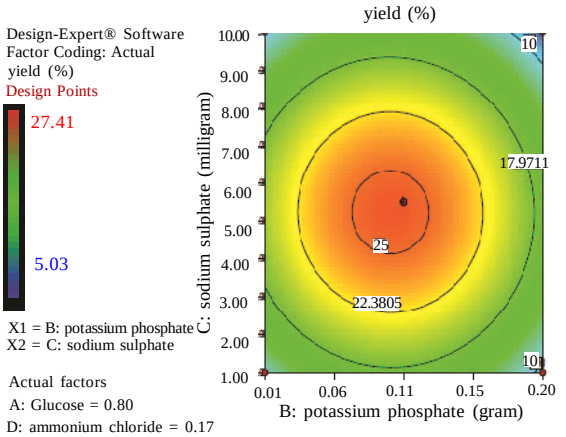

Fig 6: Contour plot – showing interaction of KH_2PO_4 and Na_2SO_4 towards yield

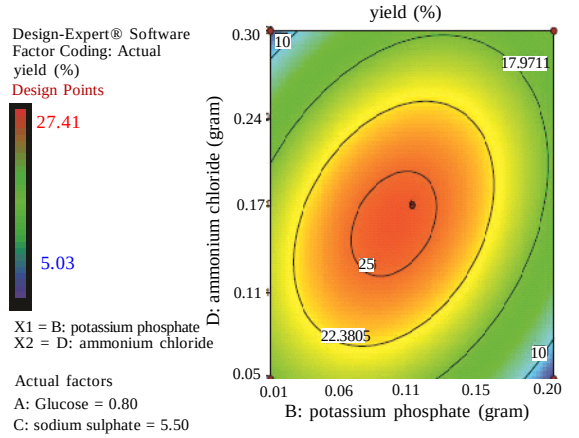

Fig 7: Contour plot – showing interaction of KH2PO4 and NH4Cl towards yield

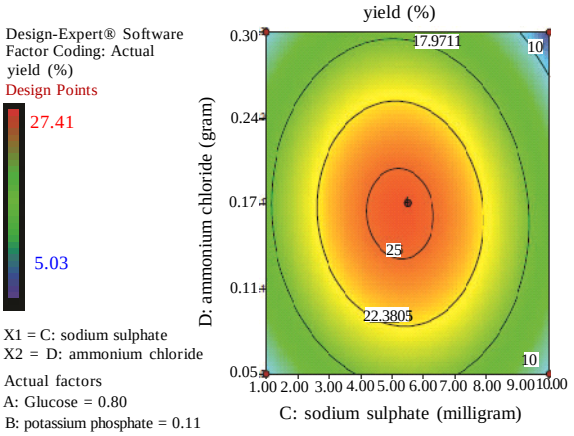

Fig 8: Contour plot – showing interaction of Na2SO4 and NH4Cl towards yield

Table 3: Actual and predicted values for RSM design

Run Order	Actual Value	Predicted Value
1	13.60	13.08
2	17.20	12.07
3	14.17	9.90
4	24.20	25.45
5	18.45	17.99
6	15.27	11.80
7	15.56	10.19
8	15.80	10.28
9	6.38	11.38
10	23.85	25.45
11	5.73	6.11
12	6.50	11.59
13	7.12	9.70
14	5.14	9.00
15	6.34	7.77
16	9.02	10.73
17	8.24	7.07
18	5.03	2.98
19	26.34	25.45
20	5.58	6.85
21	8.80	11.48
22	5.83	5.71
23	6.05	10.78
24	13.70	13.37
25	11.53	10.16
26	6.54	6.27
27	27.41	25.45
28	5.37	6.71

REFERENCES

Auling, G.; Pilz, F.; Busse, H.J.; Karrasch, M.; Streichan, M.; Schon, G., (1991). Analysis of polyphosphate accumulating microflora in phosphorus-eliminating, anaerobic-aerobic activated sludge systems by using diaminopropane as a biomarker for rapid estimation of *Acinetobacter spp.* Appl. Environ. Microbiol., (57): 3585-3592 **(8 pages)**.

Bond, P.L.; Erhart, R.; Wagner, M.; Keller, J.; Blackall, L.L., (1999). Identification of some of the major groups of bacteria in efficient and non-efficient biological phosphorus removal activated sludge systems. Appl. Environ. Microbiol., 65(9): 4077-4088 **(11 pages)**.

Dawes, E.A.; Senior, P.J., (1973). Energy reserve polymers in microorganisms. Adv. Microb. Physiol., (10): 178-203 **(26 pages)**.

Florentz, M.; Granger, P., (1983). Phosphorus-31 nuclear magnetic resonance of activated sludge: Use for the study of the biological removal of phosphates from wastewater. Environ. Technol. Lett., (4): 9-12 **(4 pages)**.

Fuhs, G.W.; Chen, M., (1975). Microbial basis of Pi removal in the activated sludge process for the treatment of waste water. Microbial. Ecol., (2): 119-138 **(20 pages)**.

Geissdorfer, W.; Ratajczak, A.; Hillen, W., (1998). Transcription of *ppk* from *Acinetobacter sp.* strain ADP1, encoding a putative polyphosphate kinase, is induced by phosphate starvation. Appl. Environ. Microbiol., (64): 896-901 **(6 pages)**.

Harold, F.M., (1966). Inorganic polyphosphates in biology: structure, metabolism, and Function Bacteriol. Rev., (30): 772-794 **(23 pages)**.

Jawad, A.; Hawkey, P. M.; Heritage, J.; Snelling, A. M., (1994). Description of Leeds *Acinetobacter* Medium, a new selective and differential medium for isolation of clinically important *Acinetobacter spp.*, and comparison with Herellea agar and Holton's agar. J. Clin. Microbiol., (32): 2353–2358 **(6 pages)**.

Khoi, L.Q.; Diep, C. N., (2013). Isolation and phylogenetic analysis of polyphosphate accumulating organisms in water and sludge of intensive catfish ponds in the Mekong Delta, Vietnam American. J. Life Sci., 1(2): 61-71 **(11 pages)**.

Kornberg, A., (1995). Inorganic polyphosphate: Towards making a forgotten polymer Unforgettable. J. Bacteriol., 177(3): 491-496 **(6 pages)**.

Kornberg, A.; Fraley, C.D., (2000). Inorganic polyphosphate: a molecular fossil come to life ASM. News, (66): 275-280 **(6 pages)**.

Kulaev, I.S., (1975). Biochemistry of inorganic polyphosphates. Rev. Physiol. Biochem. Pharmacal., (73): 131-158 **(28 pages)**.

Kulaev, I.S.; Vagabov, V. M., (1983). Polyphosphate metabolism in microorganisms. Adv. Microbiol. Physiol., (15): 731-738 **(8 pages)**.

Kulaev, I.S.; Vagabov, V. M.; Kulakovskaya, T.V., (1999). New aspects of inorganic polyphosphate metabolism and function. J. Biosci. Bioeng., .(88): 111-129 **(19 pages)**.

Kulakovskaya, T.V.; Vagabov, V. M.; Kulaev, I. S., (2012). Inorganic polyphosphates in industry, agriculture and medicine: Modern state and outlook. Process Biochem., (47): 1-10 **(10 pages)**.

Mino, T.; Van Loosdrecht, M. C. M.; Heijnen, J. J., (1998). Microbiology and biochemistry of the enhanced biological phosphate removal process. Water Res., (32): 3193–3207 **(15 pages)**.

Mullan, A.; Quinn, J. P.; McGrath, J. W., (2002). Enhanced Phosphate Uptake and Polyphosphate Accumulation in *Burkholderia cepacia* Grown under Low-pH Conditions. Microb. Ecol., (44): 69-77 **(9 pages)**.

Oehmen, A.; Lemos, P. C.; Carvalho, G.; Yuan, Z.; Keller, J.; Blackall, L. L.; Reis, M. A. M., (2007). Advances in enhanced biological phosphorus removal: from micro to macro scale. Water Res., (41): 2272-2300 **(29 pages)**.

Saheki, S.; Takeda, A.; Shimazu, T., (1985). Assay of inorganic phosphate in the mild pH range, suitable for measurement of glycogen phosphorylase activity. Anla. Biochem., (148): 277-281 **(5 pages)**.

Serafim, L. S.; Lemos, P. C.; Levantesi, C.; Tandoi, V.; Santos, H.; Reis, M. A. M., (2002). Methods for detection and visualization of intracellular polymers stored by polyphosphate-accumulating microorganisms. J. Microbiol. Met., (51): 1-18 **(18 pages)**.

Streichan, M.; Golecki, J.R.; Schon, G., (1990). Polyphosphate- accumulating bacteria from sewage plants

with different processes for biological phosphorus removal FEMS. Microbiol. Ecol., (73), 113-124 **(11 pages)**.

Sun, Z.Y.; Zhao, L., (2002). Feasibility of calcium polyphosphate fiber as scaffold materials for tendon tissue engineering in vitro. Zhongguo Xiu Fu Chong Jian Wai Ke Za Zhi, (16): 426–428 **(3 pages)**.

Suresh, N.; Warburg, R.; Timmerman, M.; Wells, J.; Coccia, M.; Roberts, M. F.; Halvorson, H. O., (1984). New strategies for the isolation of microorganisms responsible for phosphate accumulation [in] Enhanced Biological phosphorus Removal from Wastewater". Proceeding of the IAWPRC Post Conference, Paris.

AUTHOR (S) BIOSKETCHES

Aravind, J., Ph.D. Assistant Professor, Faculty of Biotechnology Kumaraguru College of Technology Coimbatore, India. E-mail: *dr.j.aravind@gmail.com*

Saranya, T., Kumaraguru College of Technology. E-mail: *saranbiotech007@gmail.com*

Kanmani, P., Kumaraguru College of Technology. E-mail: *kanmani.p.bt@kct.ac.in*

How to cite this article: (Harvard style)

Aravind, J.; Saranya, T.; Kanmani, P., (2015). Optimization and production of Polyphosphate from Acinetobacter towneri DSM 14962T HAM. Global J. Environ. Sci. Manage., 1 (1): 63-70.