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Optimization of microwave-assisted solid-phase oligosaccharides synthesis reaction and analysis of components and structure of synthetic product

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Abstract

Using phosphoric acid as the catalyst and glucose as substrate, microwave-assisted solid-phase oligosaccharide synthesis was studied. The parameters were optimized by orthogonal test design and statistical analysis method. Under the optimum reaction conditions, namely microwave power output of 1000 W, microwave irradiation time 9 min, adding 30% water to initiate reaction, adding 4% heteropoly acid A as the catalyst, the maximum yield ratio of product was 76.56%. The product was separated and characterized by using SephadexG-50 column, thin-layer chromatography (TLC) and high-performance liquid chromatography (HPLC). The component and proportion of synthetic oligosaccharides included disaccharides 20.81%, trisaccharides 15.50%, tetrasaccharide 10.84%, pentasaccharides 7.79%, higher oligosaccharides 21.63%. Results suggest that microwave-assisted solid-phase organic synthesis reaction could be used for the production of oligosaccharides.

Keywords: Microwave irradiation; Oligosaccharides synthesis reaction; TLC; HPLC; Separation

1. Introduction

It has been an established fact that carbohydrates can serve as structural components and energy source of the cell. More interestingly, their highly complex structure allows very specific interactions so that these biomolecules are involved in a variety of molecular recognition processes in intercellular communication and signal transduction such as cell adhesion, differentiation, development, regulation, etc. (Varki, 1993). For these reasons, great interest arose on carbohydrate-based pharmaceuticals and on the development of techniques for the analysis and synthesis of oligosaccharides. Today, numerous synthesis can be seen in the literature. Results of concluding them indicate two major classes: chemical and enzymatic ways. However, despite the recent advances (Boons, 1996), the synthesis for large-scale production of complex carbohydrates still cannot be easily performed by the classical chemical procedures which require long protection–deprotection steps for selectivity control and give low final yields. The enzyme-catalyzed synthesis of oligosaccharides seems to represent, nowadays, an interesting alternative to the classical chemical methods allowing the control of both regioselectivity and the stereochemistry of bond formation. However, the enzymatic approach often involves lots of prophase work, such as microorganisms' choice and cultivation, extraction, isolation, purification and modification of enzymes, which need a great deal of heavy repetitive work and a long wait. Moreover, their poor availability and the high cost of their substrates limit their exploitation (Nilsson, 1988).

Successful application of microwave irradiation technology to synthesis industry offers an important approach to effectively, quickly accomplish chemical synthesis procedure. In inorganic chemistry, microwave technology has been used since the late 1970s, while it has only been implemented in organic chemistry since the mid-1980s. Since the mid-1990s, however, the number of publications has increased significantly. The demand for diverse compound libraries for screening in drug discovery and materials science is a great driving-force for the development of new technologies for rapid parallel and combinatorial synthesis. One of those high-speed techniques is microwave-assisted organic synthesis, which has attracted a substantial amount of attention in the past few years (Lidstrâm, Tierney, Wathey, & Westman, 2001). The main benefits of performing reactions under microwave

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(MW) irradiation conditions are the significant rate-enhancements, cleaner reaction profiles, inexpensive reagents, simple experimental/product isolation procedures, and the higher product yields that can frequently be observed. Moreover, often when carrying out a reaction in a microwave oven, the use of a solvent can sometimes be avoided, which is important in order to make the synthesis more environmentally friendly ('green chemistry') (Santagada, Perissutti, Fiorino, Vivenzio, & Caliendo, 2001). Not surprisingly, these features have recently also attracted interest from the drug discovery and medicinal chemistry communities, for which the reaction speed is of great importance. The combination of microwave heating technology and combinatorial chemistry applications therefore seems a logical consequence of the increased speed and effectiveness offered by using microwave irradiation instead of conventional methods. Regardless of the origin/existence of a special microwave effect, it is extremely efficient and applicable to a broad range of practical syntheses (Larhed & Hallberg, 2001; Lidstrâm et al., 2001). The first true example of microwave-assisted combinatorial synthesis was reported by Khmelnitsky and co-workers (Cotterill et al., 1998) in 1998 and described the rapid parallel synthesis of combinatorial libraries of functionalized pyridines in 96-well plates by microwave irradiation in a domestic microwave oven. After that, there are lots of pertinent reports to come from around the world. Besson, Guillard, and Rees (2000) report a successful case of multistep synthesis of thiazoloquinazolines under microwave irradiation in solution which provide one of the good examples of the utility of microwaves in organic synthesis in the presence of solvents. However, advantages of microwave-assisted synthesis are mostly embodied in synthesis procedure under solid-state condition. Compared with traditional solution-phase chemistry, solid-phase synthesis in the presence of microwave irradiation has gained increasing attention from practitioners of combinatorial due to some advantages, like simplification of reaction work-up and product isolation, affinity and specificity of reaction (Bremer, Szewczyk, Baird, & Dervan, 2000), rate-enhancements and the higher product yields (Kirschning, Monenschein, & Wittenberg, 2001; Larhed & Hallberg, 2001). In a recent study by Stadler and Kappe (2001), using dedicated multimode microwave reactors for chemical synthesis, it was demonstrated that microwave irradiation can be effectively employed to attach aromatic carboxylic acids to chloromethylated polymer-supported resins via the cesium carbonate method in the absence of solvent. In the reaction, significant rate-accelerations and higher loadings were also observed when the microwave-assisted protocol was compared with the conventional thermal method. Reaction times were reduced from 12 to 48 h with conventional heating at 80 °C to 5–15 min with microwave flash heating at 200 °C in 1-methyl-2-pyrrolidone, employing open glass vessels. Importantly, no degradation of the polymer-supported resins even under prolonged exposure to microwave irradiation at 200 °C was observed. Today, we rationally conclude from lots of facts that microwave-assisted solid-phase organic synthesis technology is practical and high efficient in organic combination industry.

To the best of our knowledge, there is little literature on the microwave-assisted solid-phase oligosaccharides synthesis around the world so far. Our present study implemented oligosaccharides synthesis reaction under microwave-assisted solid-phase condition using glucose as the substrate. The study mainly focused on the investigation of effect of four crucial synthesis factors—microwave power output, microwave irradiation time, adding quantity of catalyst and adding quantity of initiator, on the yield ratio of synthetic oligosaccharides for later large-scale production, and these parameters were optimized by orthogonal test and statistical analysis method. At last, separation, identification and analysis of synthetic oligosaccharides were carried out by SephadexG-50 column, TLC and HPLC.

2. Materials and methods

2.1. Materials

2.1.1. Chemicals

Glucose ($C_6H_{12}O_6$) (AR class) from Sinopharm Chemical Reagent Co., Ltd in Shanghai, Maltose ($C_{12}H_{22}O_{11}\cdot H_2O$) (BR class) from Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences; Heteropoly acid A and all other chemicals used were of analytical grade.

2.1.2. Apparatus

Panasonic NN-S563JF frequency conversion micro-wave oven from Panasonic company, 722 spectrometry from Shanghai No. 3 instrument company; high-pressure liquid chromatogram (HPLC), Waters400E America; High Speed Tabletop Centrifuge from Shanghai Anting Scientific Instrument Co., Ltd; 722 grating spectrometry from Shanghai No. 3 instrument company.

SephadexG-50 gel column (100～300 Lm, Pharmacia product); DA201 resin (TianJing chemistry product); 60GF254 silica gel plate (product of QinDao ocean chemical plant).

2.2. Preparation of oligosaccharides

Reaction substrate (glucose) was added into a closed glass container, and then water was also added into it to initiate and catalyze. Then both were mixed in the closed glass container and subjected to microwave irradiation for a specific time with stirring. After the reaction finished, reaction mixture was cooled and dissolved into deionized water. The solution was then filtered. The filtrated solution was allowed to dry at room temperature, then crushed-up to afford synthetical product. The yield ratio of

oligosaccharides may be calculated by the following formulas:

$$y = \frac{\text{amount of initial glucose} - \text{amount of residual glucose}}{\text{amount of initial glucose}} \times 100\%$$

y the yield ratio of oligosaccharides (%).

2.3. Determining content of glucose

Total glucose concentration were measured by phenol–vitriol method with some slight modification. For the preparation of sample solution, 5 g phenol was dissolved in a little distilled water, shaken-up and distilled water was then supplied to the volume of 100 ml with a final concentration of 5%. Then 0.2 ml sample solution was accurately taken and filled into a 10 ml cuvette. Then 0.4 ml, 5% phenol was added into the cuvette and shaken-up. Then 2 ml vitriol was filled into the mixture in cuvette, shaken-up and stand for 30 min at room temperature. At last, absorbance values were recorded by 722 spectrophotometer at the wavelength of 490 nm. At the same time, the wash solution was measured as blank control in an identical way. Elution curve may be protracted by absorbance values and elution solution volume.

2.4. Optimization of reaction parameters of oligosaccharides synthesis

At first, the effect of changing single factor on synthesis efficiency was studied.

Then, the reaction parameters of HPDC were optimized by orthogonal test design. Microwave irradiation time, microwave power output, addition quantity of catalyst and addition quantity of initiator were taken as factors to be optimized. The investigated levels of each factor were selected depending on the above experiment results. Yield ratio of oligosaccharides was used as index to evaluate synthesis efficiency.

2.5. Isolation and purification of synthetic oligosaccharides by SephadexG-50 column

Synthetic oligosaccharides were ground, dissolved in distilled water and was eluted with water (pH 7.0) by gel permeation chromatography column at room temperature. The flow rate of elute was 0.5 ml/min. About 3 ml of elute was automatically collected in a tube at 8 min interval. The column was calibrated with reference calibrators and the collected fractions were spectrometrically detected at 490 nm. Eluted curve was protracted by tube number and absorbance. After elute from a single peak was collected, fractions were lyophilized for further analysis.

2.6. A6nalysis of synthetic oligosaccharides by thin-layer chromatography (TLC)

TLC may further analyze and determine the separated fractions by SephadexG-50 column. But consider that F3 of larger molecule weight cannot cause many spots, so, we only chose F2 and F1 for TLC analysis in our work.

F2 and F1 were, accurately taken 10 mg and added in 2 ml, 2 mol/l trichloro acetic acid solution in a tube. The tube was airproofed under vacuum and sample were hydrolyzed for 2 h in 130 °C. The result solution was stranded, cooled and condensed under depressure. TLC analysis of oligosaccharides was done as previously described (Song, Kim, Sung, & Cha, 2002) with some slight modification. An aliquot of the condensed oligosaccharides solution and standard sample solution were spotted onto a silica gel 60 F254 plate with a capillary tube, then the chromatogram was developed with a solvent system of butanol/ethanol/water (10:1:2, v/v/v). After irrigating twice, the TLC plate was dried and visualized by spraying 0.3% (w/v) anilin–diphenylhydrazine–trichloro acetic acid onto in it, followed by heating at 80 °C for 10 min. The sugars were quantitatively determined on the TLC plate by densitometry (Robyt & Mukerjea, 1994).

2.7. HPLC conditions

The analytical column was a Spherisorb NH_2 (4.6× 250 mm i.d). The mobile phase was acetonitrile/water (70/30 v/v) with 1 ml/min flow rate. The column oven was kept at 30 °C. The volume of the injection was 10 μl.

The analytical column was a Sugarpark1 (6.5× 300 mm i.d). The mobile phase was water with 0.4 ml/min flow rate. The column oven was kept at 80 °C. The volume of the injection was 10 μl.

3. Results and discussions

3.1. Protracting of the standard curve of glucose

Twenty-milligrams of glucose were accurately taken and added into a 500 ml capacity flask. Water was supplied to scale glucose solution . 0.4, 0.6, 0.8, 1.0, 1.2, 1.4, 1.6 and 1.8 ml prepared were, taken and in turn added into 20 cuvettes of 2 ml cubage, and then supplied water to 2.0 ml. Then 1.0 ml, 6% phenol and 5.0 ml H_2SO_4 were in turn added into cuvettes, shaken-up and stood for 20 min at room temperature. At last, absorbance values were recorded by 722 spectrophotometer at the wavelength of 490 nm. At the same time, 2.0 ml wash solution was measured as a blank control in an identical way. Standard curve of glucose may be protracted. Abscissa stands for glucose concentration (μg/ml). Vertical coordinates stand for OD value.

Standard curve of glucose is shown in Fig. 1. Regression equation: $Y = 0.9229X + 0.002$.

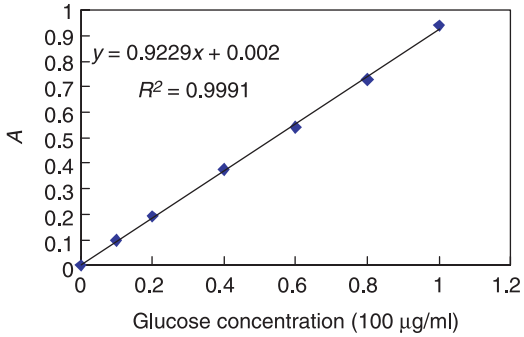

Fig. 1. The standard curve of glucose.

3.2. The conditions for polymerization

3.2.1. The effect of microwave power on the polymerization ratio

This work had studied the effect of microwave power on glucose polymerization when different microwave power (200, 400, 600 and 800 W) was set under the reaction conditions as follows: 30% initiator, 10% catalyst, 10 min microwave irradiation. The results are demonstrated in Fig. 2.

Microwave possesses the character of penetrating materials and generating efficient internal heat-transfer, resulting in even heating throughout the material irradiated. The mechanism of microwave generating heat may be primarily contributed to two major factors: dipolar polarization and conduction (Gabriel, 1998; Giguere, 1986; Langa, F., 1997; Loupy, 1998; 1997 Mingos & Baghurst, 1991;). When microwaves penetrates materials, the energy of microwaves can be absorbed by materials, which can be further translated into heat energy. The ability of a solvent to convert microwave energy into heat energy will be dependent not only the frequency, but also on the temperature (Baghurst & Mingos, 1992). When microwaves come into materials, the electromagnetic intensity and power will continuously weaken, but enhancing the power of microwaves can usually increase the capacity of penetration and the speed of the reaction. Singha, Sethia, Tewaria, Srivastavaa, and Sanghib (2003) observe that with MW power rising, yield of synthetical product will increase, which is in agreement with the result of our experiment (Fig. 2)—during the microwave irradiation, the yield ratio of synthetical oligosaccharides increased with elevating MW power. When the microwave power was over 400 W, the yield ratio of oligosaccharides could exceed 40%.

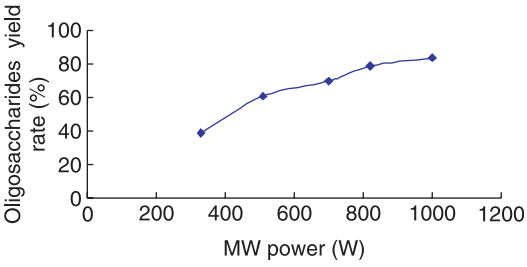

Fig. 2. Effect of microwave power on the yield of product.

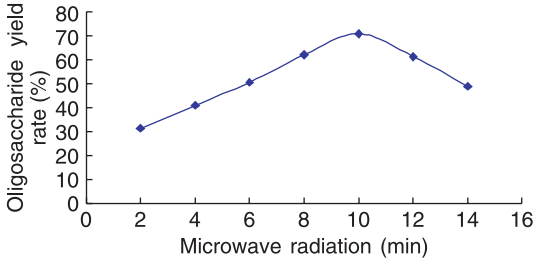

Fig. 3. Effect of microwave irradiation time on the yield of product.

In the experiment, we adopted 800 W MW power for economically accomplishing the synthesis.

3.2.2. The effect of microwave irradiation time on the polymerization ratio

This experiment in turn adopted 2, 4, 6, 8, 10, 12 and 14 min microwave irradiation to investigate the effect of microwave irradiation time on polymerization. Other experimental conditions were as follows: microwave power output of 800 W; 30% initiator; 10% catalyst.

In chemical reactions, microwave irradiation time, which may decide degree of rising temperature, is a very important factor for the yield ratio of products. Generally speaking, the yield ratio of products rises as microwave irradiation time is extended. However, when irradiation times go by certain limitation, it causes an adverse part to conversion toward synthetic product. Due to the rapid reactive speed in microwave irradiation, excessive irradiation may result in the polymerization products' coking or decomposability (Fetter et al., 2003; Zapataa et al., 2001). It could be seen in Fig. 3 that the yield ratio of polymerization gradually went-up with the microwave irradiation time's lengthening. It reached the peak value at 10 min. But after that, the yield ratio of oligosaccharides started to go down, and the color of products evidently became darker. The reason for this may be assumed that products and glucose are broken-up in such conditions as the overmuch irradiation time causing higher temperature in the reactive system.

3.2.3. The effect of the adding quantities of initiators on the polymerization ratio

Glucose in solid-state system could not effectively absorb microwave, so it is necessary to add some initiators to help reactants to absorb microwave more effectively. For rapid microwave heating, solvent selection is one of the important factors. Solvents must have a dipole to absorb the microwave energy and provide rapid super heating, therefore, polar solvents such as water, alcohols and acetic acid are optimal solvents whereas nonpolar solvents such as benzene and toluene are poor solvents (Houmes & zur Loye, 1997; Kappe, 2002, 2003; Larhed & Hallberg, 2001; Ley & Baxendale, 2002; Lidstrom, 2001; Loupy & Perreux, 2001; Wilson, Gilroy, Dolan, & Snyder, 2004;), and may make reactions get on more successfully. In theory, the quantity of initiator mostly affects initial speed of the reaction but does not affect the ongoing speed of the reaction (Wathey, Tierney, Lidström, & Westman,

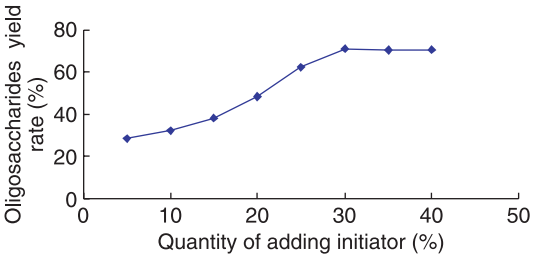

Fig. 4. Effect of initiator on the yield of product.

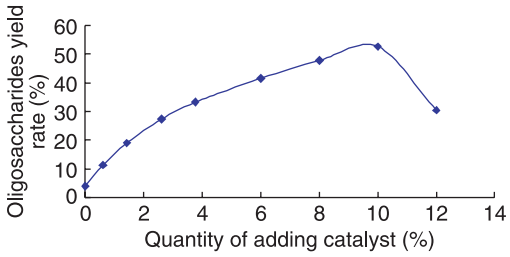

Fig. 5. Effect of catalyst on the yield of product.

2002), Once, the solid-state reaction is initiated, water which is one of the products in the reaction and behaves as a pseudo-organic at elevated temperature (Lidstrom, Tierney, Wathey, & Westman, 2001), can easily absorb microwave to make the reaction continue. Some quantities of water behaving as initiator can also avoid the products' coking or decomposability to help the reaction get on smoothly.

This experiment adopted 5, 10, 15, 20, 25, 30, 35 and 40% water as initiators to study the effect of quantities of different initiators on polymerization ratio. Other experimental conditions were as follows: 10% catalyst; 10 min microwave irradiation; microwave power output of 800 W.

Fig. 4 shows that the ratio of products that rose as the quantity of initiators increased. It reached the peak value on adding 30% initiator. The polymerization of oligosaccharides is catalyzed by the acid and it often happens in the polar-state (water-state) parts of the reactive mixture. When less initiator is added, the initiator cannot effectively absorb microwave energy for microwave irradiation can accelerate the volatilization of water. Besides this, heteropoly acid used as catalyst in the experiment play its part by absorbing on the surface of oligosaccharides in the polymerization reaction toward oligosaccharides. Less addition quantity of initiator is the cause that acid and oligosaccharides could not mix uniformly. The inhomogeneity distribution of acid concentration can usually result in coking in the sect of higher acid content under microwave irradiation, so that acid cannot play an effective part as catalyst.

It is well-known that microwave irradiation can promote the polymerization towards products. In nature, the polymerization toward oligosaccharides is a dehydration process. When microwave irradiation generates energy from the inside of molecule, water vapor rapidly generating from the inside of materials forms a kind of enormous impetus which drive water to move onto the surface in the form of water vapor and even sometimes produce very large grads of total pressures. The grad makes some water molecule arrange on the surface of materials before it is boiled away. All these quicken water volatilization. Rapid water volatilization is good for the polymerization towards products, which raises the yield ratio of products. It is the merit that conventional heating method cannot be comparable.

3.2.4. The effect of the quantity of catalyst on polymerization

This experiment adopted heteropoly acid A as the catalyst. The results suggested that the different quantities of acid as the catalyst had different effect on polymerization. We used different quantities of acid as the catalyst for the investigation of its effect on polymerization. Other experimental conditions were as follows: 30% initiator; 10 min microwave irradiation; microwave power output of 800 W.

It could be shown from Fig. 5 that the yield ratio of oligosaccharides rose as the quantity of acid increased. It approached the peak value an adding 10% initiator. When adding more than 10% acid, coking happened to the products, which led to the lower yield ratio. In addition, excessive acid addition can also make reaction to lose control. Generally speaking, in the solvent with higher concentration of oligosaccharides, acid can easily replace water in polar solvent or extend the distances between polyanions to enter liquid state. In some sense, acid absorbing a lot of polar molecule is similar to a kind of thick solvent whose state stands between solid and liquid. It is often called 'will-be liquid'. In the reactive system, glucose with shorter chains can sufficiently come-up to and mix with acid so that majority of acid becomes 'will-be liquid' and only a little becomes very dispersive solid particles. This kind of multiply state system makes the osculant areas between catalyst and glucose increase to large extent so that it accelerates the polymerization toward oligosaccharides (Seddon, 1996).

3.2.5. Result and analysis of orthogonal test

Owing to the synthesis efficiency being the highest under optimum conditions, it was very important for preparative synthesis to look for these optimum parameters. However, study of the effect of changing single factor on synthesis efficiency was not enough to judge what parameter was optimum because other factors were fixed under this condition. The optimum parameters should be obtained by using reasonable test design and mathematical analysis.

Table 1
Factors and level of orthogonal test

Level	Factors			
	A, irradiation time (min)	B, microwave power (W)	C, adding quantity of catalyst (%)	D, adding quantity of initiator (%)
1	7	330	1	10
2	8	510	2	30
3	9	700	3	50
4	10	1000	4	70

Note: Maximum microwave power: 1000 (W); 20 g catalyst or initiator are considered as 100%.

The orthogonal test was designed to optimize parameters on the basis of above experiment results. Four factors, microwave irradiation time (A) microwave power output (B) addition quantity of catalyst (C) and addition quantity of initiator, were selected for optimization. Four levels of each factor were investigated. In order to look for optimum parameters, the levels were adjusted on the basis of single factor experiment result. Yield ratio of synthetic oligosaccharides was measured after a given reaction time under the above-mentioned four variable factors and other fixed factors. The selected factors and levels are given in Table 1. The yield ratio of oligosaccharides was direct investigated index. The total evaluation index was used to analysis by statistical method. The results of orthogonal test and extreme difference analysis are presented in Table 2 and Fig. 6. The analysis of variance was performed by statistical software SPSS 12.0 and the result is listed in Table 3.

From analysis of extreme difference, the influential order of four factors on synthesis efficiency was A > C > B > D, and the optimum condition was $A_3B_4C_4D_2$. Owing to the value of *P* for A factors being extremely high, it was shown that the contribution of A factors for synthesis efficiency was the significant factor. The values of *P* for the other three factors were slightly high, so there were no significant factor. From the values of *F*, the affections were different and the order was A > C > B > D (see Table 3), which was in agreement with the result of extreme difference analysis.

Table 2
Orthogonal test design and results

No.	A	B	C	D	Yield ratio of oligosaccharides (%)
1	1	1	1	1	11.3
2	1	2	2	2	14.7
3	1	3	3	3	25.2
4	1	4	4	4	43.0
5	2	1	2	3	27.3
6	2	2	1	4	20.1
7	2	3	4	1	52.1
8	2	4	3	2	70.4
9	3	1	3	4	31.8
10	3	2	4	3	75.4
11	3	3	1	2	77.3
12	3	4	2	1	41.6
13	4	1	4	2	50.7
14	4	2	3	1	63.6
15	4	3	2	4	33.1
16	4	4	1	3	70.3
K_1	94.2	121.1	179.0	168.6	
K_2	169.9	173.8	116.7	213.1	
K_3	226.1	188.0	191	198.2	
K_4	217.7	225.0	221.2	128.0	
K_1	23.55	30.28	44.75	42.15	
K_2	42.48	43.45	29.18	53.28	
K_3	56.53	47	47.75	49.55	
K_4	54.43	56.25	55.3	32.0	
R	131.9	104	104.5	85.1	

A, irradidtion time (min); B, microwave power (W); C, adding quantity of catalyst (%); D, adding quantity of initiator (%).

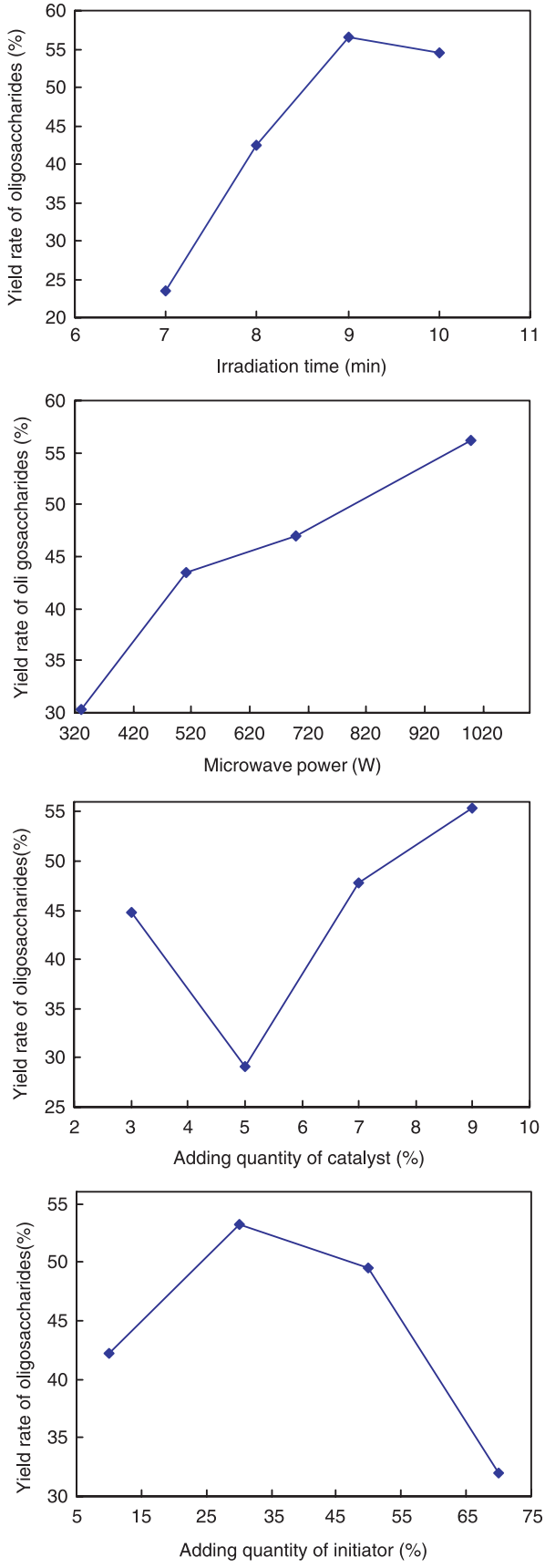

Fig. 6. Extreme difference analysis. A, irradidtion time (min); B, microwave power (W); C, adding quantity of catalyst (%); D, adding quantity of initiator (%).

Table 3
The variance analysis

Source	Sum of squares	d.f.	Mean square	*F*	Significance
A	2656	3	885.3	8.05	**
B	1423.8	3	474.6	3.31	
C	1447.3	3	482.4	3.36	
D	1025.8	3	341.9	2.38	
Error	330.2	3	110		
Total	6883.1	15			

A, irradidtion time (min); B, microwave power (W); C, adding quantity of catalyst (%); D, adding quantity of initiator (%).
** represents there was very significant ($P<0.01$) difference between two group.

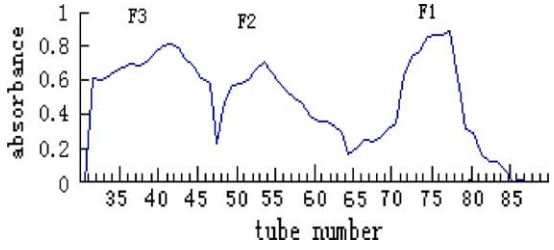

Fig. 7. GPC elution curve of the product.

Because optimal synthesis condition does not occur in orthogonal test, further validation test need to be carried out. According to $A_3B_4C_4D_2$ with other fixed factors, synthesis reaction was implemented. Result shows that yield ratio of oligosaccharides reaches 77%, so we rationally confirm the synthesis condition to be the most optimum, namely, microwave power output of 1000 W, microwave irradiation time 9 min, adding quantity of initiator 30%, adding quantity of catalyst 4%.

3.3. Isolation and purification of synthetic oligosaccharides

3.3.1. Analysis of isolation and purification of synthetic oligosaccharides by G-15 gel column

In this work, the synthetic oligosaccharides were separated by using gel permeation chromatography column (GPC). Oligosaccharides were detected from the 30th tube when total volume of washed solution reached 90 ml. GPC elution curve of the synthetic product is shown in Fig. 7. Based on Fig. 7, we could find that the product was separated into three components, and the general molecular weight range of fractions gel permeation chromatography after calibration was assessed, which were in turn named as F3 (the largest molecule weight), F2 (the middle molecule weight), F1 (the smallest molecule weight) according to the calibration results.

Corresponding eluted solution of each single peak was, respectively, collected and lyophilized under vacuum. using GPC, we could obtain Fl, F2 and F3 fractions preliminarily purified.

Due to smaller absorption of G-15 column to oligosaccharides, fractions separation are implemented simply by gel permeation chromatography using water as eluted solution, basing on the size exclusion chromatograph theory. From separation effect, we may assume that G-15 column has larger advantage in the separation of oligosaccharides of small molecule weight. Although purification of oligosaccharides fractions separated by GPC is not very high, the technology possesses the capacity of managing larger amount of sample and simple operation, which are just the merit that other separation ways cannot be comparable when purification standard is not strictly asked. Preliminary separation fractions by GPC may be further isolated and analyzed by HPLC.

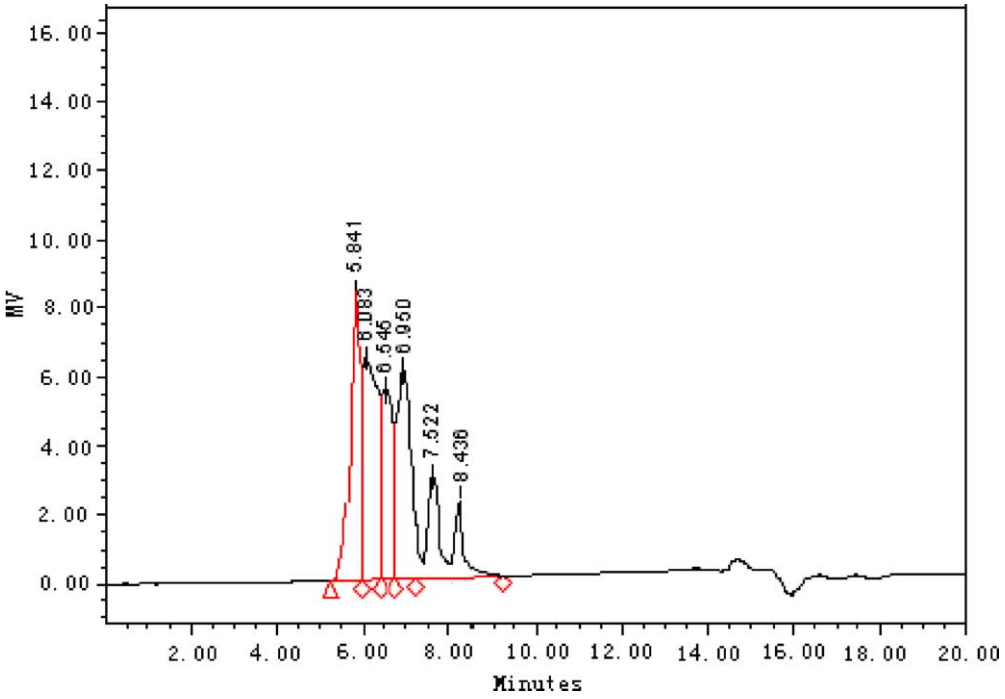

Fig. 8. The HPLC of F3 fraction.

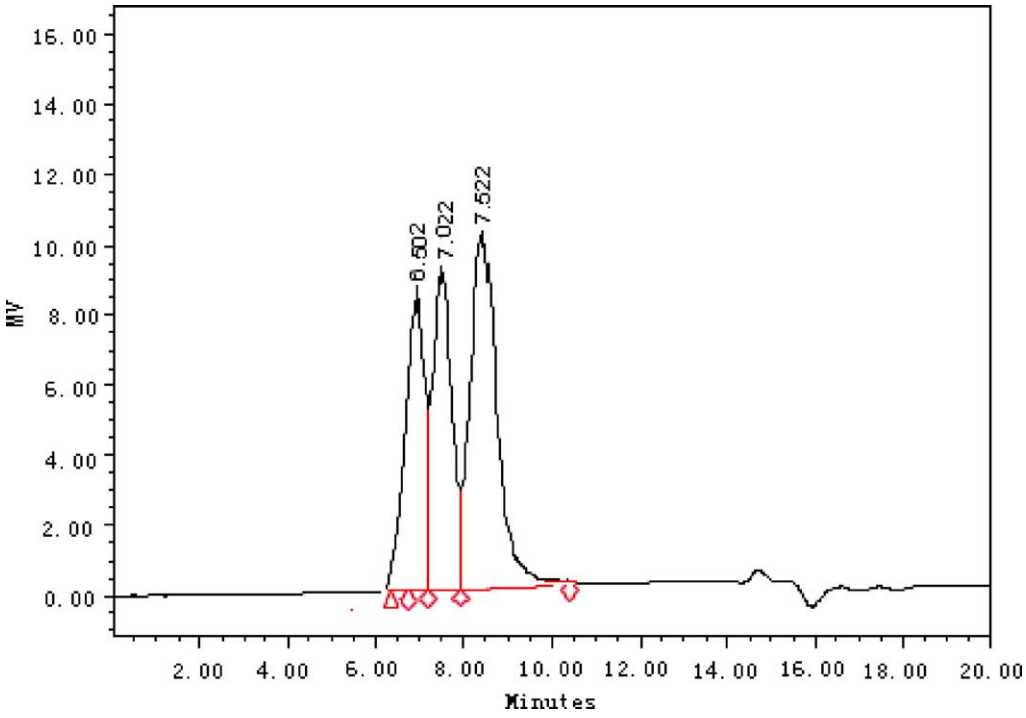

Fig. 9. The HPLC of F2 fraction.

3.3.2. Analysis of separated fractions of synthetic oligosaccharides by HPLC

Three separation fractions of synthetic product were, respectively, further characterized by HPLC, and results are in turn shown in Figs. 8–10.

According to HPLC results of Fig. 8, it may be assumed that F3 are primarily composed of pentasaccharides and higher oligosaccharides. Data from quantitative analysis indicate that F3 contains 37.8% higher oligosaccharides than hexadsaccharide, 55.4% pentasaccharides and hexadsaccharide, and 6.8% trisaccharides and tetrasaccharide.

HPLC result of Fig. 9 indicates that F2 are primarily composed of tetrasaccharides 48.4%, pentasaccharides 34.4% and higher oligosaccharides 21.8%.

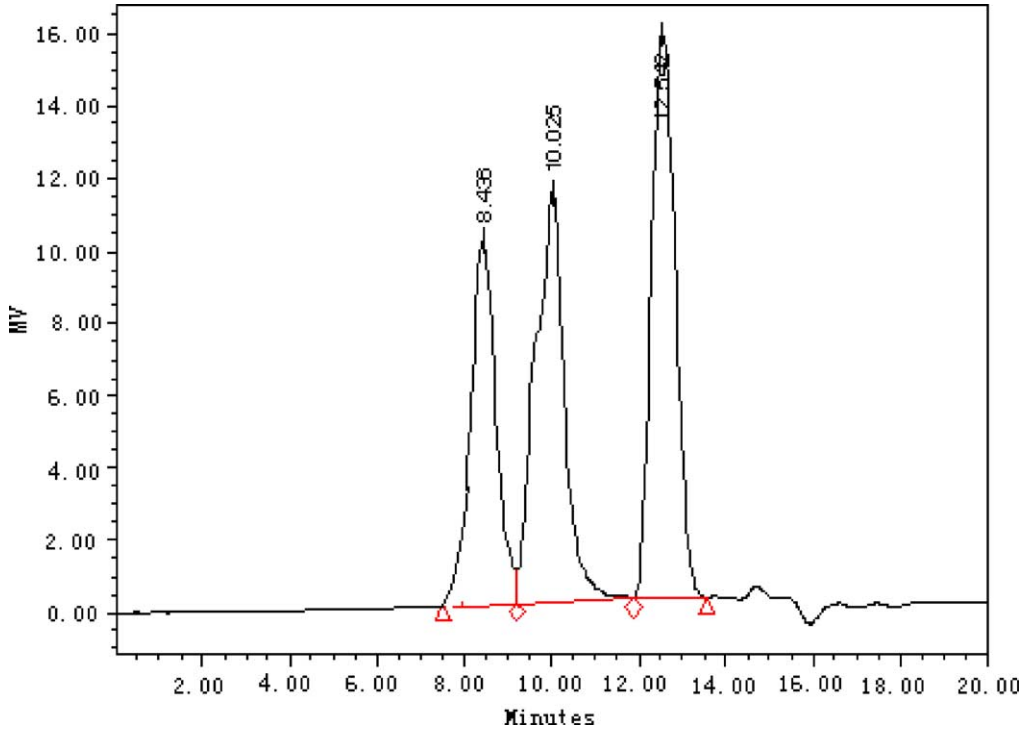

Fig. 10. The HPLC of F1 fraction.

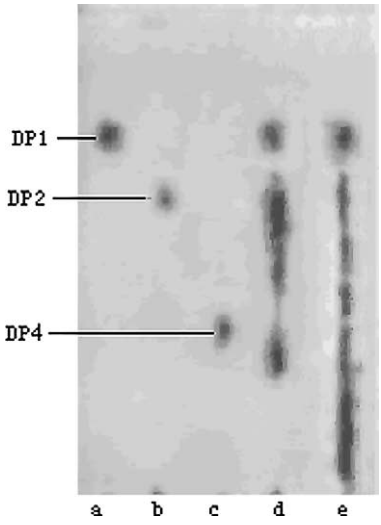

Fig. 11. Thin-layer chromatogram of the product. a, Glucose; b, maltose; c, malt-tetrasaccharide; d, synthetic oligosaccharides (F1); e, synthetic oligosaccharides (F2).

Analysis result of HPLC of F1 (Fig. 10) indicate that F1 are composed of trisaccharides 21.8%, disaccharide 36.4% and monosaccharides 41.8%.

3.3.3. Analysis of synthetic oligosaccharides by TLC

Of the chromatographic techniques, thin-layer chromatography (TLC) is still used for large-scale multiple sample screening programs (Jain, 1998; Michaud & Jones, 1980). Advantages of TLC over other methods are its simplicity, rapidity, versatility, applicability to a large number of samples, cost efficacy of operation, and ability to screen multiple samples (Jain, 2000) simultaneously which is just a good cause of its use for separation and analysis of synthetic oligosaccharides in our work.

From TLC results of F2, F1 and standard samples (Fig. 11), we discovered that mixed solution of standard sample DP1 (glucose), DP2 (maltose), DP4 (malt-tetrasaccharide) and oligosaccharides might be well separated under the identical outspread condition.

The standard samples glucose, maltose and malt-tetrasaccharide are three independent, round, clear color-spots in Fig. 11. There are no remnant of F1 observed near origin, but color-spots exist between disaccharide and tetrasaccharides. In addition, there is also a color-spot under tetrasaccharides. Color-spots of oligosaccharides are not very clear, round, especially a larger tail chain existing under disaccharide in comparison with standard sample. Therefore, we may rationally assume that F1 is primarily composed of trisaccharides, disaccharide and monosaccharides, and a little tetrasaccharides. Larger remnant of F2 existing between origin and tetrasaccharides indicates that it is primarily composed of higher oligosaccharides than tetrasaccharides.

Combining sample color-spots of TLC coincident with those of standard sample under identical conditions and result of HPLC of product confirms existence of synthetic oligosaccharides. TLC possesses promising prospect in the analysis of synthetic and isolated product for its simple operation and low cost.

3.3.4. Analysis of synthetic product by HPLC

The reaction mixture was analyzed by HPLC to detect the different sugars present. The samples were analyzed on HPLC using a Sugarpark1 column at 80 °C (Fig. 12). Main constituents of synthetical oligosaccharides could be detected and measured as following: in turn monose, disaccharides, trisaccharides, tetrasaccharide, pentasaccharides and higher oligosaccharides according to the peak order from right to left.

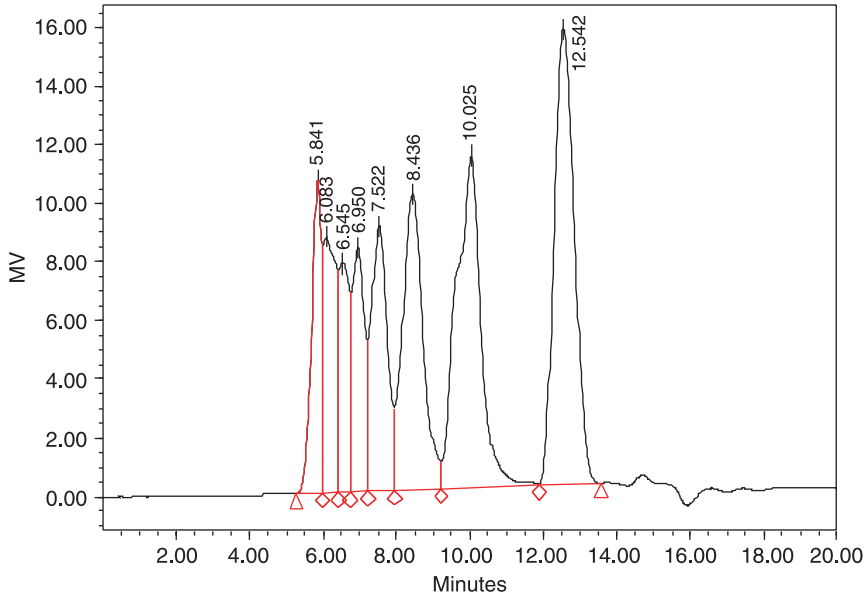

Fig. 12. HPLC of the product (Sugarpark1 column).

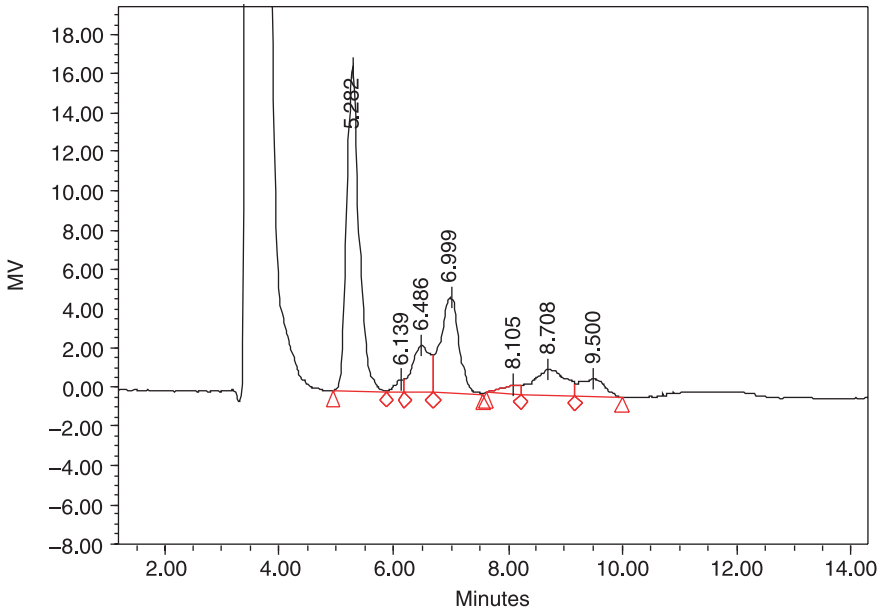

Fig. 13. The HPLC of monose, disaccharides and trisaccharides (Spherisorb NH_2 column).

Based on HPLC chromatogram (Fig. 12), the yield ratio of microwave-assisted solid-phase oligosaccharides synthesis using glucose as substrate may maximally reach 76.56%. The component percentages of synthetic product were in turn as follows: disaccharides 20.81%, trisaccharides 15.50%, tetrasaccharide 10.84%, pentasaccharides 7.79%, higher oligosaccharides 21.63%. Fig. 13 is HPLC chromatogram (Spherisorb NH_2 column, 30 °C) of monose, disaccharides and trisaccharides. It could be observed that their main constituents include glucose (49.25%), unknown component (1.48%), maltose (10.10%), isomaltose 21.78%, maltotriose 2.15%, panose 10.08% and isomaltotriose 5.16% according to the peak order. Because of poor analysis ability of Spherisorb NH_2 column to higher oligosaccharides than trisaccharides, concrete constituent and proportion of these higher oligosaccharides were unclear.

4. Conclusion

The main conclusions from this investigation can be summarized as follows:

(1) Optimal synthesis conditions for oligosaccharides are microwave power output of 1000 W, microwave irradiation time 9 min, adding 30% water to initiate reaction, adding 4% heteropoly acid A as the catalyst. Of synthetic factors, effect of microwave irradiation time on yield ratio is the most significant by statistics analysis.
(2) Synthetic product can be separated into F1, F2 and F3 fractions by G15-column.

Results of HPLC chromatogram indicate that F3 contains 37.8% higher oligosaccharides than hexadsaccharide, 55.4% pentasaccharides and hexadsaccharide, and 6.8% trisaccharides and tetrasaccharide; F2 is primarily composed of tetrasaccharides 48.4%, pentasaccharides 34.4% and higher oligosaccharides 21.8%; and F1 is composed of trisaccharides 21.8%, disaccharide 36.4% and monosaccharides 41.8%.

General components of synthetic oligosaccharides are disaccharides 20.81%, trisaccharides 15.50%, tetrasaccharide 10.84%, pentasaccharides 7.79%, higher oligosaccharides 21.63% by HPLC.

HPLC chromatogram (Spherisorb NH_2 column, 30 °C) of monose, disaccharides and trisaccharides demonstrates that their main constituents include glucose (49.25%), unknown component (1.48%), maltose (10.10%), isomaltose 21.78%, maltotriose 2.15%, panose 10.08%, isomaltotriose 5.16%.

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