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Use of statistical methods to find the polysaccharide structural characteristics and the relationships between monosaccharide composition ratio and macrophage stimulatory activity of regionally different strains of *Lentinula edodes*

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Abstract

Multiple linear regression analysis was used to deduce the correlation between the monosaccharide composition ratios of 10 regionally different strains of *Lentinula edodes* and their *in vitro* macrophage stimulatory activities. Arabinose, xylose, mannose and galactose were identified as the monosaccharides that could be related to macrophage stimulatory activities. Additional principal component analysis and factor analysis methods were used to treat the same monosaccharide composition ratio data and the compositions of arabinose, xylose, mannose and galactose were found to be important. Interestingly, glucose, although presented in large compositions in all strains presumably forms the backbone of the polysaccharide structures, is not selected as the determinant factor for either structural characteristics or that of the *in vitro* macrophage stimulatory activities.

Keywords: *Lentinula edodes*; Polysaccharide; Multiple linear regression analysis; Principal component analysis; Factor analysis; Macrophage stimulatory activity

1. Introduction

Different strains of higher Basidiomycota mushrooms are known to produce biologically active polysaccharides (PS) with different properties and chemical structures. The structures include mainly glucan and glycan normally synthesized biologically to form tree structures with various types of branched linkages [1]. Glucan and glycan play key roles in cell communication, protein interaction and immunity. Various monosaccharides form the nodes of the tree structures and with glycosidic bonds leading to branched linkages. For each monosaccaride there are six possible hydroxyl groups for linkage formation and two possible anomality 'α' or 'β' could result [2].

Polysaccharides isolated from the different strains of *Poria cocos* mycelia showed different *in vivo* and *in vitro* anti-tumor activities, depending on their monosaccharide composition, molecular mass, and chain conformation [3]. In a previous paper, we reported the monosaccharide composition, molecular weight, structural linkage, immuno-modulating and anti-tumor activities for polysaccharides extracted from different phylogenetic groups of 10 regional *Lentinula edodes* [4]. The immuno-modulating properties and anti-tumor activities of these *L. edodes* extracts were tested and the results showed that the 10 isolated *L. edodes* could be classified into three distinct groups using amplified fragment length polymorphism assay. All polysaccharides had similar molecular weight distribution between 1×10^4 and 3×10^6. The monosaccharide composition analysis revealed the presence of heterogeneous materials containing glucose, mannose, xylose, galactose, fucose, rhamnose and arabinose in different ratios. Most of the extracts exhibited significant enhancement in macrophage stimulatory activities (MSA). However, although several studies were reported concerning the immunological activity and structure of polysaccharides (mostly β-glucan) in regionally different *L. edodes*, the determining factors for the structure–function relationships remained unsolved.

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Many methods in applied mathematics and statistics are often used to summarize and/or simplify complex data and help deducing relationships among variables. Regression analyses have been broadly used to predict sources or structure–function relationships in biological sciences. Examples include the use of regression analyses to predict the distribution of tree and shrub species, terrestrial animal species [5], drug discovery [6], and culture condition of *L. edodes* [7]. However, for different areas and/or at different resolutions, it may be difficult to compare the results of these methods [5].

A simple linear regression analysis of monosaccharide (arabinose, mannose and xylose) contents in sediments was used to determine the sources of carbohydrates [8]. In addition, principal component analysis and factor analysis could be useful in describing and classifying different functional or structural groups, for the measure of glycan structures taking into account of species, for the exploration of molecular structural characteristics, for the evaluation of the polysaccharide composition in lignfied woody plant cell wall, for the evaluation of molecular lipophilicity, for the understanding of organic reaction mechanisms, for quantitative structure–retention studies in chromatography, for quantitative structure–biodegradatoion relationships, and for classification of drug-free subject and drug abuses groups using the metal contents in hair samples [1,6,9]. For example, Hori and Sugiyama used a combination of FT-IR microscopic techniques and principal component analysis method to investigate the chemical variations between softwood species as well as types of wood cell walls [10]. Hizukuri et al., applied the principal component analysis to the composition data and found that the compositions of mannose, xylose and arabinose were important characteristics that distinguished the difference between yeast, sycamore and wheat [2].

To the best of our knowledge, there have been few reports using more than one statistical methods for a comprehensive analysis of experimental data to deduce polysaccharide structure–function relationships, particularly when the amount of data are limited [6]. In this paper, we report the results of using multiple linear regression analysis method to deduce the correlation between the monosaccharide composition ratios and *in vitro* macrophage stimulatory activities of polysaccharides obtained from different mushroom strains. In addition, principal component analysis and factor analysis methods were used to find polysaccharide structural characteristics of these *L. edodes*.

2. Experimental

2.1. Materials and data

The 10 isolates of *L. edodes* obtained were: No. 135 (L24) and No. 939 (L25) from China, Tainung No. 1 “white cap” (L1) and “red cap” (L4) from Taiwan, Japanese 271 (L11, L15), Jongxing 5 (L6), Jongxing 8 (L10), Hey-King-Gang (L21) and Jong-Wen 600 (L23) from Japan. The experimental procedures on culture broth filtrate (CBF) preparation, monosaccharide composition and MSA assay’s of the mushroom strains was described in detail by Lo et al. [4]. Briefly, the monosaccharide compositions of PS from CBF (2 mg) were determined by using methods described by Blakeney and Hoebler [11,12], from standard calibration curves of individual monosaccharides [13, Table 1]. Macrophage stimulatory activity (MSA) was determined by measuring the

Table 1
Monosaccharide composition and MSA of culture broth filtrate of different strains of *Lentinula edodes*: (A) selected raw data and (B) operation data

L. edodes	Monosaccharide compositions (molar ratio)							Optical density
	Arabinose	Xylose	Mannose	Galactose	Glucose	Rhamnose	Fucose	NBT reduction
(A) Selected raw data								
L1	0.46	0.37	2.45	0.11	4.2	0	0	0.37
L4	0.79	0.75	3.46	0.36	1.62	0.02	0.04	0.35
L6	0.49	0.35	1.89	0.15	3.03	0.01	0.01	0.43
L10	0.57	0.41	1.98	0.26	4.13	0	0	0.49
L11	0.59	0.46	3.19	0.38	4.14	0.01	0.02	0.4
L15	0.59	0.42	2.17	0.15	7.47	0.03	0.01	0.52
L21	0.5	0.35	1.99	0.22	2.81	0.03	0.03	0.44
L23	0.54	0.39	1.88	0.22	3.96	0.02	0.01	0.5
L24	0.27	0.14	0.89	0	10.9	0.06	0.02	0.38
L25	0.42	0.29	2	0.12	3.59	0.03	0.02	0.36

Lentinula edodes	L1	L4	L6	L10	L11	L15	L21	L23	L24	L25
(B) Operation data										
Arabinose %	6.03	11.19	8.25	7.77	6.76	5.41	8.42	7.73	2.22	6.45
Xylose %	4.82	10.65	5.89	5.58	5.21	3.83	5.88	5.49	1.13	4.55
Mannose %	32.28	49.22	31.83	26.89	36.24	20.02	33.5	26.72	7.23	30.91
Galactose %	1.44	5.09	2.59	3.56	4.3	1.42	3.73	3.19	0.03	1.87
Glucose %	55.44	23.1	50.95	56.2	47.12	68.93	47.36	56.36	88.82	55.58
Rhamnose %	0	0.22	0.24	0	0.14	0.28	0.56	0.32	0.45	0.4
Fucose %	0	0.52	0.24	0	0.24	0.11	0.56	0.19	0.12	0.24
Exopolysaccharide content (mg mL^{-1})	0.58	0.61	0.53	0.31	0.48	0.44	0.2	0.15	0.59	0.59
MSA (% NBT reduction)	166	150	317	449	230	489	330	464	200	153

O^{2-} anion production of macrophage cell line (Mouse BALB/C macrophage, RAW 264.7) with a modified nitroblue tetrazolium (NBT, Sigma) reduction assay [4, Table 1]. The RAW 264.7 cell line was cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco) containing 10% fetal bovine serum (FBS, Hyclone). The macrophage cells were placed into wells of a 96-well microtiter plate (2.5×10^5 per well) and were treated with 20 μL of CBF or HWE for 48 h at 37 °C. After removal of the supernatant, the macrophage cell monolayers were covered with 100 μL of 2 mg mL^{-1} of NBT. The plates with stimulated cells were incubated for 4 h at 37 °C. The reduced formazans within macrophage cells were solubilized in DMSO (Merck). Optical densities were measured using an ELISA reader at 570 nm [14,15]. *L. edodes* culture medium was used for control experiments and PBS (phosphate-buffer saline) as blank solution for optical density experiments. The % NBT reduced was estimated using the equation: {[(sample average) − (blank average)]/[(control average) − (blank average)]} × 100.

2.2. Multiple linear regression analysis (MLRA)

Multiple linear regression analysis was conducted similarly to a previous reported paper with minor modifications [5]. With a multiple regression model, the relationship is described using a generalization of a straight line equation (Eq. (1)):

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \cdots + \beta_p X_p + e \quad (1)$$

where Y denotes the response variable (NBT reduction value), β_0 a constant (intercept), X_1, X_2, ..., X_p a vector of p predictor variables (monosaccharide composition value), and β_1, β_2, ..., β_p is the vector of p regression coefficients. Each predictor variable has its own coefficient, and the outcome variable is predicted from a combination of all the variables multiplied by their respective coefficients plus an error term. The relationship was determined using the least square fitting technique. A residual analysis was used to check the model fitting [16]. MLRA was performed using the computer software-Statistical Package for the Social Sciences (SPSS) and S-PLUS.

2.3. Principal component analysis (PCA)

Principal component analysis was used to transform a number of potentially correlated variables (descriptors) into a number of relatively independent variables that could be ranked based upon their contribution for explaining the variation of the whole data set [6]. Based on this method, the relatively important components of high-dimensional pattern could be successfully identified. Thus, the original high-dimensional data could be mapped onto a lower dimensional space, and therefore the complexity of a high-dimensional pattern classification problem is substantially reduced [1].

For a random vector $\boldsymbol{x}$, where

$$\boldsymbol{x} = (\chi_1, \ldots, \chi_n)^{\mathrm{T}} \quad (2)$$

The mean of the random vector is denoted by

$$\mu_x = E\{\boldsymbol{x}\} \quad (3)$$

And the covariance matrix of the same random vector is

$$C_x = E\{(\boldsymbol{x} - \mu_x)(\boldsymbol{x} - \mu_x)^{\mathrm{T}}\} \quad (4)$$

The components of $C_{\boldsymbol{x}}$, denoted by C_{ij}, represent the covariances between the random variable components χ_i and χ_j. The component C_{ii} is the variance of the component χ_i. The variance of a component indicates a measure of spread of the component values around its mean value. If two components χ_i and χ_j of the data are uncorrelated, then their covariance is zero ($C_{ij} = C_{ji} = 0$). The covariance matrix is, by definition, always symmetric. From a sample of vectors X_1, ..., X_M, the sample mean and sample covariance matrix can be calculated to estimate the mean and the covariance matrix, respectively.

From a symmetric matrix such as the covariance matrix, an orthogonal basis can be calculated by finding its eigenvalues and eigenvectors. The eigenvectors e_i and the corresponding eigenvalues λ_i are the solutions of the equation [17]:

$$C_x e_i = \lambda_i e_i, \quad i = 1, \ldots, n \quad (5)$$

For our present study, data matrix consisted of the monosaccharide compositions of 10 strains of *L. edodes* and pattern recognition based on PCA was performed using the SPSS [6,9,16].

2.4. Factor analysis (FA)

Factor analysis was used to describe the correlation among several variables in terms of a few quantities, i.e. factors. FA and PCA are two different approaches to analyze the correlation structure of a set of real valued random variables. In practice, it is often observed that the results of FA are very close to those of PCA. These two approaches are strongly related to each other [18]. FA uses an estimate of common variance among the original variables in order to generate the factor solution. A factor is the linear combination of original variables. The number of factors will always be less than the number of original variables (6). The aim of FA is to summarize the correlation structure of observed variables X_1, X_2, ..., X_p. For this purpose one constructs $k < p$ unobservable or latent variables f_1, ..., f_k, which are called the factors, and which are linked with the original variables through the equation for each $1 \le j \le p$:

$$X_j = \lambda_{j1} f_1 + \lambda_{j2} f_2 + \cdots + \lambda_{jk} f_k + \varepsilon_j \quad (6)$$

The error variables ε_1, ..., ε_p are assumed to be independent, but they have specific variances ψ_1, ..., ψ_p. The coefficients, λ_{jl}, called factor loadings, are elements of the matrix of loadings Λ [19].

Factor analyses to evaluate relationships between different strains and monosaccharide compositions were also carried out using SAS [6,20].

3. Results

3.1. Multiple linear regression analysis

The method adopted here allowed us to detect major monosaccharide composition differences between the 10 strains and to predict characteristic components of each polysaccharide immuno-competence. Using multiple regression backward analysis, the *P*-values for testing linear detected relationship were (a) arabinose < 0.001, (b) xylose < 0.001, (c) mannose < 0.001, (d) galactose = 0.007, (e) fucose < 0.001. For other monosaccharides, the *P*-values were >0.1. A *P*-value < 0.05 was considered to be statistical significant. The coefficient of determination (R^2) which measures the model fit was 0.949. To predict relationship between monosaccharide ratio and MSA, Eq. (7) was obtained:

$$Y\,(\%\ \text{macrophage activity}) = 0.138 + 1.668X_{\text{Ara}} - 1.140X_{\text{Xyl}} - 0.051X_{\text{Man}} - 0.0995X_{\text{Gal}} - 0.743X_{\text{Fuc}} \quad (7)$$

where '*X*' defines the concentration of monosaccharide measured in the extract. The positive coefficient for X_{Ara} indicates that the macrophage stimulatory activity increases with increasing X_{Ara}. The negative coefficients for X_{Xyl}, X_{Man}, X_{Gal}, and X_{Fuc} indicate that the macrophage stimulatory activity decreases with the corresponding increasing X_{Xyl}, X_{Man}, X_{Gal}, and X_{Fuc}.

The scatter plot matrix showed that arabinose and xylose were rather highly positively correlated (Pearson correlation = 0.971) and meaning that in going from one polysaccharide to the other, a higher value of X_{Ara} was found normally with a higher value of X_{Xyl}. These kind of positive correlations were also found among (a) arabinose and xylose, mannose, galactose, (b) xylose and mannose, galactose, (c) mannose and galactose, (d) glucose and rhamnose. On the other hand, negative correlations were found among (a) glucose and arabinose, xylose, mannose, galactose, (b) rhamnose and arabinose, xylose, mannose, galactose (Fig. 1). In these cases, a higher value of, e.g. X_{glc}, was accompanied by lower values of X_{Ars}, X_{Xyl}, X_{Man}, and X_{Gal}. Note that positive monosaccharide composition correlations do not necessarily lead to positive MSA activities. For example, the monosaccharide compositions of arabinose and xylose are highly positively correlated for these 10 strains of *L. edodes*; however, arabinose has a positive MAS coefficient but xylose has a negative one.

The accuracy of the multiple linear regression analysis results was further tested by residual analysis [14]. The residual is defined as follows:

$$\text{residual} = \text{observed value} - \text{predicted value} \quad (8)$$

The normal *P*–*P* plot of regression residuals using SPSS was obtained in order to assess whether the normality assumption was violated. The plot that supports the normality assumption was constructed for the cumulative proportions of residuals against the cumulative proportions of the normal distribution

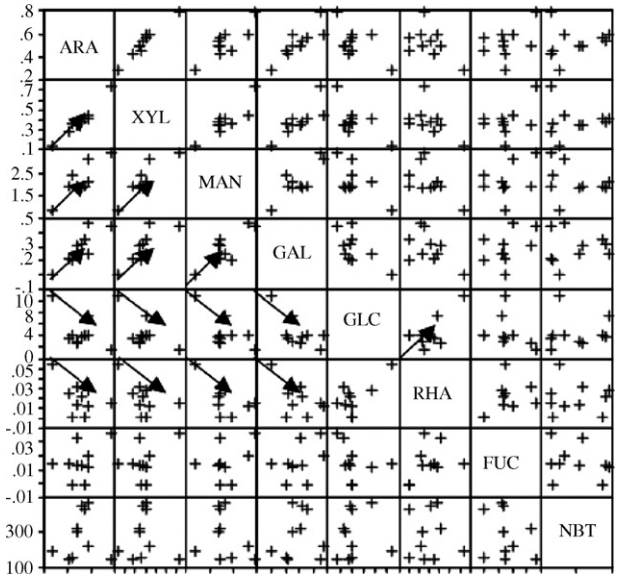

Fig. 1. 2D scatter plot of the relationship between all monosaccharide compositions and macrophage activity (NBT reduction).

(Fig. 2A). The normal probability plot showed a light tail distribution plot indicating normality is approximately valid. To check if the assumption of constant variance is not violated, the residuals were plotted against the predicted values. The change in the spread or dispersion of the plotted points could be used to detect whether the constant variance was satisfied or not. As seen in Fig. 2B, the points were dispersed in a non-systematic way, confirming the reliability of predicted model.

3.2. Principal component analysis and factor analysis

The arabinose, xylose, mannose and galactose (eigenvalue = 4.71) were the dominating features in the first principal component (Prin1) that represented 67% of the monosaccharide composition property. Monosaccharides such as rhamnose and fucose (eigenvalue = 1.39) were features in the second principal component (Prin2) that represented 20% of the monosaccharide composition property. The correlation matrix, eigenvectors and eigenvalue data were shown in Table 2A and B. The PCA two-dimensional plot (i.e. Prin1 versus Prin2, Fig. 3) revealed that L1, L4, L11, L24 and L25 were located further from the rest, which happened to be the group of relatively poorer MSA (macrophage stimulating activity). Those with better MSA, i.e. L6, L10, L15, L21 and L23, are located closer to the line of Prin1 = 0. Thus, PCA analysis, although designed to find only structural characteristics, was able to classify these polysaccharides roughly into a more active MSA group and a less active MSA one indirectly.

A third test using factor analysis (FA) indicated that the composition of arabinose, xylose, mannose and galactose were important structural characteristics among these polysaccharides (Table 3 and Fig. 4). These four monosaccharides were identical to those found for Prin1 in PCA, which further corroborated the generally believed consistent results from both PCA and FA methods.

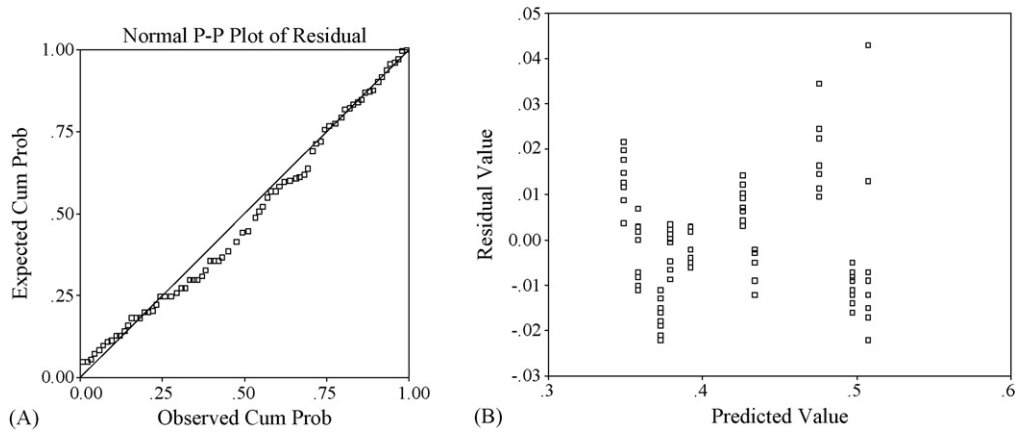

Fig. 2. (A) Normal *P–P* plot of regression residuals (dependent variable: NBT reduction). (B) Plot of residuals vs. predicted for the NBT reduction data.

Table 2
Correlation data of the correlation matrix (A) and eigenvectors (B) for the seven variables

	Arabinose	Xylose	Mannose	Galactose	Glucose	Rhamnose	Fucose
(A) Correlation matrix							
Arabinose	1	0.971	0.842	0.864	−0.628	−0.516	0.4
Xylose	0.971	1	0.886	0.815	−0.645	−0.507	0.449
Mannose	0.842	0.886	1	0.825	−0.649	−0.589	0.386
Galactose	0.864	0.815	0.825	1	−0.656	−0.51	0.439
Glucose	−0.628	−0.645	−0.649	−0.656	1	0.662	−0.319
Rhamnose	−0.516	−0.507	−0.589	−0.51	0.662	1	0.373
Fucose	0.4	0.449	0.386	0.439	−0.319	0.373	1
	Prin1	Prin2	Prin3	Prin4	Prin5	Prin6	Prin7
(B) Eigenvectors							
Arabinose	0.434	0.059	0.332	−0.185	−0.483	0.391	−0.527
Xylose	0.437	0.085	0.278	−0.462	−0.172	−0.39	0.574
Mannose	0.428	0.002	0.187	−0.069	0.814	0.339	0.001
Galactose	0.421	0.071	0.105	0.854	−0.153	−0.05	0.225
Glucose	−0.368	0.135	0.813	0.132	0.152	−0.323	−0.201
Rhamnose	−0.292	0.647	0.104	−0.016	−0.104	0.554	0.409
Fucose	0.197	0.74	−0.307	−0.024	0.134	−0.408	−0.367
Eigenvalues	4.706	1.394	0.494	0.202	0.176	0.02	0.009

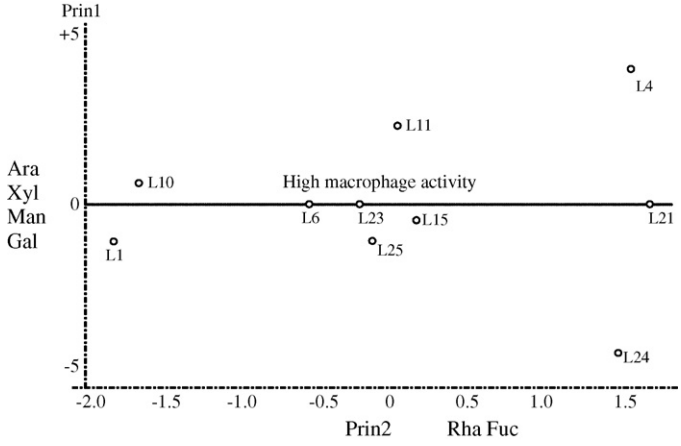

Fig. 3. PCA for composition data: relationship among different strains. Plot of Prin1 and Prin2.

Table 3
Factor pattern data for factors 1 and 2

Factors/monosaccharides	Factor 1	Factor 2
Xylose	0.947	0.1
Arabinose	0.942	0.069
Mannose	0.929	0.002
Galactose	0.914	0.084
Glucose	−0.799	0.159
Fucose	0.427	0.874
Rhamnose	−0.634	0.764

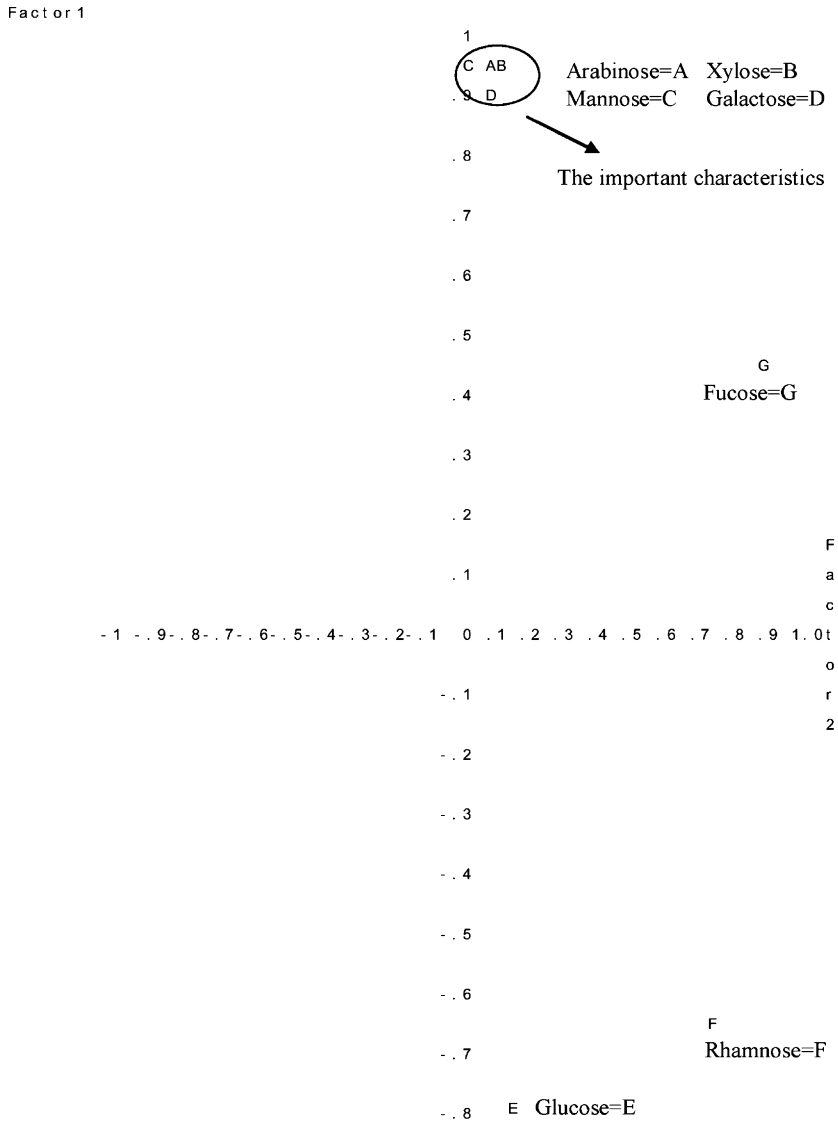

Fig. 4. Plot of factor pattern for factors 1 and 2.

4. Discussion and conclusions

Multiple linear regression analysis method was useful to deduce the correlation between monosaccharide composition and macrophage stimulating activity data of 10 different strains of *L. edodes*. The principal component analysis and factor analysis methods were useful to find polysaccharide structural characteristics. Our present data and previous reports all pointed out that arabinose, xylose, mannose and galactose were related to biological functions and were unique polysaccharide structural characteristics of *L. edodes* [2,3,21]. Specifically, the present study showed that the three different statistical methods, although applied for different purposes, generated consistent polysaccharide structural characteristics and both direct and indirect relationships between monosaccharide composition ratios and macrophage stimulating activities. Because it is not always possible to generate large quantity of experimental data, it is recommended that more than one statistical method are used for data treatment for more objective results, as shown in this study.

Although the enhanced macrophage activity was found to correlate to the complex polysaccharide structural characteristics, the actual molecular mechanisms involving cell signal transduction and more precise structure–function relationships remained to be explored. Interestingly, glucose, although presented in large compositions in all strains presumably forms the backbone of the polysaccharide structures, is not selected as the determinant factor for either structural characteristics or that of the *in vitro* macrophage stimulatory activities.

The elucidation of polysaccharide structures and biological activities normally involves complex instrumental techniques and various biological assays. For potential applications of this present study, it is possible to simplify the above-mentioned complexity by just determining the monosaccharide composition ratios and predicting the macrophage stimulatory activity using the established structure–function relationships.

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