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Methylation in methanol–water mixtures: The effect of solvent composition and high pressure

Marieke E. Bruins *, Katelijne M. Bekers, Anja E.M. Janssen, Remko M. Boom

Food and Bioprocess Engineering Group, Department of Agrotechnology and Food Sciences, Wageningen University and Research Centre, P.O. Box 8129, 6700 EV, Wageningen, The Netherlands

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Abstract

The effect of pressure (0.1 to 400 MPa) and solvent composition (methanol concentration of 5 to 30%) on the synthesis of β-methylgalactoside was studied. Galactose was used as a reactant and the reaction was catalyzed by β-galactosidase from *Aspergillus oryzae*. Under the applied conditions the enzyme was sufficiently stable and the reaction equilibrium was reached. Higher methanol concentrations obviously influenced the product yield positively due to an increase in reactant concentration but also due to a solvent effect. This solvent effect can be explained by measurement of the activities of galactose and methylgalactoside. These results may be generalized to other methylations in methanol–water systems, where methanol positively affects synthesis yields. Pressure had a small, negative effect on synthesis yields.

Keywords: β-galactosidase; High hydrostatic pressure; *Aspergillus oryzae*; Reaction volume; Equilibrium; Reverse hydrolysis

1. Introduction

In general, increased yield and selectivity in equilibrium reactions can be achieved by i) altering physical parameters like temperature or pressure, ii) changing the concentration of reactant or product, by e.g. product removal, or iii) altering the medium in which the reaction takes place. Medium effects can be due to changes in pH, addition of ions or the use of a different solvent mixture. Solvents can affect properties of reactants and products in solution. An improved understanding in solvent effects on reactions will help to choose a suitable solvent for a particular chemical transformation in a rational way. In this article we studied the use of methanol as a solvent.

Methanol–water mixtures have unique solvent behavior and cannot be regarded as ideal mixtures. Methanol exhibits both a hydrophobic and hydrophilic character. It has an –OH group that can bind with the surrounding water molecules and a methyl group that is hydrophobic. At high water content, methanol molecules polarize the local water arrangement, but the water hydrogen-bond network remains preserved. Water is still fully hydrogen-bonded with the –OH group from methanol while the hydroxyl group of methanol is hydrogen-bonded with the –OH group from water. At equimolar concentrations of methanol and water, the methanol–water mixture has a negative excess molar volume of -1.0 cm^3 mol^{-1} [1].

Considering methylation methanol not only acts as solvent, but also serves as reactant. High methanol concentrations are favorable since at higher methanol concentrations the equilibrium position is shifted towards synthesis, away from hydrolysis. Under these conditions, hydrolytic enzymes can catalyze condensation reactions if the enzyme retains its catalytic activity.

In this paper we report on the influence of pressure and solvent composition on methylation in methanol–water mixtures, influencing reaction yields. For arguments we studied the interaction between reactant or product and the methanol–water mixture. Since pressure affects methanol and water differently, pressure effects on the reaction equilibrium were quantified and explained by the reaction volume and the partial molar volumes of the components in the reaction mixture.

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* Corresponding author.
E-mail address: Marieke.bruins@wur.nl (M.E. Bruins).

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2. Enzymatic synthesis of β-methylgalactoside as a model system

Carbohydrate derivatives play a key role in many biological processes and are used as therapeutics, diagnostics, foods and cosmetics. Two methods are used in enzymatic galactosylation: thermodynamically controlled direct galactosylation (often referred to as reversed hydrolysis) and kinetically controlled transgalactosylation. In the first case water is acting as the leaving group, in the second, the aglycon is not water but for instance glucose (when lactose is used as a reactant). A thorough review on glycosidase-catalyzed synthesis of alkyl glycosides has been written by van Rantwijk et al. [2].

For our research, we chose a condensation reaction as a relatively simple model reaction. Condensation does not introduce a new component (the aglycon) in our system as a transglycosylation would, but simply water, which is already present as a solvent in our reaction system. Galactose is used as a reactant and methanol is used both as reactant and as solvent. The condensation of these two reactants will yield β-methylgalactoside and water.

$$\text{galactose} + \text{methanol} \leftrightarrow \beta\text{-methylgalactoside} + \text{water}$$

This reaction can be catalyzed by the β-galactosidase from *Aspergillus oryzae*. This enzyme has been isolated and characterized at the end of the 1970s. Enzyme activity is maximal at ±50 °C and pH 5. At the conditions of our atmospheric experiments (pH 5.5, and 25 °C) the enzyme is fully stable and its activity is about 40% of the maximum enzyme activity. [3,4] The β-galactosidase from *A. oryzae* can be used for hydrolysis and synthesis. The enzyme is rather unspecific and can be used to catalyze the galactosylation of various reactants, including sugars [5,6], alcohols [5,7–9], but also reactants like antibiotics [10] and cardiac genins [8]. β-Galactosidase from *A. oryzae* can also be used as a β-mannosidase [11].

3. Equilibrium constant as a function of pressure

The equilibrium constant (*K*) for a reaction is defined by the activities of all reaction components.

$$K = \frac{a_{\text{methylgalactoside}} \cdot a_{\text{water}}}{a_{\text{methanol}} \cdot a_{\text{galactose}}} = \frac{\gamma_{\text{methylgalactoside}} \cdot \gamma_{\text{water}}}{\gamma_{\text{methanol}} \cdot \gamma_{\text{galactose}}} \cdot \frac{x_{\text{methylgalactoside}} \cdot x_{\text{water}}}{x_{\text{methanol}} \cdot x_{\text{galactose}}} \tag{1}$$

In which a is the activity (−), γ the activity coefficient (−) and x the mole fraction (mol mol^{-1}) of a component. For any chemical reaction, the equilibrium constant is related to the Gibbs free energy (ΔG) by

$$\Delta G = -RT\ln K \tag{2}$$

In which R is the gas constant and T is the absolute temperature (in K). The Gibbs free energy is pressure (P in MPa) dependent according to

$$(\partial \Delta G / \partial P)_T = \Delta V \tag{3}$$

In which ΔV is the reaction volume change of the system in cm^3 mol^{-1} that is equal to the difference of the partial molar volumes of products and reactants.

$$\Delta V = V_{\text{products}} - V_{\text{reactants}} \tag{4}$$

These partial molar volumes include both intrinsic (van der Waals volumes) and solvational (contraction of the solvation shell and change in volume of the cavities) constituents. When pressure is the sole varied parameter in a system, Eqs. (2) and (3) can be combined, resulting in

$$(\partial \ln K / \partial P)_T = -\Delta V / RT \tag{5}$$

In which R equals 8.3145 cm^3 MPa K^{-1} mol^{-1}. This equation implicates that the application of pressure to a system in equilibrium will force the equilibrium to the state having the smallest volume. Thus a negative reaction volume shifts the equilibrium to the product side of the reaction.

4. Methanol–water mixtures and pressure

Easteal and Woolf [12] studied the densities of water–methanol mixtures at different temperatures and pressures. They used their results to calculate partial molar volumes, isothermal compressibilities and thermal expansivities. Fig. 1 illustrates the influence of pressure and solvent composition on the partial molar volumes of methanol and water. With this data it is possible to calculate part of the reaction volume for methylations. The partial molar volume of water, a product in the reaction, subtracted by the partial molar volume of methanol, which is acting as a reactant, can be expressed as a function of pressure and solvent composition. These values can be used for methylations in water–methanol mixtures in general. Under atmospheric conditions the difference between the two volumes is the smallest at approximately 10% methanol and largest at 100% methanol. Under pressure the “minimum” shifts to higher values. At 275 MPa the smallest difference between the two volumes is found at approximately 30% methanol. For all

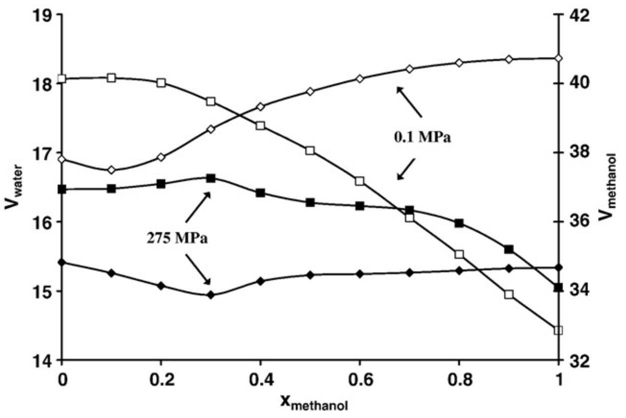

Fig. 1. Partial molar volumes of water (□) and methanol (◇) in {x methanol+(1−x) water} at 298 K, 0.1 MPa open symbols, 275 MPa closed symbols, lines to guide the eye, x is the mole fraction. Data from Easteal and Woolf [12].

methanol–water mixtures, the absolute difference between the partial molar volume of water and methanol decreases with pressure.

5. Materials and methods

5.1. Materials

All chemicals and the β-galactosidase from *A. oryzae* (E.C. 3.2.1.23, Sigma code: G5160) were purchased from Sigma. Methanol with a purity of 99% was used and MilliQ was used for water.

5.2. Density measurements

The density of various solutions was measured using a density meter (DMA 5000, Anton Paar) at 25 °C, with an uncertainty of $5 \cdot 10^{-6}$ g cm^{-3}. The density meter was calibrated with air and water at 20 °C. Calibration was checked with a water measurement at 25 °C. Solutions of different concentrations were freshly prepared prior to the measurement. Galactose and β-methylgalactoside were dried overnight in vacuum before use. The measured densities were used to calculate molar volumes by using Eq. (6), where ρ and ρ^* are the densities of the carbohydrate solution and the solvent respectively, m is the molality of the carbohydrate and M is the molar mass of the carbohydrate.

$$V_\varphi = \frac{\rho^* - \rho}{m\rho\rho^*} + \frac{M}{\rho} \tag{6}$$

5.3. Enzyme activity

β-Galactosidase activity was assayed at atmospheric pressure using *p*-nitrophenyl-β-D-galactopyranoside (*p*NPG) as an artificial substrate. 10 μl of diluted enzyme solution was added to a standard reaction mixture that was equilibrated at room temperature to make a 1.0 ml solution of 2.0 mM *p*NPG in 0.05 M 2-Morpholinoethanesulfonic acid (MES) buffer pH 5.5. The reaction was terminated after 10 min by addition of 1.0 ml 1.0 M sodium bicarbonate. The increase in absorbance at 405 nm as a result of *p*-nitrophenol formation was measured spectrofotometrically.

5.4. HPLC analysis

Samples from the reaction mixture were inactivated by the addition of NaOH to a molarity of 0.1. After mixing, the enzyme was removed by filtration over a microfilter (Millipore Ultracel YM-30). The filter was subsequently washed with half a volume of 0.2 M HCL to remove all traces of sugar. The two fractions were pooled to neutralize the final sample.

The samples were quantified by HPLC. An amine phase column (Luna 5u NH_2 100A, Phenomenex) was used at 40 °C to separate the sugars, with 80% acetonitrile/20% MilliQ as eluent at a flow rate of 1.5 ml min^{-1}. Detection was done with RI. The β-methylgalactoside concentration was determined from the peak area using a calibration curve. The measured methylgalactoside concentration was subtracted from the initial weighed galactose concentration to calculate residual galactose.

5.5. Enzymatic synthesis and hydrolysis of β-methylgalactoside

The equilibrium synthesis of methylgalactose from galactose and methanol was studied at different pressures and varying concentrations of methanol. A standard reaction mixture contained 100 mM galactose and 0.025 g g^{-1} enzyme in a mixture of methanol and 0.05 M MES buffer. As a control that equilibrium was reached, hydrolysis experiments were performed. For hydrolysis experiments reaction mixtures were prepared in which 100 mM galactose was replaced with 100 mM methylgalactoside. Experiments were included in the interpretation when hydrolysis experiments gave the same outcome as synthesis experiments, indicating that equilibrium was reached.

The buffer pH of the aqueous medium before methanol addition was set at pH 5.5 at room temperature and atmospheric pressure. Although the pH of a MES buffer is hardly influenced by high pressure, it is not totally pressure stable [13]. The pressure dependence of the pH of the MES buffer can be calculated using the equation of Elyanov and Hamann [14]. The ionization volume of MES is 3.9 cm^3 mol^{-1} [15]. At 400 MPa the pKa will change from 6.1 to 6.3, causing an increase in pH from 5.5 to 5.7. Therefore, the standard reaction mixture was tested at three pH values (5.5, 6.0 and 6.5) at atmospheric pressure to check the influence of pH on equilibrium.

All reactions were conducted at 25 °C in volumes of 0.5 to 2 ml. Samples were taken in time for HPLC analysis and enzyme activity tests.

5.6. High-pressure experiments

Reactions at high pressure were performed in a multivessel high-pressure apparatus (Resato FPU 100-50, Resato International B.V., Roden, The Netherlands), which was kindly made available by Agrotechnology and Food Innovations of Wageningen University and Research Centre. The reaction mixtures were transferred into polyethylene bags, which were closed by sealing. As pressure medium a glycol mixture was used. The vessels were individually pressurized by an electronic driven hydraulic pump and a high-pressure intensifier unit. The pressurization rate was approximately 200 MPa min^{-1}. The vessels were kept at a constant temperature by a water bath set at 25 °C. During pressurization, the temperature in the vessels increased due to adiabatic heating, but did not exceed 36 °C. The reaction was kept under pressure for 1 or 7 days, to ensure that equilibrium was reached. After that the pressure valves were opened. Pressure release was rapid and almost instantaneous. Samples were taken for HPLC analysis and enzyme activity tests.

5.7. Equilibrium data

Although fundamentally the equilibrium constant (K) for a reaction is defined by the activity of all reaction components we

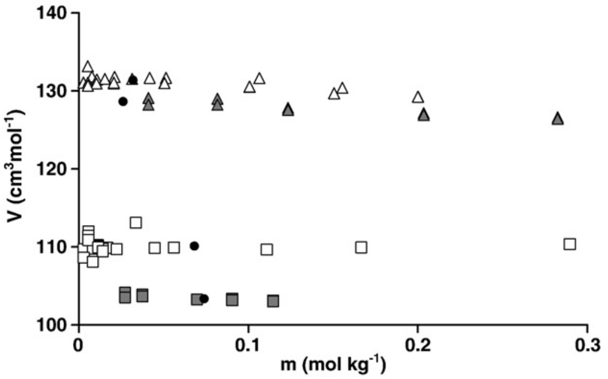

Fig. 2. Molar volumes of galactose (□) and methylgalactoside (Δ) in water (open symbols) or in 20% (w/w) methanol–water (closed symbols). The circles represent the values that were taken for the calculation of the reaction volumes in Table 1.

choose to also use a more practical (apparent) equilibrium constant (K^{app}), defined by the concentrations of the reaction components to plot our data.

$$K^{app} = \frac{x_{methylgalactoside} \cdot x_{water}}{x_{methanol} \cdot x_{galactose}}; \; K = K^{app} \cdot \frac{\gamma_{methylgalactoside} \cdot \gamma_{water}}{\gamma_{methanol} \cdot \gamma_{galactose}} \qquad (7)$$

5.8. Solubility and activity of the reaction components

In order to calculate the true equilibrium constant, the activities or activity coefficients of all components involved need to be known. Activities of methanol–water mixtures can be obtained from literature, e.g. from Bell et al. [16]. However the activity (coefficient) of methylgalactoside and galactose in water–methanol mixtures is unknown. For estimation, we measured the solubility of these components in water, methanol, and in a 20% water–methanol mixture. Galactose consists of α and β anomers that are not equally soluble. In saturated solutions, the activity coefficient is related to the mole fraction solubility (x^0) according to [17]

$$\gamma = \frac{a^0}{x^0} \qquad (8)$$

In which a^0 is the activity of the pure solid. This activity is a constant, which is independent on the nature of the solvent. It can therefore be taken out of the equation.

$$cK = \frac{a^0_{galactose}}{a^0_{methylgalactoside}} K = \frac{a_{water}}{a_{methanol}} \cdot \frac{x^0_{galactose}}{x^0_{methylgalactoside}} \cdot \frac{x_{methylgalactoside}}{x_{galactose}} \qquad (9)$$

For the determination of the solubility of galactose and methylgalactoside, solutions were prepared of galactose and methylgalactoside in water, methanol and a 20% methanol–water mixture. The mixtures were stirred overnight. Samples were taken and centrifuged for 2 min at 3000 rpm to remove non-dissolved sugar. Subsequently, the samples were weighed and freeze-dried to determine the amount of sugar per gram solution.

6. Results and discussion

6.1. Reaction volume based on molar volumes

The density of solutions of β-methylgalactoside or galactose in water or 20% methanol–water was determined. Eq. (6) was used to calculate the corresponding molar volumes and the results are given in Fig. 2. Volumes were measured over a range of carbohydrate concentrations and interpolated to the concentration that was reached in the equilibrium experiments (shown as black dots in Fig. 2).

Hypothetical yields for an equilibrium reaction can be calculated from the molar volumes of the products and reactants using Eq. (4). Here we calculate the reaction volumes for two different reaction compositions. The results are given in Table 1. The molar volumes of water and methanol were taken from literature ([12], see also Fig. 1). In a 20% methanol–water mixture both galactose and methylgalactoside have a lower volume compared to that in water. However, the volume decrease of galactose is higher than that of methylgalactoside. Therefore the reaction volume changes from 1.6 to 5.3 when water is replaced by 20% methanol–water as a solvent.

6.2. Enzyme stability

The residual enzyme activity was tested after each experiment. Reaction mixtures with 30% (w/w) methanol did not show any activity loss after 1 day. At 40% methanol approximately half of the activity was lost after 1 day. Pressurized samples with 30% methanol hardly showed any activity loss after 1 day at pressures upto 275 MPa. At 400 MPa approximately 40% of the activity was retained in the 30% methanol mixture after 1 day of incubation. Experiments were included in the interpretation when hydrolysis and synthesis experiments gave similar results, indicating that equilibrium was reached prior to complete enzyme inactivation.

Athes and Degraeve [18] studied the pressure stability of this enzyme and found no significant decrease of catalytic activity after 1 h at 450 MPa. Degraeve and Rubens [19] also studied the stability of the enzyme as a function of pressure. No significant activity loss took place after 1 h incubation below 300 MPa. However, at 450 MPa half of the initial activity was lost following a 1 h incubation period.

Table 1
Molar volume of all components in cm^3 mol^{-1} at 25 °C and atmospheric pressure

	Water	20% methanol
V^2_{water}	18.07	18.01
$V^2_{methanol}$	37.81	37.87
$V^1_{galactose}$	110.1	103.4
$V^1_{\beta\text{-methylgalactoside}}$	131.4	128.6
ΔV	1.6	5.3

The reaction volume is calculated from these. Concentration of β-methylgalactoside is given in mM.
Data from ^{1}own measurements and ^{2}literature ([12]).

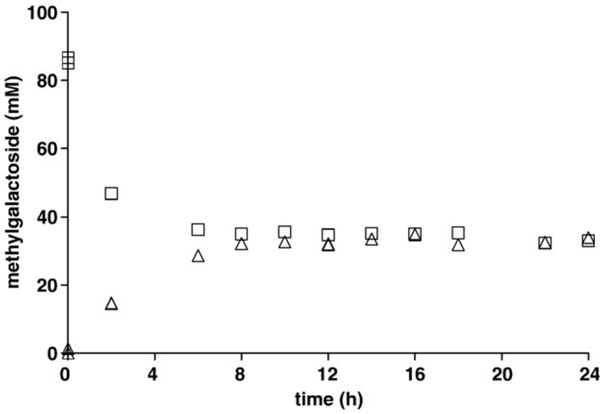

Fig. 3. β-Methylgalactoside concentration in time, either from β-methylgalactoside synthesis (Δ) or hydrolysis (□).

6.3. Enzymatic synthesis of β-methylgalactoside at atmospheric pressure

β-Methylgalactoside formation was measured at atmospheric conditions. Whether equilibrium was reached was checked by measuring the hydrolysis of β-methylgalactoside and synthesis from galactose under comparable circumstances. In most case only the final situation was measured. In Fig. 3, the kinetics of the reaction is plotted for both the hydrolysis and synthesis reaction under atmospheric conditions. The reaction was also performed at different pH values. No influence of pH on the final equilibrium was found. The minor pH-shift that takes place under pressure was therefore neglected.

6.4. The influence of methanol on β-methylgalactoside formation

The influence of the methanol concentration on the β-methylgalactoside formation at atmospheric pressure is depicted in Fig. 4a. Since methanol is one of the reactants, the product concentration positively correlates with the methanol concentration. However, the apparent equilibrium constant also increases (Fig. 4b).

An increasing apparent equilibrium constant is not uncommon when methanol–water is used as a solvent. Studies on solvent effects of a series of monohydric alcohols including methanol on the keto-enol tautomeric equilibrium of pentane-2, 4-dione also showed a positive correlation between the alcohol concentration and the equilibrium constant [20].

6.5. Incorporation of activities in the equilibrium constant

The apparent equilibrium constant based on concentrations is very useful to estimate product yield at different water–methanol compositions. To explain solvent effects it is also neccesary to determine the influence of the water–methanol composition on the activities of all components. The activity data can be used to calculate the true equilibrium constant.

To estimate the activity of galactose and methylgalactoside their solubiltities were measured at different solvent compositions. The solubility of methylgalactose divided by the solubility of galactose is plotted in Fig. 5a. A straight line is fitted throug the datapoints to intrapolate the results for all different methanol concentrations that were used. Higher methanol concentrations lead to an improved relative solubility of methylgalactose. The activity of water and methanol in different methanol–water mixtures were taken from literature [21,22]. The relative activity coefficients are depicted in Fig. 5b. Again a straight line is fitted to intrapolate the results for all different methanol mole fractions used. The activities were used to calculate the true equilibrium constant (K) from Eq. (9) and depicted in Fig. 4b. The true equilibrium constant seems more constant than K^{app}, which is based on concentrations. The main contribution to this is not found in the water or methanol activity, but in the large relative difference between the galactose and methylgalactoside activities at higher methanol concentrations.

6.6. High-pressure enzymatic synthesis of β-methylgalactoside

Reaction mixtures with different methanol concentrations were pressurized. Product formation decreased with increasing pressure. In Fig. 6 the apparent equilibrium constant is plotted against pressure. K^{app} is dependent on the methanol concentration in a similar way as shown in Fig. 4b for the results at atmospheric pressure. The effect of pressure can also be seen. Above 100 MPa,

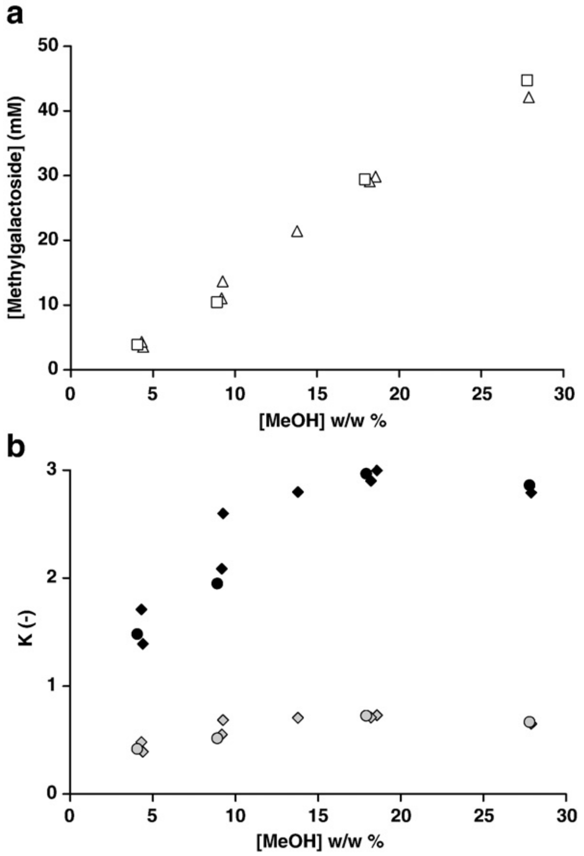

Fig. 4. a) Influence of methanol–water concentrations on β-methylgalactoside concentration (Δ: synthesis, □: hydrolysis). b) Influence of methanol–water concentrations on the apparent equilibrium constant K^{app} (◆: synthesis, ●: hydrolysis) and the true equilibrium constant K (◆: synthesis, ●: hydrolysis) at atmospheric pressure.

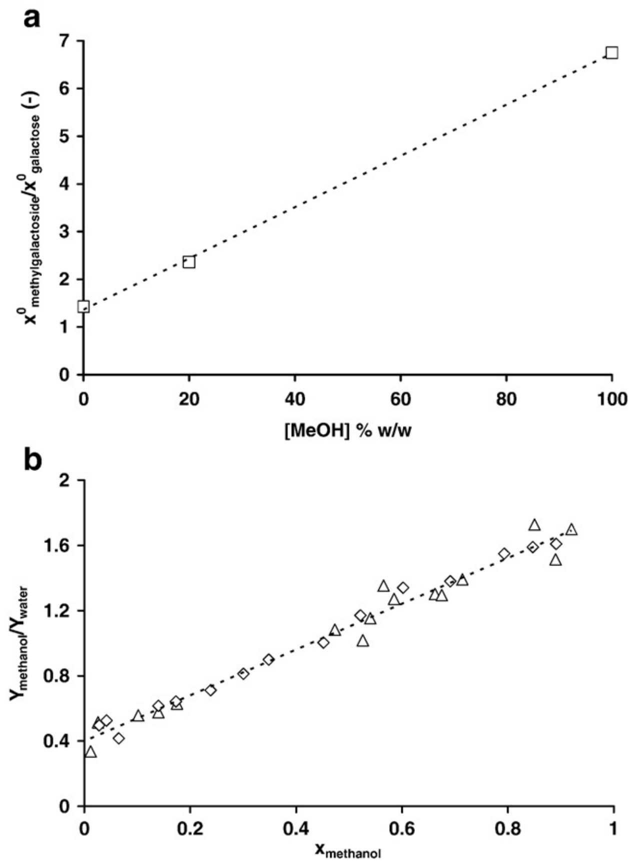

Fig. 5. a) Relative solubility of methylgalactoside compared to galactose in different methanol–water mixtures. b) Relative activity coefficients for methanol versus water in different methanol–water mixtures. Data from Δ Maripuri and Ratcliff [21] and ◇: Kohoutová et al. [22].

increasing pressure decreases the amount of β-methylgalactoside that is formed, which is shown as a decrease in the equilibrium constant.

6.7. Calculation of reaction volume based on equilibrium measurements

To quantify the influence of pressure on the equilibrium constant (K_p), the reaction volume is calculated from Eq. (5). As a reference the equilibrium constant at the corresponding methanol concentration at atmospheric pressure (K_0) is taken. The results are shown in Fig. 7. The average reaction volume is 1.8 ± 1.3 cm^3 mol^{-1}. The large inaccuracy in this number is mainly due to the values for the equilibrium constant at lower pressures. When only values of higher pressures are considered the confidence interval is halved. The reaction volume is independent on the methanol concentration, which means that under the applied conditions methanol–water is a pressure independent solvent system.

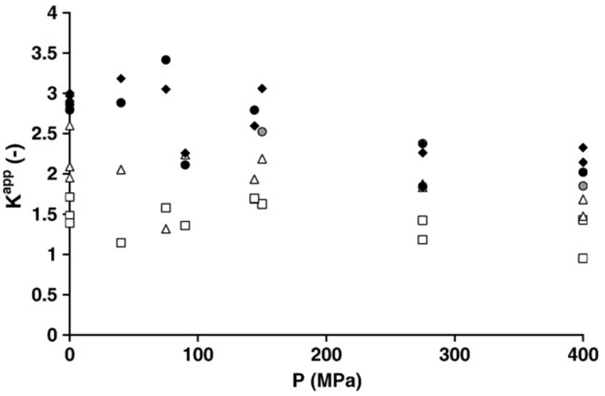

Fig. 6. Apparent equilibrium constant as a function of pressure at different methanol concentrations (☐ 4%, Δ 9%, ● 13%, ◆ 18%, ● 28%).

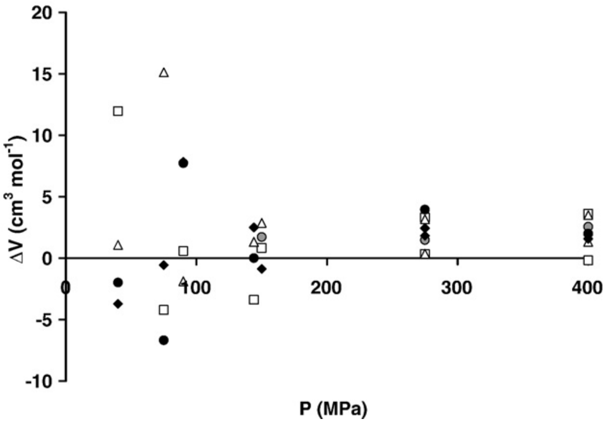

Fig. 7. Reaction volume calculated at different pressures for different methanol concentrations (☐ 4%, Δ 9%, ● 13%, ◆ 18%, ● 28%).

7. Conclusions

The influence of pressure and solvent composition on methylation in methanol–water mixtures was shown. A higher methanol concentration results in a higher product concentration since methanol is one of the reactants. In this research we found an additional increase in product concentration due to a solvent effect. These effects can be attributed to the increase in the ratio of activity coefficients of galactose and methylgalactoside at higher methanol concentrations, as determined by solubility tests. The true equilibrium constant remains the same; however the activities of the different components in the reaction mixture change, having an effect on the final product concentration. This effect can be generalized for other methylations in methanol–water reactions, since a methyl group is more hydrophobic than the hydroxide-group that it replaces, and the product will fit better in a methanol rich solvent than its corresponding reactant.

Pressure had a small, negative effect on synthesis yields. To check for added influence of the solvent to the equilibrium under pressure, all equilibrium data from pressure experiments were normalized using the equilibrium constant at atmospheric pressure at the corresponding methanol concentration. The results showed that the reaction volume was independent of the solvent composition. Synthesis was less when pressure was increased. This could be described by a reaction volume of 1.8 cm^3 mol^{-1}. This value is consistent with the calculated reaction volume of 1.6 cm^3 mol^{-1} in water. However, the reaction volume based on molar volumes at 20% methanol was much higher: 5.3 cm^3 mol^{-1}. This would have led to a much more pronounced decrease of the equilibrium constant due to pressure. For a correct comparison also the compressibility of all components has to be taken into account. This may have an important effect under pressure that counteracts the methanol effect. The scatter on our synthesis data, especially at lower pressures, keeps us from drawing more firm conclusions.

The use of an enzyme sets the boundaries for the conditions that were applied in our research. Within these conditions it is difficult to separate the absolute effect of a variable such as pressure on the reaction behavior, from the effects of other variables like the solvent composition. Moreover, changes in one variable can influence several other variables. Measurements at higher methanol concentrations were not possible due to enzyme inactivation, but may be conducted on equilibrium systems that do not need enzymes as a catalyst.

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