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Chemo-characterization and optimization of macro and micro nutrients for exopolysaccharides and mycelia growth in pleurotus tuberregium (RUMPH, EX FR)

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Abstract

Exoploysaccharides are potential nutraceutic, probiotic, phamarceutic and cosmesceutic natural products in fungi. *Pleurotus tuberregium* is an underutilized tropical fungus that is useful as food with recent reports of pharmacological activities. The present study evaluated and characterized environmental conditions and nutrients for optimal *P. tuberregium* mycelia growth (PtMG) and exopolysaccharides (PtEPS) production using batch culturing and diverse chemometric methods, respectively. Rank-sum analysis categorized the assessed conditions and nutrients into six stimulatory classes and most suitable nutrients and conditions for PtMG and PtEPS were co-identified with analysis of variance. Glucose, yeast, sodium chloride (NaCl) and tricalcium diphosphate ($Ca_3(PO_4)_2$) were excellent stimulant (ES) for both PtEPS and PtMG under culturing condition of pH 6 at 30 °C for 15 d. The Chemo-phene trees generated by cluster analysis allowed visualization and clear-cut demarcation of the six stimulatory classes. The information embedded in the present study is useful for improving and developing culturing media for optimal PtMG and PtEPS yields, enabling maximal *P. tuberregium* utilization as food and medicine.

Keywords: chemometrics; environmental condition and nutrients; *P. tuberregium* exopolysaccharides (PtEPS); *P. tuberregium* mycelia growth (PtMG); stimulant.

Practical Application: Industrial utilization of *P. tubberregium* for production of exopolysaccharide and fungus biomass.

1 Introduction

Exopolysaccharides (EPS) are membrane bound polysaccharides found in large family of lower organisms including fungi and released as slime/capsules into immediate environments. Some roles ascribed to EPS in fungi are substrate adhesion, defense, adsorption and storage of nutrients (Wotton, 2004). EPS are high molecular weight polymers of diverse homo or heterosugars (Zhang et al., 2004; Zhao et al., 2014). The immunomodulatory, antimicrobials, antioxidant, antitumor, hypoglycemic and anti-tyrosinase properties of EPS have been reported (Hwang, et al., 2005; Borges et al., 2013; Barakat & Sadik, 2014; Zhao et al., 2014). Therefore, EPS has potential applications in pharmaceutical, neutraceutical, and probiotical for many devastating diseases such as diabetes and cancer. Bae et al. (2005) reported potentials of EPS in cosmetics, cosmeceutical, and nutricosmetical industries for skin treatment. In addition, EPSs are use industrially, as emulsifiers, thickeners, stabilizers, and gelling agents (Mahapatra & Banerjee, 2013).

Exopolysaccharides have been isolated in diverse fungi using different techniques and biomass materials and still possess similar medicinal properties (Borges et al., 2013; Zhao et al., 2014; Anike et al., 2015). Advantages of bio-exopolysaccharides over chemosynthetic EPS are: they are renewable, non-toxic and biodegradable (Freitas et al., 2011). Fungi of the *Pleurotus* genus are nutritious because of their reported high content of proteins, fibers, low calorie, vitamins and minerals content (Reis et al., 2012). Their extracts in various solvents and bioactive have been tested for various pharmacological properties for therapeutic and preventive purposes and these properties are associated to their polysaccharides content (Hwang, et al., 2005; Borges et al., 2013; Barakat & Sadik, 2014; Zhao et al., 2014).

Pleurotus tuberregium (Fr.), the king tuber mushroom, is a lesser known edible fungus that belongs to the Basidiomycotina and mainly distributed in tropical and subtropical regions such as Africa, Australia, and China with history of use as food and medicine (Oso, 1977). In Nigerian village settings, it is use mainly as a substitute for meat protein in stew and soups, and is cherish as a delicacy in many dishes as a flavor and important nutrients in urban. It can be cooked alone as mushroom soup or with other vegetables or assorted porridge dishes. Extracts from *P. tuberregium* have been associated with antitumor and superoxide radical scavenging activities and for treating headaches, fever and stomach upsets (Zhang et al., 2004; Oyetayo, 2011). Its EPS has been shown to possess pharmacological activities such as antihyperglycemic, antihyperlipidemic and antioxidant properties (Huang et al., 2012; Bamigboye et al., 2016).

Nevertheless, the potential of *P. tuberregium* to produce EPS and fungus biomass for food and medicinal uses is underexplored. In addition, few studies have been conducted to identify the best cultivation conditions and extensive nutrient

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supplements for its EPS production in submerged fermentation. Furthermore, chemometric methods such as Duncan multiple range test, rank-sum and cluster analysis for characterization and identification of most suitable culturing conditions and nutrients in fungi are underexplored. Therefore, characterization, identification using chemometrics and optimizing environmental conditions and nutrient supplements for optimal PtEPS and PtMG in submerged fermentation is sought in the present study to maximize *P. tuberregium* utilization for EPS and MG production.

2 Materials and methods

All chemical reagents were of AOAC standard and distilled water was prepared in the Department of Chemical Sciences Laboratory, Tai Solarin University of Education, Ijagun, Ijebu-Ode, Nigeria, where all experiments were carried out in batch culturing mode in three replicates.

2.1 Microorganism sample

Sclerotia of *Pleurotus tuberregium* used in this study were purchased from Bodija market, Ibadan, Oyo State, Nigeria. The sclerotia were washed thoroughly with tap water to remove adhering debris, dried at 30 °C to constant weight and stored at room temperature (25 ± 2 °C). A hundred gram sclerotia was soaked in dil. H_2O for 30 min, incubated for 3 d and finally, plated on 4% malt extract agar medium at 25 °C.

2.2 Assessment of environmental culturing conditions

Basal medium (pH 7.0) used contained 3 g yeast extract, 3 g malt extract, 1 g potassium dihydrogen phosphate hydrate ($KH_2PO_4.H_2O$), 50 g glucose, 5 g calcium nitrate ($Ca(NO_3)_2$), 1 g nicotinic acid, and 1 g magnesium sulphate ($MgSO_4$) per litre and served as positive control throughout the study. Incubating periods of 3, 6, 9, 12 and 15 d; cultivating temperatures of 20, 25, 30, 35, 40 and 45 °C; and pH 4, 5, 6, 7, 8, 9, and 10 were assessed for optimal yield of PtEPS and PtMG.

2.3 Assessment of nutritional supplements

For selection of most suitable nutrients for optimal yield of PtEPS and PtMG, 50 g glucose in the positive control medium was separately substituted with 50 g of fructose, xylose, sucrose, lactose, dextrose, and maltose. Three grams, each of yeast and malt extract in control medium were replaced with each of 10 g/L yeast, peptone, ammonium oxalate ($(NH_4)_2C_2O_4$), ammonium sulphate ($(NH_4)_2SO_4$), ammonium nitrate (NH_4NO_3) and ammonium chloride (NH_4Cl). For minerals, $KH_2PO_4.H_2O$ was swop with tricalcium phosphate ($Ca_3(PO_4)_2$) and sodium dihydrogen phosphate (NaH_2PO_4); and sulphate ion as $MgSO_4$ was replaced with hydrates of iron (ii) sulphate ($FeSO_4.7H_2O$), zinc sulphate ($ZnSO_4.7H_2O$) and copper (ii) sulphate ($CuSO_4.7H_2O$); and 1g each of Na, Ca, K and Mg chloride salt was added-value. Thirty millimeters of each experimental medium was sterilized at 121 ^{0}C for 15 min at 96 psi and 0.3 ml of 10% lactic acid was added to suppress bacterial growth after sterilization. Each 30 ml medium was inoculated with a 12 mm agar plus disc of vigorously growing *P. tuberregium* mycelium.

2.4 Determination of PtMG and total PtEPS in dry weight

The dry weight yield of PtMG was determined by filtration of the mycelium cultured medium through No. 1. Whatman filter paper. The residue was air dried to a constant weight using a sensitive weighing balance (Baran scientific and instrument company, England). The crude PtEPS from the various cultures was isolated using precipitation method. Ten milliliters of each cultured sample were transferred into a clean test tube and centrifuged at 4500 rpm for 15 min using Cole Centrifuge model 0414-1. Five milliliters of the supernatant was carefully transferred into a clean test tube with an addition of 10 ml 96% ethanol and left overnight at 4 °C. The precipitate was recovered by centrifugation at 4500 rpm for 15 min and washed successfully with 96% ethanol/H_2O (1:4 v/v), lyophilized (Labconco Vacutec) and weighed.

2.5 Analysis of data

Precision measures, ANOVA, and correlation analyses were carried out using SAS v. 9.2 (Statistical Analysis System, 2002). Characterization of environmental conditions and nutrients for PtMG and PtEPS yield was by chemometrics: Duncan multiple range tests, rank-sum analysis according to Moyib et al. (2015b), and Chemo-phene trees by dissimilarity analysis representative for windows (DARwin, Perrier & Jacquemoud-Collet, 2006).

3 Results and discussion

3.1 Optimizing environmental conditions

The pattern of trends shown in Figure 1 indicate favorable acidic medium, long incubating periods and increasing cultivating temperature till a peak at 30 °C for high yield of PtMG and PtEPS. Medium acidity of pH 6, culturing temperature of 30 °C and incubating period of 15 d were most suitable environmental conditions for production of both PtMG and PtEPS in *P. tuberregium*. Analysis of varance statistics shows various initial medium pHs, incubating periods and cultivating temperatures were highly significant for both the PtMG and PtEPS ($p < 0.0001$, $r^2 = 0.99$) except for non-significant difference among cultivating periods of 20, 25 and 35 d for PtEPS and Pearson correlation analysis shows a strong inverse relationship among level of pH with PtMG ($p<0.0001$, $r^2 = -0.82$) and PtEPS ($p<0.0001$, $r^2 = -0.81$) yields. Therefore, as the culturing medium acidity increases, levels of PtMG and PtEPS were decreasing, supporting a favorable acidic medium for *P. tuberregium* growth and metabolic activities as noted earlier. Incubating period had positive relationships with PtMG ($p<0.0001$, $r^2 = 0.84$) and PtEPS ($p<0.0001$, $r^2 = 0.81$), supporting lengthy incubation period. Cultivating temperature showed inverse relationships with both PtMG ($p<0.05$, $r^2 = -0.59$) and PtEPS ($P<0.001$, $r^2 = -0.65$) and apparently, indicates higher levels of PtMG and PtEPS at low temperatures, but highest level was obtained with a median temperature (30^{o}C) and hence, the low r^2 (0.59). The present results are similar to previous reports of acidic medium, a cultivating temperature above room temperature and above 10 d incubating period in fungi (Nehad & El-Shamy, 2010; Adejoye, et al. 2012; Joshi et al., 2013; Lai et al., 2014).

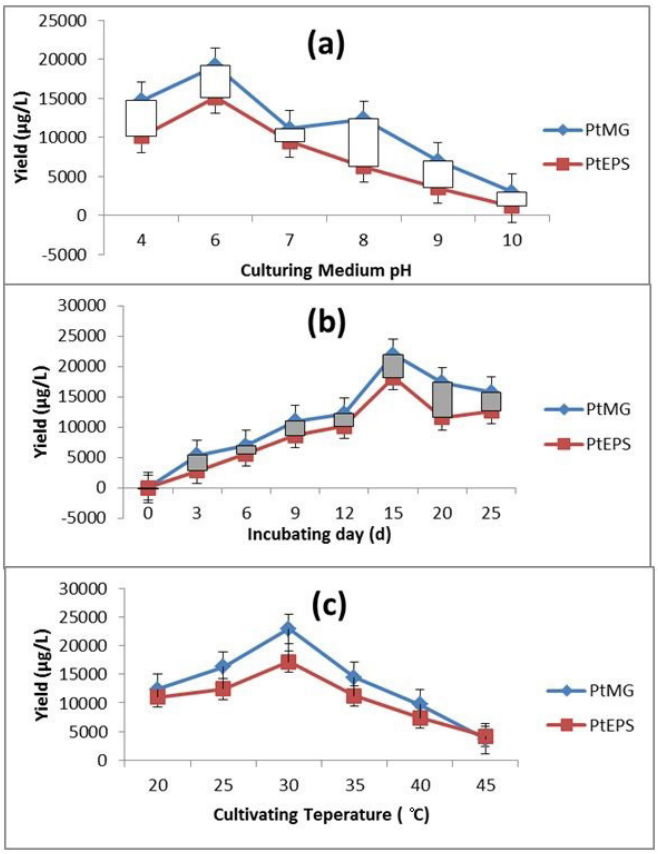

Figure 1. Trend of PtMG and PtEPS among environmental conditions in *P. tuberregium*. (a) Pattern of PtMG and EPS among various assessed media pH (b) pattern of PtMG and PtEPS among incubating periods (c) pattern of among cultivating temperatures.

3.2 Identifying suitable macro and micro nutrient sources

Optimal carbon sources for energy

Figure 2a shows glucose and lactose with highest and lowest yields, respectively, for both PtMG and PtEPS. Adejoye et al. (2012) suspicious of galactose' positive role in lactose for *L. squarrosulus is* somehow supported with its fourth position to glucose. The very low levels of PtMG and PtEPS observed in the negative control indicate *P. tuberregium* could survive in no-sugar medium but with low metabolic activities. Yields of PtMG and PtEPS were significantly distinct among the various carbon sources ($p<0.0001$, $r^2= 0.99$). The present choice of glucose as optimal carbon source for both PtMG and PtEPS is similar to reports in fungi (Nehad & El-Shamy, 2010; Barakat & Sadik, 2014; Lai, et al., 2014) and contrasts to starch, sucrose, maltose and xylose in some other studied fungi (Joshi et al., 2013; Mahapatra & Banerjee, 2013; Anike et al., 2015). Though, many diverse explanations such as affinity of a particular fungus for a specific sugar and preference between mono- and disaccharides were given but many lack biochemical elucidation. The present results are expected for glucose, one, as the only starting and direct sugar for glycolytic pathway that generates ATP in cells respiration (Berg et al., 2007). Two, the transportation barrier varies from facilitated simple diffusion for monosaccharides to active transport for maltose (Garrett & Grisham, 2005; Berg et al., 2007). Three, enzymes

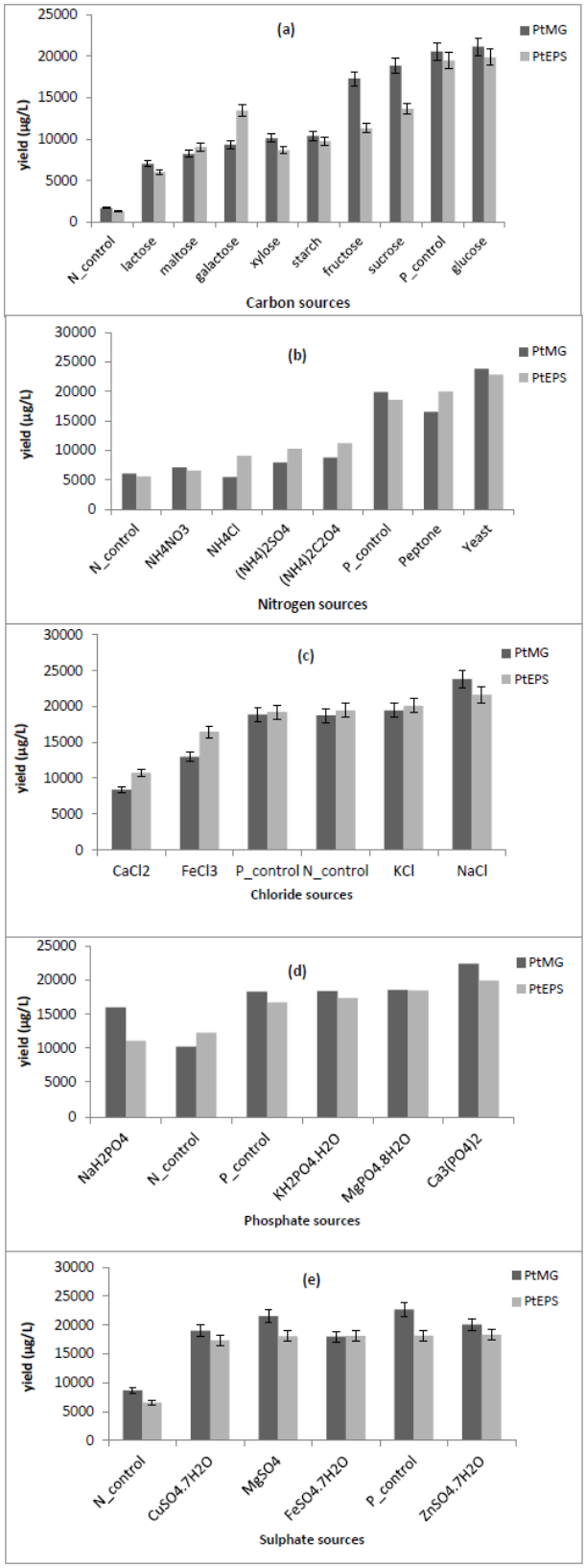

Figure 2. Trend of PtMG and PtEPS among carbon, nitrogen, and micro nutrients sources in *P. Tuberregium,* (a) carbons (b) nitrogen (c) chlorides (d) phosphates (e) sulphates. P_Control, positive control medium; N_control, basal medium without (a) glucose, (b) yeast and malt extract, (c) chloride, (d) KH_2PO_4 and (e) $MgSO_4$.

affinity for substrate suggests greater affinity of hexokinase to hexoses than glucosidase to disaccharides for breaking glycosidic bond (Garrett & Grisham, 2005; Berg et al., 2007). Therefore, the present result infers rate of metabolism of sugars, perhaps, as a potential limiting factor in MG and EPS production, which requires biochemical investigation.

Optimal nitrogen source for protein

Yeast showed highest induction and closely followed by peptone for PtEPS and PtMG yields (Figure 2b). Chemo-nitrogen sources were at the bottom level indicating their lower productivity to bio-nitrogen sources in *P. tuberregium*. The very low levels of PtMG and PtEPS observed in negative control indicate non-absolute reliance of *P. tuberregium* on nitrogen. High significant variation was observed among the assessed nitrogen sources for both PtMG and PtEPS ($p<0.0001$, $r^2 = 0.99$). The present result supports and establishes the importance of yeast as the most utilized nitrogen source and complex organic nitrogen are preferable over chemo-nitrogen by fungi (Nehad & El-Shamy, 2010; Joshi et al., 2013; Mahapatra & Banerjee, 2013; Anike et al., 2015).

Optimal chloride for macromineral

Figure 2c shows NaCl as the optimal chloride for both PtMG and PtEPS and the comparative levels obtained in the controls suggest low importance of chlorides for production of PtMG and PtEPS. The lowest level of $CaCl_2$ suggests an inhibitory effect, which could be explained by its larger molecular size, lattice energy and the amount used in term of either micro or macro source. The present result supports NaCl as a choice of chloride salt for MG while few mentioned $CaCl_2$ but when used as micro mineral (Lai et al., 2014; Boumaaza et al., 2015). The present result indicates low demand for chlorides as obtainable in NaCl while $CaCl_2$ and $FeCl_3$ weren't favorable and their dosage, perhaps, caused an osmotic pressure in the culturing media and might led to cytotoxicity (Turkkan, 2013).

Optimal sulphate and phosphate for micronutrient

According to Figure 2d, $Ca_3(PO_4)_2$, induced the highest level of both PtMG and PtEPS. Figure 2e shows $MgSO_4$ as the optimal source for PtMG and $ZnSO_4$ was followed by $FeSO_4.7H_2O$ and $MgSO_4$ for PtEPS production. The high levels of assessed sulphates signify importance of SO_4^{2-} for PtMG and PtEPS production and are in good utilizable forms (Srivastava, 1968). The assessed phosphate and sulphate salts were significantly demarcated for PtMG and PtEPS yields ($p<0.0001$, $r^2 = 0.99$). $Ca_3(PO_4)_2$ as the most suitable source could replace KH_2PO_4 in culturing medium for *P. tuberregium*. Since, $MgSO_4$ was optimal among sulphates for PtMG and followed $ZnSO_4$ for PtEPS, $MgSO_4$ was concluded as a choice of sulphate for both PtMG and PtEPS for convenience of further experiments. Similar studies merged phosphate and sulphate sources for assessment of optimal minerals and results reported so far differ greatly for comparison with the present observations due to non-similar mineral salts assessed (Srivastava, 1968; Turkkan, 2013; Lai et al., 2014).

3.3 Characterization of various environmental condition and nutrients for optimal production of PtMG and PtEPS

The 52 assessed environmental conditions and nutrients were ranked for their stimulatory effects on PtMG and PtEPS yields according to Moyib et al. (2015b). Six stimulant classes were generated, excellent stimulant (ES), good stimulant (GS), stimulant (S), fair stimulant (FS), poor stimulant (PS) and non-stimulant (NS) (Table 1). For examples, yeast, NaCl and $Ca_3(PO_4)_2$ were classified as ES for both PtMG and PtEPS; glucose was ES for PtEPS but GS for PtMG (detailed classification is provided in Table 1). A chemo-phene tree was generated to reveal relationships and visual groupings among the 52 environment and nutrient conditions (Figure 3). Figure 3 reveals six clusters for PtEPS and each cluster was distinct for each stimulant class in Table 1 except for few odds. Examples, for PtEPS, cluster 2 consists ES with an odd GS class (-vechloride), and cluster 4 has S class with an odd ES (pH6).

Noteworthy, the odd nutrients/conditions identified by the chemo-phene tree were observed at barrier between two sequential classes in Table 1 and preferably, they should be right class in their present clusters (for example, pH 6 should be S class for PtEPS). The chemo-tree for PtMG follows similar demarcations for PtEPS but with little dissimilarity (Figure not shown). Therefore, glucose and fructose should be right placed as ES and GS, respectively. Therefore, clustering is an added value that identified misclassification of odd conditions and nutrients in rank-sum analysis and also, rank-sum analysis was able to reduce the large groupings in Duncan grouping to a manageable group size and they complement one another.

Classification of nutrients and environmental conditions for their stimulatory effects on MG and EPS is scanty in fungi.

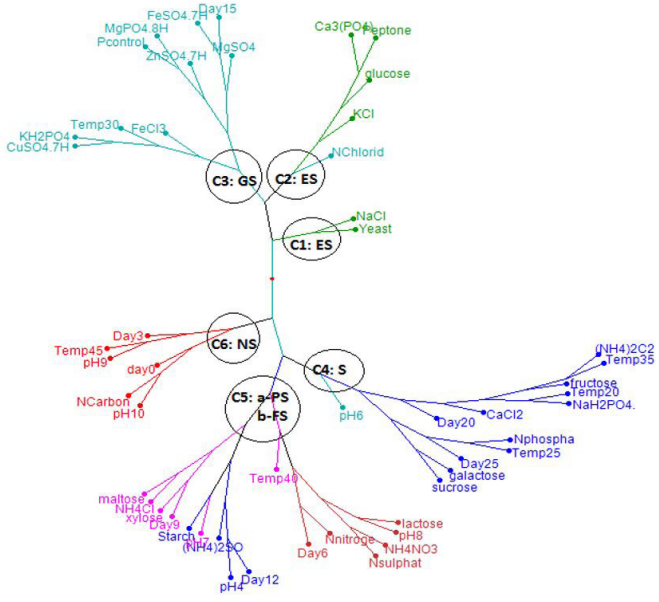

Figure 3. A chemo-phene tree for six stimulatory classes among 52 environmental conditions and nutrients for PtEPS by Cluster analysis in Darwin. Deep green = ES (Excellent stimulant), bluish-green = GS (Good stimulant), blue = S (stimulant), lilac = FS (fair stimulant), brown =PS (poor stimulant), and red = NS (non-stimulant).

Table 1. Rank-sum estimates and stimulatory classes among the environmental conditions and nutrients for PtMG and PtEPS.

Rank	Nutrient	PtEPS	Dev	Stdzed	PtEPS‡ Class	Rank	Nutrient	PtMG	Dev	Stdzed	PtMG Class
1	Yeast	22841.23	-25.5	-3.4	ES	1	Yeast	23827.62	-25.5	-3.4	ES
2	NaCl	21627.94	-24.5	-3.2	ES	2	NaCl	23822.45	-24.5	-3.2	ES
3	KCl	20116.1	-23.5	-3.2	ES	3	Temp30°C	22893.88	-23.5	-3.2	ES
4	Peptone	19993.62	-22.5	-3	ES	4	$Ca_3(PO_4)_2$	22455.62	-22.5	-3	ES
5	$Ca_3(PO_4)_2$	19936.66	-21.5	-2.8	ES	5	Day15	21929.92	-21.5	-2.8	ES
6	Glucose	19850.37	-20.5	-2.8	ES	6	$MgSO_4$	21538.02	-20.5	-2.8	ES
7	NChloride	19467.02	-19.5	-2.6	GS	7	Glucose	21148.59	-19.5	-2.6	GS
8	$MgPO_4.8H_2O$	18467.09	-18.5	-2.4	GS	8	$ZnSO_4.7H_2O$	20099.97	-18.5	-2.4	GS
9	+ve control	18427.2	-17.5	-2.4	GS	9	+ve control	20066.99	-17.5	-2.4	GS
10	$ZnSO_4.7H_2O$	18341.94	-16.5	-2.2	GS	10	KCl	19427.52	-16.5	-2.2	GS
11	Day15	18168.28	-15.5	-2	GS	11	pH6	19184.85	-15.5	-2	GS
12	$FeSO_4.7H_2O$	18136.83	-14.5	-2	GS	12	$CuSO_4.7H_2O$	19055.56	-14.5	-2	GS
13	$MgSO_4$	18054.6	-13.5	-1.8	GS	13	Sucrose	18832.84	-13.5	-1.8	GS
14	KH_2PO_4	17378.01	-12.5	-1.6	GS	14	NChloride	18730.12	-12.5	-1.6	GS
15	$CuSO_4.7H_2O$	17355.83	-11.5	-1.6	GS	15	$MgPO_4.8H$	18547.41	-11.5	-1.6	GS
16	Temp30°C	17168.52	-10.5	-1.4	GS	16	KH_2PO_4	18388.51	-10.5	-1.4	GS
17	$FeCl_3$	16460.64	-9.5	-1.2	GS	17	$FeSO4.7H_2O$	18034.29	-9.5	-1.2	GS
18	pH6	15147.28	-8.5	-1.2	GS	18	Day20	17350.35	-8.5	-1.2	GS
19	Sucrose	13661.79	-7.5	-1	S	19	Fructose	17276.82	-7.5	-1	S
20	Galactose	13439.05	-6.5	-0.8	S	20	Peptone	16536.63	-6.5	-0.8	S
21	Day25	12633.52	-5.5	-0.8	S	21	Temp25	16307.27	-5.5	-0.8	S
22	Temp25°C	12404.55	-4.5	-0.6	S	22	NaH_2PO_4.	15963.21	-4.5	-0.6	S
23	NPhosphate	12238.83	-3.5	-0.4	S	23	Day25	15719.05	-3.5	-0.4	S
24	Day20	11536.47	-2.5	-0.4	S	24	pH4	14726.56	-2.5	-0.4	S
25	Fructose	11291.79	-1.5	-0.2	S	25	Temp35	14437.51	-1.5	-0.2	S
26	Temp35	11216.3	-0.5	0	S	26	$FeCl_3$	12982.07	-0.5	0	S
27	$(NH4)_2C_2O_4$	11201.24	0.5	0	S	27	Temp20	12395.9	0.5	0	S
28	NaH_2PO_4.	11042.09	1.5	0.2	S	28	pH8	12365.01	1.5	0.2	S
29	Temp20	11012.26	2.5	0.4	S	29	Day12	12215.66	2.5	0.4	S
30	$CaCl_2$	10762.75	3.5	0.4	S	30	pH7	11115.64	3.5	0.4	S
31	$(NH4)_2SO_4$	10248	4.5	0.6	S	31	Day9	11002.16	4.5	0.6	S
32	pH4	10125.06	5.5	0.8	S	32	Starch	10380.8	5.5	0.8	S
33	Day12	10115.54	6.5	0.8	S	33	NPhosphate	10170.4	6.5	0.8	S
34	Starch	9751.94	7.5	1	S	34	Xylose	10109.12	7.5	1	S
35	pH7	9500.85	8.5	1.2	FS	35	Temp40	9734.44	8.5	1.2	FS
36	Maltose	9062.07	9.5	1.2	FS	36	Galactose	9354.61	9.5	1.2	FS
37	NH4Cl	9058.31	10.5	1.4	FS	37	$(NH4)_2C_2O_4$	8756.26	10.5	1.4	FS
38	xylose	8682.31	11.5	1.6	FS	38	NSulphate	8661.7	11.5	1.6	FS
39	Day_9	8613.31	12.5	1.6	FS	39	$CaCl_2$	8375.56	12.5	1.6	FS
40	Temp_40	7377.19	13.5	1.8	FS	40	maltose	8234.56	13.5	1.8	FS
41	NSulphate	6547.48	14.5	2	PS	41	$(NH4)_2SO_4$	7935.08	14.5	2	PS
42	NH_4NO_3	6524.36	15.5	2	PS	42	NH_4NO_3	7073.8	15.5	2	PS
43	pH8	6283.32	16.5	2.2	PS	43	Lactose	7040.13	16.5	2.2	PS
44	Lactose	6057.06	17.5	2.4	PS	44	pH9	7008.98	17.5	2.4	PS
45	Day6	5583.75	18.5	2.4	PS	45	Day6	6962.6	18.5	2.4	PS
46	NNitrogen	5562.12	19.5	2.6	PS	46	NNitrogen	6077.63	19.5	2.6	PS
47	Temp45	4166.4	20.5	2.8	NS	47	NH_4Cl	5473.39	20.5	2.8	NS
48	pH9	3578.86	21.5	2.8	NS	48	Day3	5391.33	21.5	2.8	NS
49	Day3	2756.46	22.5	3	NS	49	Temp45	3678.85	22.5	3	NS
50	NCarbon	1331.34	23.5	3.2	NS	50	pH10	3028.94	23.5	3.2	NS
51	pH10	1174.75	24.5	3.2	NS	51	NCarbon	1707.71	24.5	3.2	NS
52	day0	0	25.5	3.4	NS	52	day0	0	25.5	3.4	NS

P. tuberregium exopolysaccharides (PtEPS); *P. tuberregium* mycelia growth (PtMG); Dev, deviation (deviation of rank from Grand mean; Stdzed, standardized mean (deviation of rank over standard deviation); NChloride, nochloride; NPhosphate, no phosphate; NSulphate, no sulphate; NNitrogen, no nitrogen; NCarbon, no carbon; ‡ES = Excellent stimulant, GS = Good stimulant, S = Stimulant, FS = Fair stimulant, PS = Poor stimulant, and NS = Non stimulant.

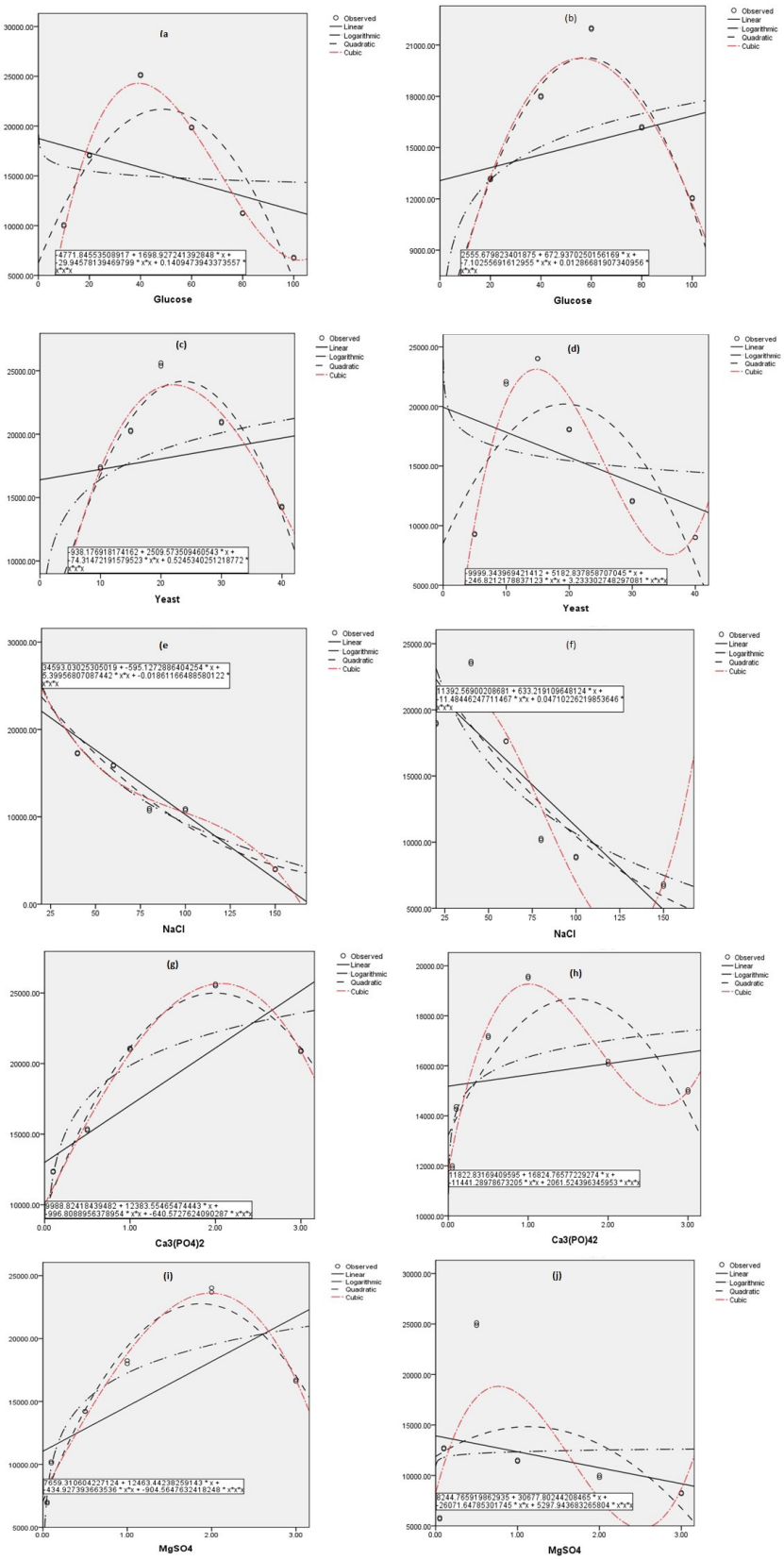

Figure 4. Optimizing glucose, yeast, NaCl, tricalcium diphosphate, and magnesium sulphate for PtMG and PtEPS and their curve fitness (a) PtMG curve fitness for glucose, (b) PtEPS curve fitness for glucose, (c) PtMG curve fitness for yeast, (d) PtEPS curve fitness for yeast, (e) PtMG curve fitness for NaCl, (f) PtEPS curve fitness for NaCl, (g) PtMG curve fitness for $Ca_3(PO_4)_2$, (h) PtEPS curve fitness for $Ca_3(PO_4)_2$, (i) PtMG curve fitness for$MgSO_4$, and (j) PtEPS curve fitness for $MgSO_4$. *P. tuberregium* exopolysaccharides (PtEPS); *P. tuberregium* mycelia growth (PtMG); the white area in the curve is the corrected equation for the best fitted cubic model curve depicted in red color in the grey area.

However, such classification has been reported for proper utilization in food crops (Moyib et al., 2015a, 2015b) and at present extended to *P. tuberregium*. Nonetheless, selection of optimal conditions and nutrients based on ANOVA, response surface methodology, orthogonal matrix method, and Plackett-Burman design, for optimization of EPS production have been successful used in fungi (Borges et al., 2013; Joshi et al., 2013; Mahapatra & Banerjee, 2013) and the present chemometrics, Duncan multiple range test, rank-sum procedure and clustering analysis are also suffice, robust, simple, informative and self- explanatory in nature.

3.4 Optimization of dosages of glucose, yeast, NaCl, $MgSO_4$ and $Ca_3(PO_4)_2$

Figure 4a and b show increasing glucose level from 10 to 100 g/L in a culture medium containing 10 g yeast, 1.0 g nicotinic acid, 20 g NaCl, 1 g $MgSO_4$ and 1 g $Ca_3(PO_4)_2$ per liter caused an increasing in the production of PtMG till 40 g/L glucose and PtEPS production increases till 60 g/L glucose, after which both fell gradually till 100 g/L glucose. Glucose has been reported in varying amounts that ranged between 40 and 100 g/L for optimal production of EPS, of which the present selected concentrations of 40 (PtMG) and 60 g/L (PtEPS) are within the range reported for many fungi. Such high demand of glucose for EPS has been previously observed, even at higher NaCl levels (Nehad & El-Shamy, 2010; Lai et al., 2014). The higher glucose required for PtEPS over PtMG should be expected, since glucose is one of the basic units of EPS as glucans and mannogalactans (Zhang et al., 2004; Huang et al., 2012).

For yeast, 5 to 40 g/L dosages were evaluated, PtEPS and PtMG responded positively to increasing the dosage of yeast till 15 g/L and 20 g/L, respectively, after which, both fell gradually with increasing yeast dose till 40 g/L (Figure 4c, d). Therefore, in the presence of 50 g glucose and 20 g NaCl, a moderate dose of 15 g/L and 20 g/L yeast favored PtEPS and PtMG production, respectively and are comparable to 25 g/L chosen for MG and EPS, in *L. squarrosulus* (Anike et al., 2015) while lower concentrations of yeast have been reported in some other fungi (Nehad & El-Shamy, 2010; Joshi et al., 2013).

Concentrations of NaCl for optimization was tested from 20 to 150 g/L in a medium containing 50 g glucose, 20 g yeast, 1g each of $Ca_3(PO_4)$, $MgSO_4$ and nicotinic acid. Figure 4e shows increasing NaCl dose beyond 20 g/L reduced PtMG production, and growth was halted at 100 g/L, indicating salt saturation with a low yield at highest dose of 150 g/L. PtEPS production was favorable till 40 g/L NaCl and depreciated with further increasing dose (Figure 4f). The present result suggests NaCl is a value-added nutrient but at moderate concentration that shouldn't exceed 40 g/L, beyond which the growth of *P. tuberregium* could be stunted and PtEPS production truncated (Turkkan, 2013; Boumaaza et al., 2015).

Surprisingly, level of PtEPS increases gradually with increasing dose of PO_4^{3-} from 0.05g to 1.00 g/L and reduces sharply at increasing dose beyond 1.0 g/L while PtMG increases sharply till a dose of 2 g/L and fell at 3 g/L (Figure 4g, h). The result shows that PO_4^{3-} supported production of both PtEPS and PtMG but at different doses. Production of PtMG increases with increasing dose of $MgSO_4$ till 2 g/L and fell sharply at 3 g/L (Figure 4i, j). PtEPS showed a different pattern, it increases with increasing dose till 0.5 g and dropped sharply at 1.0 g and then gradually till 3 g/L, indicating higher requirement of sulphate for PtMG than PtEPS. The present results signifies $Ca_3(PO_4)_2$ and $MgSO_4$ are important nutrients require in minute quantity as expected of microminerals and are within the reported range in fungi (Mahapatra & Banerjee, 2013; Lai et al., 2014). Generally, the patterns of the curves generated for PtMG and PtEPS production using regression analytical tool in SPSS (v. 17) were best fitted with cubic model.

4 Conclusions

The present chemometrics, Duncan multiple range test, rank-sum analysis and hierarchical clustering in the given successive sequential order, complement one another, and were able to classify assessed conditional and nutrient sources into useful stimulatory classes for optimal yields of PtEPS and PtMG and allowed robust selection of most suitable nutrients for PtEPS and PtMG. The present results are useful for production of PtMG and PtEPS for pharmaceutical, food, cosmetics and any applicable industrial purposes.

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