The Integrated Enzymatic Production

and Downstream Processing

of Glucosides

Bartholomeus Mattheus de Roode

Promotores:	dr. ir. R.M. Boom Hoogleraar in de Levensmiddelenproceskunde bij het departement Agrotechnologie en Voedingswetenschappen aan Wageningen Universiteit
	dr. Ae. de Groot Hoogleraar in de Bio-Organische Chemie bij het departement Agrotechnologie en Voedingswetenschappen aan Wageningen Universiteit
Co-promotores:	dr. ir. A. van der Padt Voormalig universitair docent bij het departement Agrotechnologie en Voedingswetenschappen, sectie proceskunde, thans werkzaam als Senior Researcher bij Corporate Research van Friesland Coberco Dairy Foods
	dr. M.C.R. Franssen Universitair hoofddocent bij het departement Agrotechnologie en Voedingswetenschappen, Laboratorium voor Organische Chemie

Samenstelling promotiecommissie:

Prof.dr.ir. A.B. de Haan
Technische Universiteit Twente
Prof.dr.ir. A.P.G. Kieboom
Universiteit Leiden
Prof.dr.ir. E.J.R. Sudhölter
Wageningen Universiteit
drs. J. de Slegte
Friesland Coberco Dairy Foods, Deventer

De enzymatische directe glucosideringsreactie omschrijven als 'reversed hydrolysis' is zoiets als 'stijgen' omschrijven met 'omgekeerd dalen'.
Vrijwel alle literatuur over de enzymatische glucosideringsreactie met uitzondering van dit proefschrift.

De hydrolysereactie van β-glucosiden door beta-glucosidase beschrijven met een uni-uni kinetiekmodel is onjuist.
Vrijwel alle literatuur over de enzymatische hydrolyse van glucosiden.

Alvorens hun uitvoerige NMR-studie van ledol te rechtvaardigen door de bestaande literatuur zeer verwarrend te noemen, hadden Kaplan *et al.* beter eerst kennis kunnen nemen van het werk van Gijsen *et al.* en Pakrashi *et al.*
Kaplan, M.A.C., Pugialli, R.L., Lopes, D., Gottlieb, H.E. Phytochemistry, 55, 749-753 (2000).
Gijsen, H.J.M., Wijnberg, J.B.P.A., Stork, G.A., de Groot, Ae, Tetrahedron 48, 2465 (1992).
Pakrashi, S.C., Ghosh Dastidar, P.P., Chakrabarty, S., Achari, B., Journal of Organic Chemistry 45, 4765-4567 (1980).

Alhoewel het erg vriendelijk is, ondermijnt het met naam en toenaam bedanken van alle reviewers van het afgelopen jaar enigszins de anonieme status van de reviewer.
Biocatalysis and Biotransformation, 18 (2000).

In tegenstelling tot wat de auteurs beweren, duidt het ontstaan van het NMR-signaal van een α-glucoside bij de glucosidering van slechts 1 van de substraat/cosolvent-combinaties met een β-glucosidase juist *wel* op een artefact.
Huneke, F-U, Baily, D., Nucci, R.,Cowan, D., Biocatalysis and Biotransformation 18, 291-299 (2000)

De keuze om in de bioreactor een relatief grote hoeveelheid water te gebruiken is logisch, maar ondermijnt de door de auteurs genoemde voordelen van micro-encapsulatie van het enzym.
Yi, Q, Sarney, D.B., Khan, J.A., Vulfson, E.N., Biotechnology and Bioengineering, 60, 385-390 (1998)

Ook plagieren van eigen werk zou bij wet verboden moeten worden.
Iborra, J.L., Castellar, M.R., Canovas, M. & Manjon, A. Biotechnology Letters 14, 475-480 (1992).
Iborra, J.L., Castellar, M.R., Canovas, M. & Manjon, A. Enzyme Microb. Technol. 15, 780-784 (1993).
Vulfson, E.N. Biotechnology Letters 12, 397-402 (1990).
Vulfson, E.N. Enzyme Microb. Technol. 12, 950-954 (1990).

Het is eerlijker om de opbouw van no-claim korting bij autoverzekeringen per gereden kilometer i.p.v. per tijdseenheid te berekenen.

Na generatie NIX en de patatgeneratie is het wachten op de D-generatie.

Een sportieve auto dient uitgerust te zijn met trappers.

Stelling elf:
Het vooroordeel dat exacte wetenschappers buiten de maatschappij staan wordt nog eens bevestigd door een maximum te stellen aan het aantal maatschappij-relevante stellingen.
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- 2-4 wetenschappelijke stellingen buiten het terrein van het onderwerp van het proefschrift;
- 2 maatschappij-relevante stellingen"

Stellingen behorend bij het proefschrift "The integrated enzymatic production and downstream processing of glucosides"
Matthew de Roode, 14 maart 2001.

Bartholomeus Mattheus de Roode

The Integrated Enzymatic Production and Downstream Processing of Glucosides

Proefschrift
ter verkrijging van de graad van doctor
op gezag van de rector magnificus
van Wageningen Universiteit
prof.dr.ir. L. Speelman
in het openbaar te verdedigen
op vrijdag 15 juni 2001
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Dit proefschrift is opgedragen aan Jannie Buitelaar

en Sarah de Roode

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Ik hebbe by yder operatie, die het na mijn oordeel nodig had, met aanmerkingen verrykt, in welke ik my in geen meiningen heb ingelaten, alſ die op de ervaring ſteunden.

Ik heb alle vlyt aangewend, om alle duiſtere woorden te myden, op dat ik verſtaanbaarder ſoude weſen alſ die voor my geſchreven hebben. Want de meeſte namen, die ik gebruikt heb, ſyn gemeen; ook iſ er geen konſt—woord, dat ik myn aanmerkingen niet ontlede.

Ik en wil my omtrent myn operatien geenſinſ voor ietſ ongemeenſ uitgeven, want ik diergelyke by andere meer heb geſien, in welke ik nietſ en vond dat verbeterenſ waardig waſ. Maar de manieren van opereren ſulje by my zo verwart niet vinden, alſ wel by ſommige.

N.Lemery, Voor-reden uit: Het philosoophische Laboratorium of der Chymisten Stookhuys, 1692

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CHAPTER 1

INTRODUCTION

GLYCOSIDES

Natural occurrence and function

Glycoside is the general name for a covalently coupled aglycon (literally: 'not sugar') to a sugar moiety. Glycosides are widely distributed in nature and can be found in nearly any living organism.[1, 2] Aglycon structures vary from simple medium-chain alkanols to proteins, while the sugar moiety can vary from monosaccharides to polysaccharides.

In plants, glycosides are believed to play a role in accumulation, storage, and transport of hydrophobic substances. In comparison to the free aglycon they show enhanced water solubility and a decreased reactivity. This allows better storage within the plant vacuole and protects the plant from any form of toxicity exhibited by the free aglycon. A few examples of storage and transport of hydrophobic aglycons are given below.

Typical examples of aglycon storage forms are cyanogenic glycosides, which play a role in the plant defense mechanism (see figure 1). At least 2500 cyanogenic glycoside containing taxa are known.[3] The main purpose of these glycosides is the defense of the plant against herbivores. Damage to the plant tissue will result in their β-glucosidase-mediated hydrolysis and the subsequent formation of HCN by the action of an oxynitrilase. Compartmentalization of the glycosides and these enzymes prevents hydrolysis in intact plant tissue.

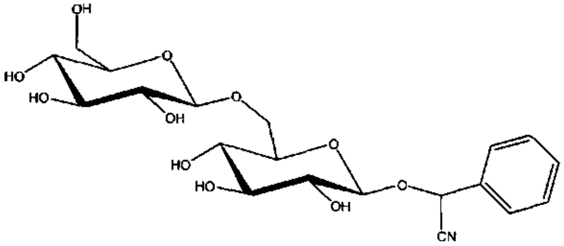
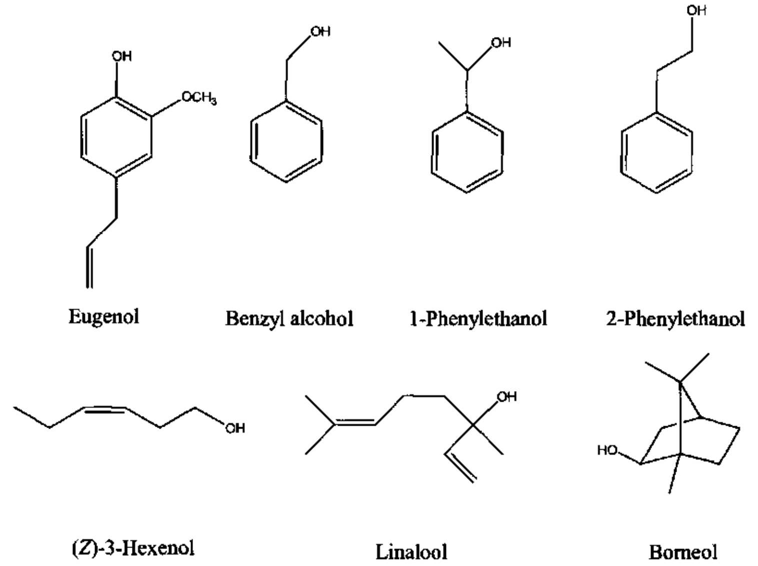


Figure 1: Amygdalin. An example of a cyanogenic glycoside from bitter almonds.

Another function of glycosides in plants is as precursors for flower fragrances (see figure 2).[4] An increase of several aroma glycosides was found during the development of the flower buds. Just before flower opening a maximum of glycosides was found, after which the levels decreased again within 24 h.



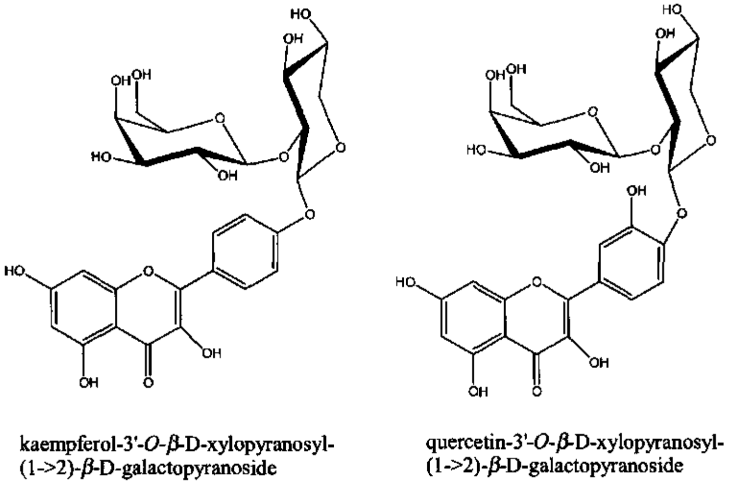
Eugenol Benzyl alcohol 1-Phenylethanol 2-Phenylethanol

(*Z*)-3-Hexenol Linalool Borneol

Figure 2: Aglycons from glycosidic precursors as found in flower buds of *Gardenia jasminoides*.

Although the exact mechanism is not elucidated yet, β-glucosidase activity in the flowers increased and decreased in the same time span.

Glycosides from flavonoids, such as quercetin and kaempferol (see figure 3), play a role in the UV-B radiation protection of white clover (*Trifolium repens* L.).[5]



kaempferol-3'-*O*-*β*-D-xylopyranosyl-(1->2)-*β*-D-galactopyranoside

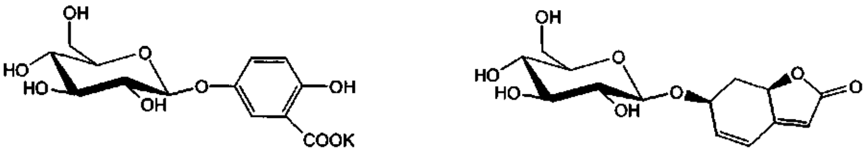
quercetin-3'-*O*-*β*-D-xylopyranosyl-(1->2)-*β*-D-galactopyranoside

Figure 3: Flavonoid based glycosides from *Trifolium repens* L.

The aglycons were released (probably with involvement of a suitable enzyme) after induction with UV-B light.

Glycosides are also known to play a role in plant leaf movements of various plant species (see figure 4).[6]

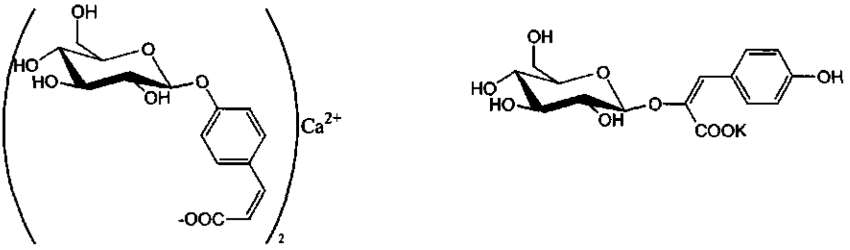
Leaf-closing glycosides



Potassium 5-*O*-*β*-D-glucopyranosylgentisate
(*Mimosa pudica* L.)

Phyllanthurinolactone
(*Phyllanthus urinaria* L.)

Leaf-opening glycosides



Calcium 4-*O*-*β*-D-glucopyranosyl *cis*-*p*-coumarate)
(*Cassia mimosoides* L.)

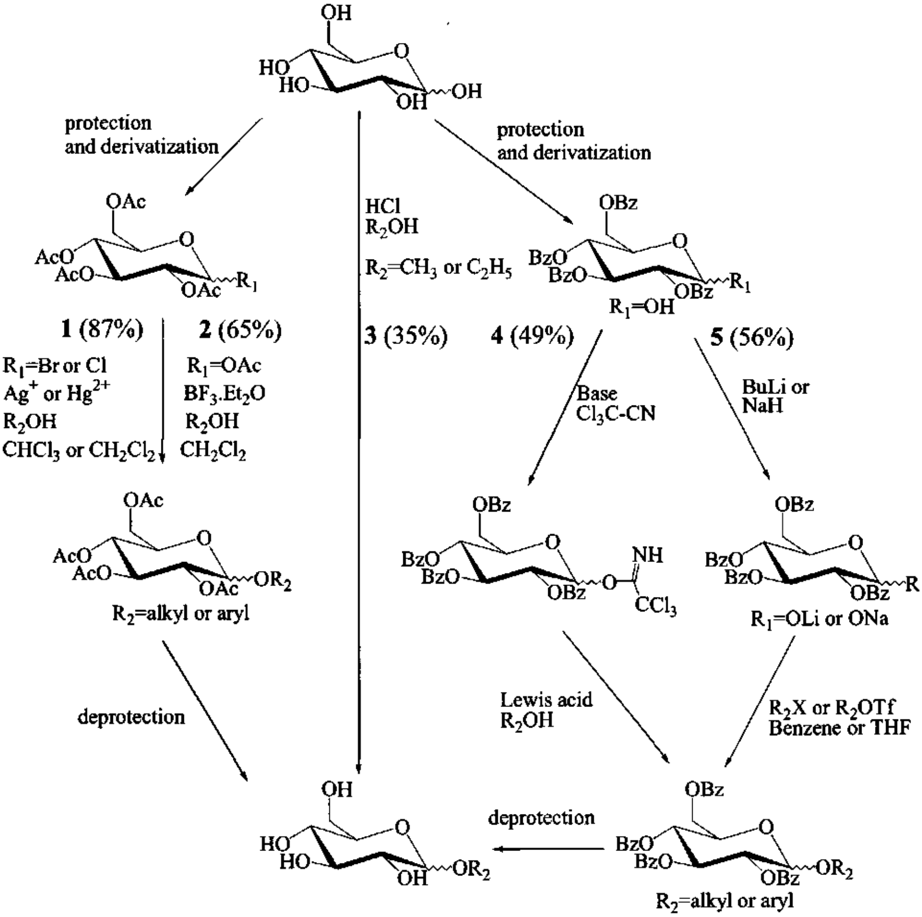
Potassium lespedezate
(*Lespedeza cuneate* G. Don)

Figure 4: Isolated leaf-movement glycosides. The glycosides and their corresponding aglycons were isolated as a pair from the same plant. Between brackets: source plant.

In this case, the glycosides are more active than their aglycons. The glycoside is deactivated by a β-glucosidase, which activity is controlled by a biological clock. Based on the activity of the glycoside and the corresponding aglycon the leaf is closed or opened.

Chemical preparation

The first method for the preparation of glycosides was developed by Fischer in 1893 and since then various glycosylation methods have been developed.[7] A schematic summary is given in scheme 1.



Scheme 1: Schematic summary of glycoside syntheses. 1. Koenigs Knorr method,[8] 2. Smits method,[9] 3. Fischer method,[10] 4. Trichloroacetamidate method,[11] 5. 1-*O*-alkylation.[12] The glucopyranoside structure is taken as an example; for most reactions other sugars can be used as well. Ac = acetyl, Bz = benzyl. Between brackets: maximum yield obtained.

With the exception of the Fischer method, all these methods require a sufficiently protected and activated sugar donor. In one case (reaction 5) even an activated sugar acceptor is necessary. In addition, in all reactions but reaction 3 toxic solvents and/or toxic and expensive catalysts are used. Only the Fischer method (reaction 3) is based on the unprotected sugar donor and acceptor. However, this reaction is limited to the glycosylation of short chain alkyl alcohols.

The overall glucoside yields given in scheme 1 are based on methanol, and will alter (most likely decrease) if other aglycons are used. The products are in all cases a mixture of the two anomeric forms α and β. Complete anomeric selectivity is in most reactions possible by tuning the reaction conditions, but this will compromise the yield.

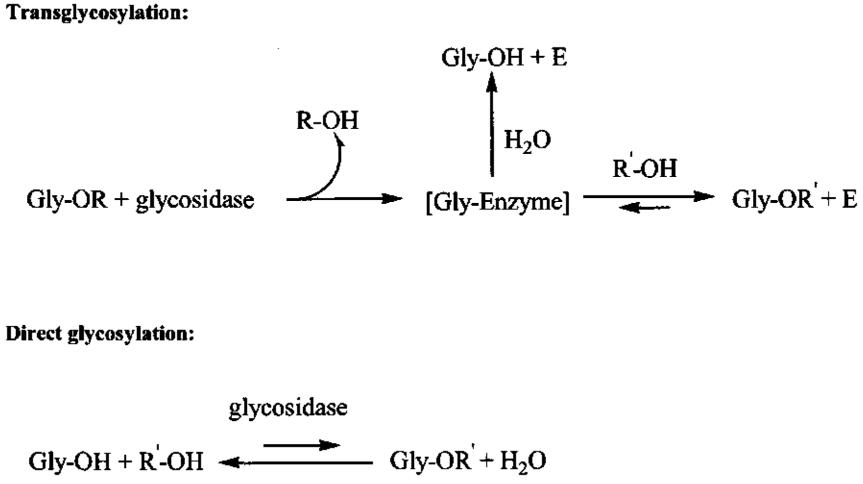
Enzymatic preparation

Glycosides can be prepared enzymatically with glycosyl transferases or glycosidases (see table 1).[13-15]

Table 1: Properties of carbohydrate converting enzymes[16]

	Glycosyl transferases	Glycosidases
Availability	Generally not available commercially or very expensive	Inexpensive, available in large quantities
Specificity	Very specific towards both sugar and aglycon	Display very low specificity towards sugar acceptor
Substrates	Oligosaccharides, nucleotide-diphosphate glycosides, etc (may be very expensive)	Mono- & disaccharides, simple alkyl and aryl glycosides
Stability	Little is known, a general conclusion cannot be drawn	Generally stable, some thermostable species are commercially available

In vivo, oligosaccharides and glycosides are synthesized by a variety of glycosyl transferases, which selectively transfer a sugar from an activated intermediate, such as a sugar nucleotide, to an aglycon. In this case, large-scale synthesis in bioreactors is only feasible when a regeneration system for these expensive nucleotides can be developed. Furthermore, the high selectivity of these enzymes makes them rather inflexible in their application. Therefore, glycosidases are generally used for glycosylation reactions. The natural function of glycosidases is to hydrolyze glycosides. Hence, they have been used for the screening of glycosidically bound volatiles in fresh plant material[17, 18], and to release aroma compounds.[19, 20] However, by tuning the reaction conditions it is possible to use glycosidases for the reverse reaction, as was already shown in 1914 with β-galactosidase.[21] Glycosylation with glycosidases can be done in two ways (see scheme 2).


Transglycosylation:

Direct glycosylation:

E = enzyme
R'-OH = aglycon
Gly-OH or Gly-OR = glycosyl donor
Gly-OR' = desired glycoside

Scheme 2: Two different ways of glycosylation with glycosidases

Depending on the sugar donor, the glycosylation reaction can be kinetically controlled (transglycosylation) or thermodynamically controlled (direct glycosylation*). In the transglycosylation reaction, higher yields can be reached in a shorter period of time than in the direct glycosylation due to an 'overshoot' of the equilibrium in the first reaction.[22] During batch transglycosylation, the glycoside yield is at a maximum, when the velocities of glycosylation and hydrolysis are equal. At this point the reaction has to be terminated, because thermodynamic control will take over and the glycoside yield will decrease. A drawback of the transglycosylation reaction is the use of, sometimes expensive, sugar donors. Furthermore, the formation of by-products from the sugar

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* In the literature the term "reversed hydrolysis" is frequently used.

donors (the alcohol- or not reacted sugar) may hamper the downstream processing. Therefore, the direct glycosylation is preferred if a high yield can be achieved. In this case the sugar donor is a monosaccharide. Different methods can be used to realize a high product yield, such as working at high substrate concentrations,[23-25] working at low water activities,[26-30] or selective removal of the product.[31, 32]

A comparison between the chemical methods and the enzymatic methods and between the individual enzymatic methods was made on the basis of space-time yield and the waste production for each method (see table 2). The Koenigs-Knorr method, having the highest overall yield, was used as a typical example for the chemical methods.

Table 2: Space-time yield and waste production for different glycosylation methods.[14] Glcβ = coupled to glucose via a β-linkage, Galβ = coupled to galactose via a β-linkage.

Method	Sugar donor	Product	Space-time yield ($g.l^{-1}.d^{-1}$)	Waste production (kg waste per kg product)
Koenigs-Knorr	Glucose	Glcβ-$OCH(CH_3)CH{=}CH_2$	36	20
Direct glucosylation	Glucose	Glcβ-$OCH(CH_3)CH{=}CH_2$	10	2
Direct glucosylation	Glucose	Glcβ-$OCH_2CH{=}CH_2$	21	0.7
Transglycosylation	Lactose	Galβ-$OCH_2CH{=}CH_2$	580	4
Direct glucosylation	Glucose	Glcβ-$O(CH_2)_6OH$	5.6	0.5
Transglycosylation	Lactose	Galβ-$O(CH_2)_6OH$	2380	3

Although the space-time yield for the Koenigs-Knorr method is reasonable, the method is a real waste generator. Moreover, this waste mainly consists of toxic solutions and heavy metals, whereas the waste from the enzymatic methods mainly consists of mono- and disaccharides. When comparing the space-time yield and the waste production of the enzymatic methods it is evident that transglycosylation is by far the most attractive method. Although the waste production is somewhat higher, the space-time yield of the transglycosylation method outnumbers the space-time yield of the direct glycosylation completely. However, as already discussed, the formation of by-products may interfere with the downstream processing of the product.

The fact that all examples in table 3 are based on lactose or glucose as sugar donor is not surprising, since these substrates are cheap and, therefore, most suitable for industrial application. The choice of only these two sugar donors narrows down the accessible enzymes for these reactions to glucosidases (in this case no specific anomeric configuration is necessary) and β-galactosidases.

Industrial applications

In most cases the type of aglycon determines the industrial applicability of a glycoside. The coupling of a sugar with a long alkyl chain as aglycon results in a glycoside with good surfactant and emulsifying properties, and therefore these glycosides are applied in detergents[33-35] and cosmetics.[36-38] Glycosides with unsaturated alkyl chains, like terpenes, are claimed to have antifungal and antimicrobial activity,[39, 40] although it is unclear why the activity of these aglycons is improved by glycosylation. Glycosides of peptides and steroids are used in antitumor formulations[41] and cardiac related drugs,[42] respectively. Finally, glycosides of flavors and fragrances are used as 'controlled release' compounds. The slow hydrolysis of the glycosides, sometimes enhanced by a suitable enzyme which is added to the application, results in a delayed release of flavors and fragrances in food[43] and, very creatively, in underwear.[44]

BIOREACTOR SYSTEMS

Glycosidase reactors

The majority of studies concerning glycosidase reactors were based on the hydrolysis reaction. In these reactions, the bioreactor choice is mostly dependent on the physical state of the enzyme. Many studies focussed on increasing the enzyme stability by immobilization and, logically, packed-bed[45, 46] and fluidized-bed[47, 48] reactors were chosen. Immobilized enzymes in packed-bed reactors were also used in studies that concentrated on production.[49, 50] However, free enzymes are more often used in these cases. Consequently, stirred tank reactors were applied,[51] sometimes in combination with a membrane[52, 53] for enzyme retention and as downstream process. In addition, stirred tank reactors in combination with free enzymes were used in a study of an intensive stirring technique induced by an electromagnetic field,[54] and in an enzyme stability study under high pressure.[55]

Although rather scarce, bioreactors were used for the glycosylation reaction as well. A stirred tank reactor in combination with a membrane was used for the production of β-thujaplicin 2-*O*-β-D-glucoside with free, water-insoluble, UDP-glucosyl transferase (see figure 5).[56]

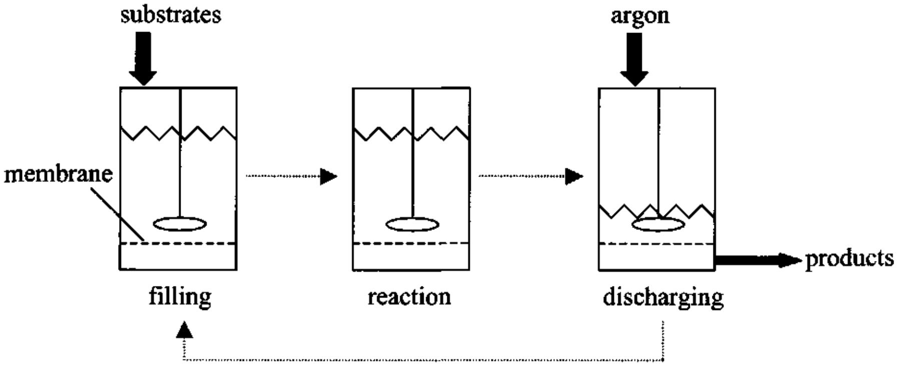

Figure 5: Semi-continuous glucosylation of β-thujaplicin 2-*O*-β-D-glucoside in a membrane reactor system.

The process was operated semi-continuously by sequentially filling and discharging the reactor, with retention of the enzyme via membrane ultrafiltration. With this bioreactor system a glucoside production of 0.11 mg.(g enzyme)$^{-1}$.h^{-1} was possible in four successive batches without loss of enzyme activity. Although the product was purified with HPLC, this production was based on the analytical yield. Due to the use of expensive UDP-glucose, the limited availability of this enzyme, and the laborious downstream process, the industrial applicability of this reactor may be questioned. However, the demand for this product and an improved reactor design to eliminate the sequential filling and discharging of the reactor could make these arguments irrelevant.

A bioreactor system with more potential for an industrial application is a stirred tank reactor with immobilized almond β-glucosidase for the production of hexyl glucoside (see figure 6).[32]

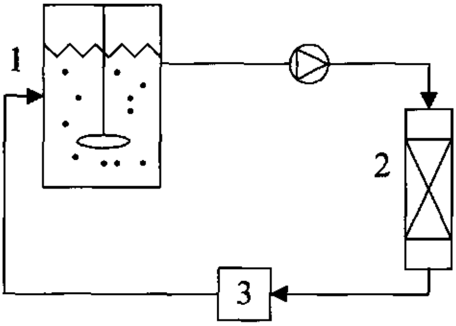

Figure 6: Bioreactor system for the semi-continuous production of hexyl glucoside. 1. Stirred tank reactor with immobilized enzyme, 2. Alumina adsorption column, 3. Hexanol hydrating step.

The combination of a stirred tank reactor with an immobilized enzyme seems unusual, but in this way it was possible to work at very low water concentrations. The enzyme requires a minimum amount of water and, therefore, hexanol was saturated with buffer. Glucose and the enzyme were both encapsulated together in microcapsules. Although the stability of the enzyme in this environment seemed to be enhanced, the continuous production of hexyl glucoside was unfeasible with this system, because it is impossible to supply glucose to the microcapsules during the reaction. Therefore, the semi-continuous production was performed with a hexanol phase/water phase ratio of 10, bypassing the initial objective of working at very low water concentrations. With this system an initial production rate of 0.20 $mg.U^{-1}.h^{-1}$ was accomplished. Besides the mentioned shortcoming it is noteworthy that this is one of the few literature examples of a glycosidase reactor in which the product stream is actually processed via an alumina adsorption column.

Downstream processing of glycosides

The nature of the aglycon determines for an important part the downstream process. In case of a hydrophobic aglycon, the produced glycoside will most likely migrate towards the aglycon phase. This migration behavior is of course dependent on the hydrophobicity of the aglycon, and an increase in aglycon hydrophobicity will lead to an increased migration of the glycoside to the aglycon phase. This allows a separation step based on the polar sugar part of the glycoside. Thus, in some cases back-extraction with water, and selective adsorption come into focus. Extraction with water was used for the extraction of chemically produced C_8 to C_{12} alkyl glycosides from a toluene mixture.[57] Selective adsorption was used for the purification of various glycosides with silica[29, 58-60] and alumina[32, 61] as adsorbents. Another possibility for the purification of glycosides is to make use of their surfactant properties. Glycosides from hydrophobic aglycons (C_{12}-C_{22} alkyl chains) do have surfactant properties and are applied as foaming agent.[62] This foaming characteristic could be used for purification purposes. Finally, if the aglycon is volatile, the large difference in boiling point of the aglycon and the corresponding glycoside can be used to separate the aglycon/glycoside mixture by distillation.[63-65]

Purification of a glycoside of a hydrophilic aglycon is more complex, since the glycoside has to be recovered from a mixture of highly similar components. Adsorption on two different columns to subsequently remove the sugars and the glycoside from the aglycon was used for the purification of glyceryl glucoside.[66] Distillation could be an option if the aglycon is volatile enough. However, after distillation the distillate will contain the unreacted sugar as well as the glycoside.

OBJECTIVE AND JUSTIFICATION

Glycosides are of commercial interest for the industry in general and for the pharmaceutical and food industry, specifically. Chemical preparation of glycosides is not applicable in the food industry, and therefore the enzyme-catalyzed reaction is a good alternative. However, until now the low yield in the enzymatic reaction prevents the production of glycosides on a commercial scale. Therefore, high yields should be established by a combination of optimum reaction conditions and a continuous removal of the product. Since the reaction should be based on cheap substrates in practice only lactose and glucose can be used as sugar donor, which limits the enzyme choice to glucosidases and β-galactosidases. The expected downstream processing problems due to the formation of by-products when using lactose induced us to use glucose as a substrate. Consequently, a glucosidase has to be used, and being an inexpensive and commercially available enzyme, almond β-glucosidase was selected.

Currently, a reactor for the commercial scale production of glucosides is not available. Therefore, the objective of this study is also the design of a production process that combines the optimum reaction conditions for the enzymatic glucosylation with a selective removal of the desired product.

OUTLINE OF THE THESIS

In this thesis, a glucosylation reaction mediated by almond β-glucosidase has been developed, together with a reactor that integrates the enzymatic production and downstream processing of glucosides. The specificity of the enzyme towards certain aglycons is discussed and explained in chapter 2. The glucosylation reaction is investigated and described in chapter 3. Successively, the optimum reaction conditions for operational stability of the enzyme during the glucosylation reaction (chapter 3.1), and the performance of the enzyme in an one-phase reaction (chapter 3.2) are described. The optimum reaction conditions have been applied in a reactor system that is presented in chapter 4. In chapter 4.1, the downstream processing based on adsorption is integrated with the enzymatic production of hexyl glucoside. The glucosylation of geraniol (an industrially relevant aglycon) is described in chapter 4.2, using the same reactor system. Furthermore, alternative downstream processes are introduced. Final considerations are given in chapter 5. Attention is given to alternative enzymes for the glucosylation reaction, the observed stability of the produced glucosides, an evaluation of the downstream processes, and the industrial applicability of the reactor system.

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CHAPTER 2

WHY ARE SOME ALCOHOLS EASY TO GLUCOSYLATE WITH β-GLUCOSIDASES WHILE OTHERS ARE NOT? A COMPUTATIONAL APPROACH.[*]

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ABSTRACT

A method is presented to predict the reactivity of alcoholic aglycons in the β-glucosidase mediated glucosylation reaction. The successful enzymatic glucosylation of an aglycon appears to be mainly dependent on the nucleophilicity of the aglycon. Vinylic and phenolic aglycons are not enough nucleophilic to be glucosylated enzymatically, although their chemical glucosylation is facile. By using PM3 and AM1 semi-empirical methods, the magnitude of this nucleophilicity can be calculated and was found to correlate with the charge on the reacting atom of the aglycon. Based on this trend, the aglycons can be divided in reacting and non-reacting. The orbital related parameters seem to have a limited influence on the reaction behaviour. In addition to these calculations, the energy of the transition state of two enzymatic reactions has been calculated using a simplified model of the enzyme active site for both an experimentally reacting and an experimentally non-reacting aglycon (cyclohexanol and phenol, respectively). The activation energy for the cyclohexanol complex was computed to be 1.3 kcal.mol^{-1}, while the calculated activation energy for the phenol complex is 15.8 kcal.mol^{-1}. This difference can indeed explain the fact that cyclohexanol is easily glucosylated while phenol is not.

Finally, it is pointed out that facile and fast calculation methods can be used to make a confident prediction about the reaction behaviour of aglycons without performing the actual laboratory experiments.

INTRODUCTION

β-Glucosidases

β–Glucosidases are widely applied in food technology, biotechnology and organic chemistry. These enzymes have been used for the hydrolysis of glucosides and for the glucosylation of various alcohols. In the hydrolysis reaction, β–glucosidases are commonly used to screen glucosidically bound volatiles in fresh plant material.[1] Through hydrolysis of the corresponding glucosides, a variety of aglycons like monoterpenols[2, 3] or phenols[4, 5] are easily released. By tuning the reaction conditions, for example by working at high substrate concentrations, β–glucosidase can also glucosylate alcohols[6-9]. However, this enzymatic pathway suffers from low yields in comparison to chemical glucosylation.

If we compare the aglycons that are used for the enzymatic glucosylation reaction with the volatiles that are released in the enzymatic hydrolysis reaction, it is apparent that the hydrolysis reaction is not in all cases reversible. Such reported glucosylation reactions mainly deal with linear alcohols[10-12] and sugars[13, 14], while phenols have never been enzymatically glucosylated, to our knowledge. In line with this, only the aliphatic hydroxyl group of hydroxyalkyl phenols is glucosylated[15], whereas phenolic glucosides are readily hydrolysed. To understand this one-sided behaviour of β-glucosidase, the mechanism for the reaction, proposed in 1953[16], has to be examined (see schematic view in Figure 1).

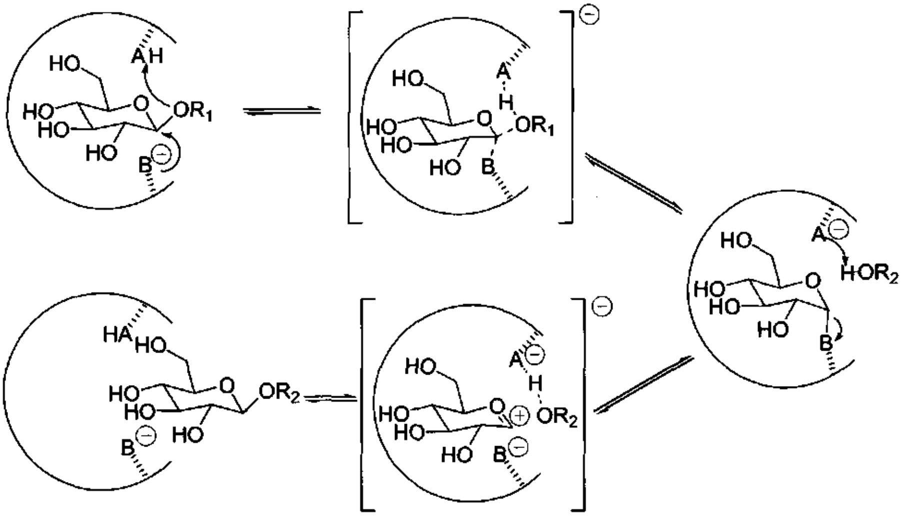

Figure 1: Schematic view of the reaction mechanism of β-glucosidase. R_1OH = water or aglycon, R_2OH = aglycon or water. AH = acidic amino acid side chain. B^- = basic amino acid side chain

In the first step, the capacity of the aglycon to act as a leaving group is enhanced by protonation, after which the anomeric centre of the glycon is attacked by a nucleophilic group from the enzyme. Formation of the covalent α-glucose-enzyme intermediate[17] (proved for an *Alcaligenes faecalis* β-glucosidase) proceeds *via* a transition state with some oxocarbonium ion character[18, 19] (determined from kinetic isotope effect and *ab initio* studies). The nucleophilic attack of an aglycon or water upon the anomeric centre proceeds *via* a transition state with a more pronounced oxocarbonium ion character[20-22] (derived from kinetic isotope effect and inhibition studies). The newly formed β-glucoside leaves the active site, returning the enzyme to its original state. Studies on almond β-glucosidase[23, 24] show evidence of a similar mechanism. In a recent study of the reaction mechanism the position of the acidic amino acid in the active site (AH in Figure 1) is discussed. It seems that the aglycon is not protonated "from above" (as in Figure 1), but "from the side" (the non-dissociated amino acid is positioned at the same side as the basic amino acid B).[25]

In the proposed reaction mechanism, which has been substantiated by several studies[19, 26], the nature of both glycon and aglycon is important. The glycon is important for the recognition of the glucoside by the enzyme, whereas the aglycon mainly determines the reaction rate in both hydrolysis and glucosylation reactions. In studies with an *A. faecalis* β-glucosidase, the first step in the reaction mechanism was found to be rate-determining for the hydrolysis of glucosides having relatively poor leaving groups ($pK_a > 8$).[20] If the leaving group is a relatively strong acid ($pK_a < 7$), the second step becomes rate-determining, as was shown with almond β-glucosidase.[23]

It is assumed that the second step in the mechanism proceeds *via* an oxocarbonium ion-like transition state, in which the charge development on the oxygen atom can be, to some degree, substrate dependent. This may lead to the conclusion that the differences in the aglycon reactivity should not be as extreme (reacting or non-reacting) as they appear to be. The nucleophilicity of the aglycon clearly is important in this step of the mechanism. Therefore, a method to determine the nature of the nucleophilicity of the aglycon would be helpful to explain why some aglycons cannot be glucosylated. With the aid of computational electronic structure methods it is possible to calculate a set of parameters which are related to the nucleophilicity of the aglycon.

Computational quantum chemistry in enzyme catalysis

Computational quantum chemistry plays an increasingly important role in the description of enzyme catalysis. *Ab initio* and semi-empirical calculation methods are mainly used to elucidate the transition state of the enzyme reaction.[27] Studies on the mechanisms of β-lactamase[28], glucosaminidase[29], glutathione reductase[30], serine hydrolase[31] and glycosidases[32] are typical recent examples for this type of calculations. Another type of studies involve structure-activity relationships based on computationally derived reactivity parameters. In many cases, the reactivity can be correlated with frontier orbital parameters[33], i.e. the energy and relevant orbital coefficients of the HOMO (Highest Occupied Molecular Orbital) and LUMO (Lowest Unoccupied Molecular Orbital), and the charges on the atoms directly involved in the reaction. HOMO or LUMO energies of the reactants display a good correlation with the enzymatic reaction rates of peroxidase[34], glutathion *S*-transferase[35], catechol-1,2-dioxygenase[36] and cytochrome P450.[37]

Although the mechanism of β-glucosidase has been thoroughly investigated in literature, to our knowledge, the glucosylation reaction has never been studied from a theoretical point of view. The aim of this chapter is to give a theoretical background for the observed differences in reactivity of β-glucosidase towards aglycons in glucosylation reactions. To this end, the orbital coefficients and LUMO and HOMO energies will be calculated, completed with electrostatic potential charge and orbital-based atomic charges for 16 nucleophiles which have been subjected to the glucosylation reaction. Furthermore, the ESP (ElectroStatic Potential) and orbital-based atomic charges will be calculated for a variety of successfully glucosylated aglycons from the literature. The importance of the different parameters with respect to the glucosylation reaction will be evaluated. In addition, the reaction path for a successfully glucosylated aglycon and a non-reacting aglycon will be simulated. From these data the transition state for the glucosylation of both systems will be calculated. Finally, we will show that the calculation of these parameters is a useful tool to rapidly predict the behaviour of aglycons in the β-glucosidase-mediated glucosylation reaction.

EXPERIMENTAL

Calculations

The structures of all aglycons were pre-optimised using the MM2 force field as implemented in the ChemOffice Ultra program (Cambridge Scientific Computing). These structures were subsequently fully optimised with semi-empirical methods (both AM1 and PM3), to obtain the HOMO/LUMO energies, orbital coefficients, orbital-related net atomic charges, and the electrostatic potential charges. In all cases solvent models were used to mimic the effects of water, with a dielectric constant $\varepsilon = 78.5$. Within MOPAC97 (part of ChemOffice Ultra) this was performed using the COSMO routine.[38] The AM1 results differed marginally from those calculated with the PM3 hamiltonian, but the same trend was observed. Therefore, the results from the AM1 calculations will not be discussed.

For two cases transition state optimisations were performed (PM3 computation in Gaussian 94[39]). First, a reaction path for the stepwise approach of the reacting oxygen atom of the aglycon anion and the reacting carbon atom of the glucose-glutamic acid complex was calculated (from 3.0 Å to 1.4 Å , stepsize r(C-O) = 0.1 Å). Secondly, transition state optimisations were carried out with the QST2 option implemented in Gaussian 94. The transition state was in both cases characterised by performing vibrational frequency analysis (one imaginary frequency, corresponding to movements along the reaction path, was found). The transition states of the complexes with neutral aglycons were estimated by following the reaction path for the stepwise approach of the reacting oxygen atom of the neutral aglycon and the reacting carbon atom of the glucose-glutamic acid adduct (from 1.70 Å to 1.65 Å, stepsize r(C-O) = 0.01 Å). The energies between the optimised structures where the $C\text{-}O_{\text{leaving group}}$ bond was broken ($C\text{-}O_{\text{leaving group}}$ distance equals 1.55 Å for both structures) were used as a representative estimate of the transition state energy.

Chemicals

Glucose, cyclohexanol and nerol were obtained from Sigma. Ethyl acetate, petroleum ether, acetonitrile (HPLC-grade) and methanol (HPLC-grade) were obtained from Lab-Scan. *t*-Amyl alcohol, *p*-methoxyphenol and geraniol were obtained from Acros. Citronellol, vanillin, eugenol, cinnamin alcohol, phenethyl alcohol, benzyl alcohol, furaneol and 4-hydroxybenzaldehyde were obtained from Aldrich. 1-Hexanol and 1-octanol were obtained from Merck. Phenol was obtained from Brocades. Deionized water was used in all experiments. Citric acid and sodium phosphate from Sigma were used to prepare the buffer solution.

Citrate/phosphate buffer pH 6.0 was prepared by dissolving 7.74 g citric acid and 45.23 g sodium phosphate in 1 l water.

Enzyme

Almond β-glucosidase (E.C.3.2.1.21) was obtained from Sigma (5.6 $U.mg^{-1}$).

Equipment

Incubations were carried out in thermostated New Brunswick Scientific G24 or Innova 4080 incubator shakers. The HPLC system was composed of a Gynkotek pump and auto sampler with a Gynkotek UV-detector and a Sedex Evaporative Light Scattering Detector. Samples were eluted with acetonitrile/water (1 $ml.min^{-1}$, 90/10 v/v %) on a Spherisorb S5-amino column (150x4.6mm). Activity measurements were carried out using a Perkin Elmer Lambda 18 UV/Vis spectrophotometer. NMR measurements were carried out on a Bruker AC 200. Mass spectra were measured with a Finnigan Mat 95 mass spectrometer. Preparative scale separations were carried out with Bond Elut LRC NH_2 columns from Varian.

Enzymatic glucosylation

The enzymatic reaction was optimised according to the results from chapter 3.1. The enzymatic reaction was carried out in a well-capped 20 ml vial containing 0.5 ml of citrate/phosphate buffer pH 6.0 (0.16 M) with an almond β-glucosidase activity of 0.4 $U.ml^{-1}$ to 30 $U.ml^{-1}$ buffer solution, 500 g glucose per kg of buffer solution and an organic phase (4.5 ml) consisting of pure aglycon or of various concentrations of the aglycon in *t*-amyl alcohol. The two-phase system was shaken at approximately 280 rpm at 50 °C. At regular time intervals samples for HPLC analysis were taken from the organic layer of the reaction mixture.

Preparative glucosylation of geraniol, nerol and citronellol

The enzymatic reactions were carried out as described above, using 4.5 ml of pure aglycon and 20 U enzyme per ml buffer solution. After reaching equilibrium, the reactions were stopped. The two-phase system was evaporated under reduced pressure at 40 °C. The liquid residue was filtered over a funnel with a plug of cotton in order to remove insolubles. The clear mixture was separated over a preparative amino column. The column was activated with 3 ml $CHCl_3$ after which 3 ml of the reaction mixture was applied. Then, the column was rinsed with three portions of 1 ml $CHCl_3$. Finally, 3 ml of methanol was used to elute the glucoside from the column. This methanol phase was evaporated to yield the pure glucoside.

Geranyl-β-D-glucopyranoside FAB-MS: 339 (M+Na), 163, 145, 137, 113, 95, 81, 69; ^{1}H NMR (200 MHz, D_2O) δ 1.39 (s, 3H, $H_{9'}$), 1.46 (s, 6H, $H_{8'}$, $H_{10'}$), 1.90 (br s, 4H, $H_{4'}$, $H_{5'}$), 3.02 (t, J = 8.3 Hz, 1H, H_2), 3.13 (br s, 1H, H_5), 3.43 (m, 2H, H_3, H_4), 3.65 (br s, 2H, H_6), 4.10 (d, J = 7.4 Hz, 2H, $H_{1'}$), 4.22 (d, J = 7.9 Hz, 1H, H_1), 4.96 (m, 1H, $H_{6'}$), 5.15 (m, 1H, $H_{2'}$)

Neryl-β-D-glucopyranoside FAB-MS: 339 (M+Na), 315 (M-H), 163, 137, 95, 81, 69, ^{1}H NMR (200 MHz, D_2O) δ 1.39 (s, 3H, $H_{9'}$), 1.55 (s, 6H, $H_{8'}$, $H_{10'}$), 1.95 (br s, 4H, $H_{4'}$, $H_{5'}$), 3.00 (t, J = 8.3 Hz, 1H, H_2), 3.18 (br s, 1H, H_5), 3.50 (m, 2H, H_3, H_4), 3.65 (br s, 2H, H_6), 4.10 (d, J = 7.7 Hz, 2H, $H_{1'}$), 4.21 (d, J = 7.9 Hz, 1H, H_1), 5.00 (m, 1H, $H_{6'}$), 5.18 (m, 1H, $H_{2'}$)

Citronellyl-β-D-glucopyranoside ^{1}H NMR (200 MHz, D_2O) δ 0.89 (s, 3H, $H_{9'}$), 1.42 (s, 6H, $H_{8'}$, $H_{10'}$), 1.47 (m, 1H, $H_{2'}$), 1.65 (br s, 4H, $H_{4'}$, $H_{5'}$), 3.01 (t, J = 8.2 Hz, 1H, H_2), 3.18 (br s, 1H, H_5), 3.50 (m, 2H, H_3, H_4), 3.65 (br s, 2H, H_6), 4.17 (d, J = 7.9 Hz, 2H, H_1), 4.36 (d, J = 7.1 Hz, 1H, H_1), 4.95 (m, 1H, $H_{6'}$)

Activity measurements

All non-reacting aglycons were monitored for their inhibitory effect on the enzyme. For this purpose, enzyme reactions were carried out as described above. At regular time intervals, samples were taken from the water phase for activity measurements. The activity of β-glucosidase was measured by monitoring the hydrolysis of *para*-nitrophenol glucoside (pNP glucoside) with UV-vis spectrometry. A known amount of β-glucosidase was added to 2.5 ml of a 2 mM solution of pNP glucoside in a citrate/phosphate buffer pH 6.0. The increase of *p*-nitrophenol in time was measured at 405 nm at 40 °C.

RESULTS

Glucosylation

First, enzymatic glucosylation experiments with phenol, eugenol and vanillin were performed (see Figure 2, structures 1, 2 and 3), but no glucosides could be obtained from these reactions.

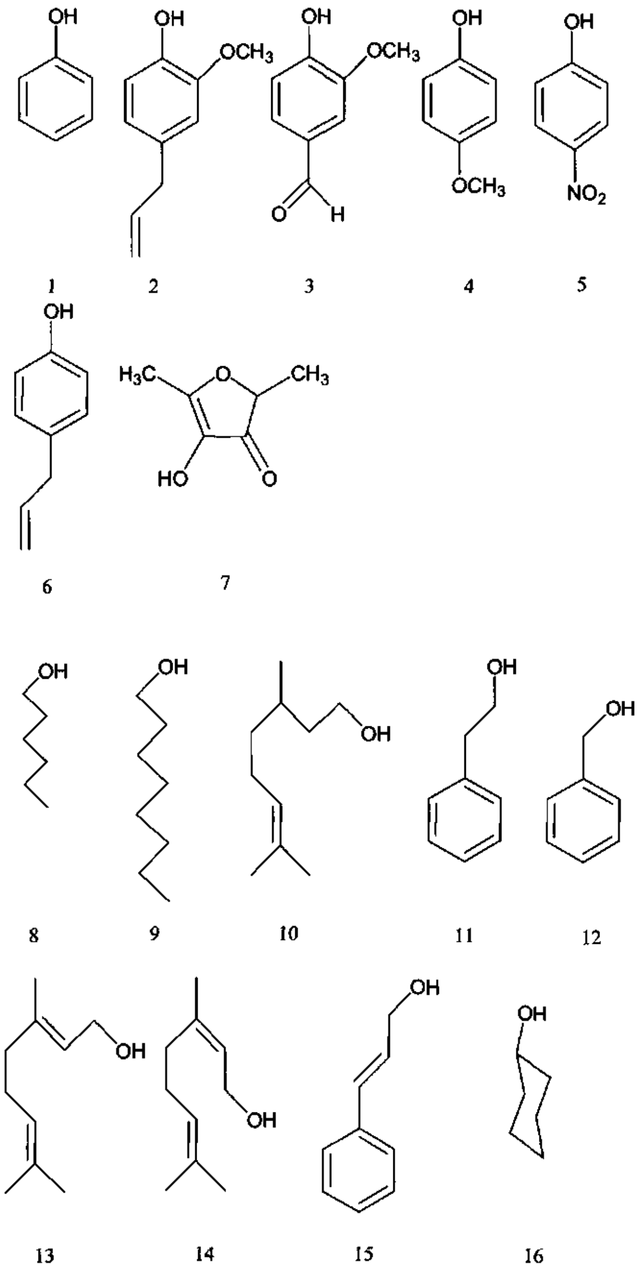

Figure 2: 16 Aglycons which have been subjected to the glucosylation reaction with β-glucosidase.
1. Phenol, 2. Eugenol, 3. Vanillin, 4. *p*-Methoxyphenol, 5. *p*-Nitrophenol, 6. Chavicol, 7. Furaneol, 8. Hexanol, 9. Octanol, 10. Citronellol, 11. Phenethyl alcohol, 12. Benzyl alcohol, 13. Geraniol, 14. Nerol, 15. Cinnamin alcohol, 16. Cyclohexanol

The successful glucosylation of primary (Figure 2, structures 8 to 12), allylic (Figure 2, structures 13 to 15) and secondary (Figure 2, structure 16) alcohols induced us to search for the reason why the first group of aglycons could not be glucosylated. The nucleophilic character of the non-reacting aglycons was modified by substituting the phenol ring with electron-donating (Figure 2, structure 6) or electron-withdrawing (Figure 2, structures 4 and 5) groups. These modifications did not lead to the desired glucosides either. In addition, an enolic furan derivative (Figure 2, structure 7) was tried, but this was also not glucosylated.

These results suggest that the nucleophilicity of the aglycon is an important reactivity parameter. Therefore, to investigate the nature of the nucleophilicity of all tested aglycons, we have calculated the charges and relevant orbital-related parameters of the atoms that are directly involved in this reaction for the ground state of the molecules in figure 2.

Semi-empirical calculations

As there is no unambiguous quantum mechanical operator to calculate the charge on an atom, several methods to approximate this charge are available. Since the charge on the oxygen atom of the aglycon is likely to be important for the reactivity, and to minimise the possibility of any method-dependent result, two very different electron-density division schemes were used to obtain two types of charges: net atomic charges (based on an orbital-division technique) and ESP charges (derived from the electrostatic potential around the molecule). The calculated charges on the oxygen atom of the aglycons and the relevant parameters for the active frontier orbitals are shown in Table 1.

Table 1: Calculated values of net atomic and ESP charge (Q), orbital coefficient of the reacting atom in the HOMO (c) and the molecular orbital energy (E_{HOMO}) for different aglycons. **Bold** = Successfully glucosylated by β-glucosidase, Plain = Unable to glucosylate with β-glucosidase

Aglycon	Q_{nuc}		c_{nuc}^2 (-)	E_{HOMO} (eV)
	Net Atomic	ESP		
Octanol	-0.408	-0.417	0.364	-11.08
Hexanol	-0.407	-0.420	0.359	-11.08
Citronellol	-0.405	-0.417	0.003	-9.52
Geraniol	-0.404	-0.405	0.006	-9.56
Cinnamyl alcohol	-0.403	-0.426	0.010	-9.25
Phenethyl alcohol	-0.403	-0.415	0.002	-9.77
Cyclohexanol	-0.400	-0.430	0.260	-11.08
Nerol	-0.399	-0.398	0.002	-9.62
Benzyl alcohol	-0.397	-0.362	0.020	-9.94
p-Methoxyphenol	-0.300	-0.314	0.107	-9.08
Phenol	-0.299	-0.302	0.141	-9.47
Chavicol	-0.299	-0.321	0.126	-9.33
Eugenol	-0.284	-0.269	0.125	-9.35
Vanillin	-0.279	-0.281	0.104	-9.31
p-Nitrophenol	-0.278	-0.291	0.155	-9.89
Furaneol	-0.266	-0.261	0.114	-9.60

The charge on the reactive atoms was also calculated for successfully glucosylated aglycons from the literature (see Table 2).

Table 2: Calculated Net atomic and ESP charges on the reactive oxygen atom of successful glucosylated aglycons with β-glucosidase according to literature

β-glucosidase source	Aglycon	Q_{nuc}	
		Net atomic	ESP
Almond[40]	2-Acetamidopropanol	-0.399	-0.402
	Methyl-2-acetamido-3-hydroxypropanoate	-0.393	-0.348
	trans 1-Acetyl-4-hydroxy-L-proline	-0.391	-0.406
	Methyl-2-azido-3-hydroxypropanoate	-0.387	-0.360
	Methyl-2-acetamido-3-methyl-3-hydroxy-propanoate	-0.383	-0.401
Almond[41]	Pent-3-en-2-ol	-0.403	-0.409
	But-3-en-2-ol	-0.402	-0.420
	Pent-1-en-3-ol	-0.400	-0.420
	Hex-1-en-3-ol	-0.395	-0.421
Sulfolobus solfatoricus[42]	3-Hydroxymethyl-4-hydroxypentanol	-0.403	-0.402
Almond[12]	6-Trifluoro-6-acetamidohexanol	-0.407	-0.413
Almond[43]	2-Trimethylsilylethanol	-0.419	-0.420
	Hexane-1,6-diol	-0.407	-0.420
	Pent-4-en-1-ol	-0.402	-0.411

Determination of the enzyme model

The applicability of initial state calculations to explain the reactivity of different aglycons is, of course, limited to reactions in which the initial state resembles the transition state in a sufficient degree for the purpose at hand. However, for reactions with an early transition state, this technique allows a very fast determination of reactivity trends.[44-46] The optimisation of the geometry of the transition state itself provides more direct information, and can give a more detailed insight into the observed differences in the aglycon reactivity. Therefore, a model of the enzyme-glucose intermediate has to be made. For this, it is important to determine to what extent the active site of the enzyme can be simplified, without losing specific reaction characteristics. Various studies concerning the active site of β-glucosidases have been performed to elucidate the role of the non-covalent interactions between the enzyme and the substrate glycon. The importance of hydrogen bonding at the different positions of the glycon has been studied for *A. faecalis* β-glucosidase and compared with earlier studies on β-glucosidases from *E. coli, A. wertii, A. oryzae* and mammalian lactase.[47] Interactions with all oxygen atoms of the glycon play a role in the recognition of the substrate, the stabilisation of the ground state and the transition state of the substrate-enzyme intermediate. However, the most important interaction for stabilising the transition state for all β-glucosidases seems to be the interaction at the 2-position of the glycon.[47]

Mutagenesis experiments with *A. faecalis* β-glucosidase[48] and the crystal structure of a white clover β-glucosidase[49] have identified glutamate as the attacking nucleophile in the active site. Based on this information, approximate models of the structure of the glucose-enzyme intermediate can be made, and two of these (model systems I and II) are presented in Figure 3.

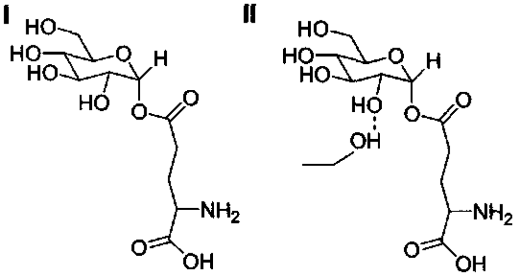

Figure 3: Two models of the covalent glucose-enzyme intermediate as used in the calculations. I. γ-(α-glucosyl) glutamate. II. I with a hydrogen bond on the 2-position of glucose. For reasons of simplicity, ethanol was taken as the hydrogen bond donor/acceptor.

Using these enzyme models, the reaction path of an aglycon approaching the glucose-enzyme intermediate can be simulated. Since a simplified model of the glucose-enzyme intermediate is used, the obtained energy levels of the different stationary points of the glucosylation reaction will be approximations, yet useful for a comparison between the different aglycons and to indicate which parts of the model system are important for the nucleophilic substitution reaction. For this purpose the relevant charges and orbital parameters on the reacting carbon atom of these two systems and glucose are given in Table 3.

Table 3: Calculated values of the orbital and charge parameters of the anomeric carbon atom for two models of the covalent β-glucosidase intermediate

Model	Q_{elec}	c_{elec}^2 (-)	E_{LUMO} (eV)
Glucose	0.197	0.174	1.920
I	0.200	0.008	0.331
II	0.186	0.004	0.361

The differences between the orbital parameters of the two model systems I and II are minimal. However, compared to glucose there is a large difference. Apparently, the covalent bond with glutamic acid affects the electron distribution on the anomeric centre, while the hydrogen bond barely influences this distribution. The charges on the anomeric centre of model I and glucose are almost identical. The hydrogen bond does have a small effect on the charge of the anomeric carbon atom, as was expected. This difference will have an influence on the results of the calculations of the transition state, but since this effect is similar for all approaching aglycons this will not significantly affect our final conclusions. These models represent the electrophile as present in glucosidase better than glucose. However, since the difference in charge and orbital parameters on the anomeric centre of the two models is small, further calculations have been done with intermediate model I to obtain a compromise between completeness of representation and computational efficiency.

Transition state optimisation

Although the reaction mechanism of the glucosylation by β-glucosidase is investigated thoroughly with a variety of experimental techniques, the degree of concertedness of bond breaking and bond making in the transition state is still unclear.[25] Evidence based on kinetic isotope effect studies exists for a transition state with a S_N2-like character in the first step of the mechanism.[18, 21] For the second step in the mechanism larger kinetic isotope effects were observed, supporting a more oxocarbonium-like intermediate.[20]

Therefore, it is difficult to predict the character of the transition state *a priori*. The transition state of the glutamic acid-glucose-aglycon complex was optimised with a non-reacting (phenolate) and a reacting aglycon (cyclohexanolate). These anions were chosen based on their similar steric effects (Van der Waals area of 62.5 Å^2 and 63.5 Å^2, respectively), but significantly different reactivity.

First, the reaction path of both aglycons reacting with model system I was determined, *via* a stepwise reduction of the bond length between the oxygen-atom of the aglycon and the anomeric carbon atom with full optimisation of all the geometrical features (see Figure 4).

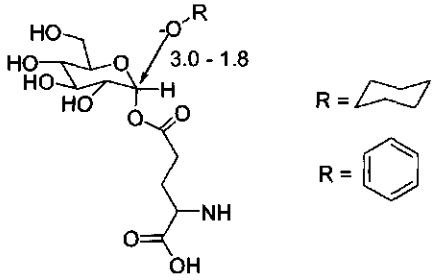

Figure 4: Approach of a deprotonated aglycon towards the anomeric centre of the enzyme-glucose model.

The structure with maximum enthalpy on this approximate reaction path was used as a starting point for the full geometry optimisation of the corresponding transition states. Optimised transitions states are presented in Figure 5, together with selected geometrical features.

Although the conjugation of the hydroxyl group with a phenyl ring will diminish its nucleophilicity, several cases are known in which phenols can be glucosylated efficiently. For example, the chemical glycosylation of tyrosine has been successful in an acid-catalysed condensation reaction between peracetylated glucose and a suitably protected tyrosine residue[54] and a variety of phenols have been glucosylated based on the same glucose donor, using boron trifluoride etherate as catalyst.[55] Since this nucleophilicity factor did not yield a clear answer, it was investigated in more detail.

Calculation of parameters related to the nucleophilicity of the aglycons

The nucleophilicity of the tested aglycons can be described with a confined number of parameters that can easily be calculated with semi-empirical quantum chemical calculations. From the parameters in Table 1, a correlation between the charge on the reacting atom of the aglycon and the reactivity thereof can be inferred. Apparently, vinylic and phenolic aglycons are not enough nucleophilic to be glucosylated by almond β-glucosidase. This is clearly caused by the reaction path taken by the enzyme, since chemical glycosylation of all substrates is facile. The charge on the directly involved oxygen atom in reactive aglycons is in all cases calculated to be more negative than that in non-reacting aglycons, and this conclusion is independent of either of the three methods of charge calculations. In fact, the computed charge differences between reactive and non-reactive aglycons are quite substantial: the net atomic charge on the oxygen atom of the reacting aglycon with the lowest negative charge (benzyl alcohol) and the non-reacting aglycon with the highest negative charge (*p*-methoxyphenol) differ almost 0.1. Similarly, for the ESP charge, a threshold negative charge is found for reacting aglycons. Apparently, if the negative charge on the reacting atom of the aglycon is above a threshold value (*vide infra*) glucosylation becomes energetically unfavourable.[*]

The orbital coefficient and the energy of the HOMO do not show any correlation with success of glucosylation. On the basis of frontier MO theory it is to be expected that a

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[*] This outcome is independent of the computational method used. The electron density on the oxygen atom of benzyl alcohol and *p*-methoxyphenol was also calculated as NPA- and Merz-Kollman (ESP) charges by B3LYP/6-31G(d) and B3LYP/6-31+G(d) computations. Numerically the values differed from those obtained with the semi-empirical calculations discussed in the text, but the same trend was observed, although in a far more time-consuming manner.

high orbital coefficient and a E_{HOMO} which is close to the E_{LUMO} of the electrophile are favourable for the reaction.[33] However, aglycons with a high orbital coefficient and a relatively high E_{HOMO} have been found among the group of non-reacting aglycons (e.g., vanillin, phenol and thiophenol). Conversely, aglycons with a low orbital coefficient and a low E_{HOMO} have been found among the reacting aglycons (e.g., geraniol and benzyl alcohol). The fact that these two orbital-related parameters do not correlate with the reactivity of the aglycons is in agreement with the principles of hard and soft acids and bases.[56] Since both the negatively charged nucleophilic aglycons and the positively charged electrophilic enzyme-glucose intermediate are hard in terms of the HSAB-theory, the reaction is expected to be charge controlled.[33] Therefore, the parameter with the major influence on the reaction is the charge on the reacting atom of the aglycon. The other parameters have only minor influence on the reactivity.

This correlation between successful glycosylation and the negative charge on the directly involved oxygen atom is also perfect for all available literature data as tabulated in table 2. The difference between the lowest net atomic charge value on the reactive oxygen atom (-0.383 for methyl-2-acetamido-3-methyl-3-hydroxypropanoate) and the first non-reacting aglycon is over 0.08. Based on the results from Tables 1 and 2, the (PM3-calculated) threshold values for successful glucosylation are -0.383 for the net atomic charge and -0.348 for the electrostatic potential charge.

Transition state optimisation

In both transition states, presented in figure 5, glucose is in a skew boat conformation, which is in accordance with the proposed conformation in the reaction mechanism[20, 22] and *ab initio* calculations on ring distortion.[32] The phenolate transition state differs from the cyclohexanolate transition state both in structure and in energy with respect to the separate reactants. These differences are expressed in the distances between the reactive atoms and in the charges on the reactive atoms. The charge on the anomeric carbon atom in the transition state is 0.335 with cyclohexanolate, while it is computed to be 0.414 in the case of phenolate. This difference in charge is related to the $C\text{-}O_{\text{leaving group}}$ distance in the transition state: 1.86 Å and 2.05 Å, respectively. The charge on the $O_{\text{nucleophile}}$ decreases with a decreasing $C\text{-}O_{\text{nucleophile}}$ distance. This is in accordance with calculations for charges on the oxygen atom of the aglycon in the hydrolysis reaction of a glucoside. The charge on the oxygen atom (in the hydrolysis reaction the $O_{\text{leaving group}}$) decreases from -0.5 to -0.79 with a decreasing C-$O_{\text{leaving group}}$ distance.[21]

The difference in the C-$O_{\text{leaving group}}$ distance between the two transition states seems to be in contradiction with the proposed oxocarbonium-like character of this step in the mechanism, since the C-$O_{\text{leaving group}}$ distance in an S_N1 reaction should be independent of the strength of the nucleophile. However, the results show that a more nucleophilic aglycon induces a decreased C-$O_{\text{leaving group}}$ distance, which seems to support more S_N2 character of the transition state in this step of the reaction mechanism.

The computed activation energies of both complexes show a striking difference. The activation energy for the phenolate complex is 15.8 kcal.mol^{-1}, while the activation energy for the cyclohexanolate complex is only 1.3 kcal.mol^{-1}, which readily explains the different reactivity of the aglycons. The analogous calculation of the activation energies of the complexes with neutral aglycons resulted in higher activation energies but still showed a difference of 7.6 kcal.mol^{-1} between that for the reacting and the non-reacting aglycon. Therefore, the direct computation of the transition state yields a result that is completely in line with the initial state computations presented in Table 1 and 2.

The origin of the difference in activation energy

As can be seen in Table 4, the energetic effect of the geometrical perturbation of the nucleophiles in the transition state is minimal. The difference between the ground state energy and the energy of the nucleophile in the transition state is only 0.8 and 1.1 kcal.mol^{-1} for cyclohexanolate and phenolate, respectively. In contrast, the perturbation of the reactant state of the glucose-glutamic acid intermediate is much larger in the phenolate transition state than in the cyclohexanolate transition state (41.5 kcal.mol^{-1} and 32.0 kcal.mol^{-1} respectively). In addition to this, the (energy-lowering) quantum mechanical resonance energy in the cyclohexanolate transition state is larger than for the phenolate transition state (31.5 kcal.mol^{-1} and 26.8 kcal.mol^{-1} respectively). Hence, the difference in activation energy of both complexes is a result of both a larger resonance energy in the cyclohexanol transition state and a larger deformation energy in the phenol transition state. The perturbation of the enzyme model is the main contributor to the deformation energy.

CONCLUSIONS

The reactivity or lack thereof of almond β-glucosidase towards 16 aglycons was explained using quantum chemical methods. A clear relation between the computed charge on the oxygen atom of the nucleophile and the success of glucosylation of the aglycon was found. If the negative charge on the reacting atom of the aglycon is above a threshold value, glucosylation becomes unfavourable. This difference in reactivity was also expressed in the difference in the computed activation energy of >15 kcal.mol^{-1} between a reacting and a non-reacting aglycon approaching an enzyme model.

Use of this method predicted that glucosylation of nerol, geraniol and citronellol by β-glucosidase should be successful, in contrast with literature reports.[57] Subsequent experimental testing indeed showed this prediction to be borne out (see experimental section), which shows that calculation of the charge on the reacting atom -by either semi-empirical or density functional methods- is a reliable method to predict the aglycon reactivity.

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CHAPTER 3

THE GLUCOSYLATION REACTION MEDIATED BY ALMOND β-GLUCOSIDASE

CHAPTER 3.1

THE INFLUENCE OF GLUCOSE ON THE STORAGE AND OPERATIONAL STABILITY OF ALMOND β-GLUCOSIDASE DURING THE GLUCOSYLATION OF CYCLOHEXANOL*

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great potential for industrial applications. All these reasons induced us to investigate the influence of glucose on the stability of this enzyme, both under storage and under operational conditions.

MATERIALS AND METHODS

Chemicals

Glucose and cyclohexanol were obtained from Sigma. Ethyl acetate, petroleum ether, acetonitrile (HPLC-grade), methanol (HPLC-grade) and dichloromethane were obtained from Lab-Scan. *t*-Amyl alcohol was obtained from Acros, triethyl amine was obtained from Janssen Chimica and *p*-nitrophenyl-β–D-glucoside (pNP-glucoside) was obtained from Aldrich. Deionised water was used in all experiments. Citric acid, sodium phosphate, sodium acetate and acetic acid from Sigma were used to prepare buffer solutions.

Citrate/phosphate buffer pH 6.0 was prepared by dissolving 7.74 g citric acid and 45.23 g sodium phosphate in 1 l water. Sodium acetate/acetic acid buffer pH 5.9 was prepared by mixing a 0.2 M sodium acetate solution and a 0.2 M acetic acid solution until the proper pH was reached.

Enzymes

β–Glucosidase was obtained by extracting almond meal (Sigma) with various buffer solutions. Purified almond β–glucosidase was obtained from Boehringer (2.45 $U.mg^{-1}$) and Sigma (5.6 $U.mg^{-1}$).

Equipment

Incubations were carried out in thermostated New Brunswick Scientific G24 or Innova 4080 incubator shakers. Activity measurements were carried out using a Perkin Elmer Lambda 18 UV/Vis spectrophotometer. The HPLC system was composed of a Gynkotek pump and auto sampler with a Gynkotek UV-detector and a Sedex Evaporative Light Scattering Detector. Samples were eluted with methanol/water 50/50 % on a Spherisorb S10-ODS2 column (240x4.6mm) or with acetonitril/water 75/25 % on a Spherisorb S5-amino column (150x4.6mm). NMR measurements were carried out on a Bruker AC 200. Mass spectra were measured with a Finnigan Mat 95 mass spectrometer.

Activity measurements

The activity of almond β–glucosidase was measured by monitoring the hydrolysis of *para*-nitrophenyl glucoside with UV-spectrophotometry. A known amount of β–glucosidase was added to 2.5 ml of a 2 mM solution of pNP-glucoside in a citrate/phosphate buffer pH 6.0. The increase of *p* -nitrophenol in time (1 μmol.min^{-1} increase of pNP = 1 unit) was measured at 405 nm at 40°C. The activity is expressed as a percentage of the initial activity.

Extraction of β–glucosidase from almond meal

1 g of almond meal was shaken with 20 ml of a sodium acetate/acetic acid buffer pH 5.9 or with a citrate/phosphate buffer pH 6.0 (0.16 M) at room temperature. After 1 hour the mixture was filtered and a clear solution was obtained. The activity of the solution varied from 0.23-0.40 U.ml^{-1} solution. An increase of the extraction time by 1 hour had no effect on the hydrolytic activity of the supernatant.

Stability measurements

The enzyme stability was measured by incubating almond β–glucosidase with known activity in 1 ml of 0.16 M citrate/phosphate buffer pH 6.0. Various amounts of glucose were added to the enzyme solution and the mixture was shaken at approximately 280 rpm at 50°C. At regular time intervals 50 μl samples were taken and the activity was measured as described above.

Enzymatic glucosylation/operational stability measurements

The enzymatic reaction was carried out in a well capped 20 ml vial containing 0.5 ml of citrate/phosphate buffer pH 6.0 (0.16 M) with a known almond β–glucosidase activity, 500 g glucose per kg of buffer solution and an organic phase (4.5 ml) consisting of pure cyclohexanol or of various concentrations of cyclohexanol in *t*-amyl alcohol. The two-phase system was shaken at approximately 280 rpm at 50°C. At regular time intervals samples for HPLC-analysis were taken from the organic layer of the reaction mixture.

Concentrations of the glucoside were calculated using an external standard of synthesised cyclohexyl glucoside. Samples for activity measurements were taken from the water layer of the reaction mixture.

Chemical synthesis of cyclohexyl glucoside

The synthesis of cyclohexyl glucoside as reference material was carried out by using an adapted literature method.[18, 19] The reaction was carried out under a nitrogen atmosphere. β-D-Pentaacetyl glucose (19.5 g, 50 mmol) and 10.4 ml (100 mmol) of cyclohexanol were dissolved in 50 ml of anhydrous dichloromethane. 6.25 ml (50 mmol) of $BF_3.Et_2O$ was added slowly to the solution. The reaction mixture was stirred at room temperature. After 24 hours the clear red solution was extracted with three portions of 50 ml of a 5% $NaHCO_3$ solution. The organic layer was washed with 50 ml of water and dried over Na_2SO_4. After evaporation under reduced pressure, the clear yellow mixture was separated on a silica column with petroleum ether 40-60/ ethyl acetate 2:1 as the eluent, giving a mixture of α– and β-1-cyclohexyl-2,3,4,6-tetraacetylglucopyranoside (4.39 g; 20%, based on β-D-pentaacetyl glucose).

To 4.13 g (9.60 mmol) of 1-cyclohexyl-tetra-O-acetyl glucose, 100 ml of a mixture of methanol:water:triethylamine (8:1:1) was added. The solution was stirred at room temperature for 24 hours. The clear yellow solution was evaporated under reduced pressure at 40°C. The yield of the obtained white powder was quantitative. The product consisted of a mixture of α- and β-cyclohexyl glucoside (β:α=1,5:1)*.

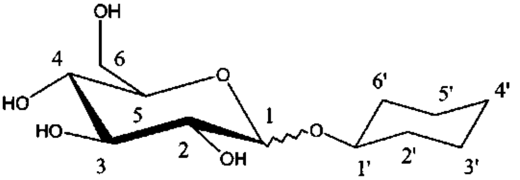

Figure 1: Cyclohexyl glucoside

CI-MS: 263 (MH^+), 163, 145.

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* Both α- and β-glucoside have the same retention time on our HPLC configuration, so the mixture can be used as reference material for our tests.

^{1}H-NMR$^\#$ (200 MHz, CDCl$_3$): δ 1.26 (m, 5H; $H_a^{3'}$, $H_a^{5'}$, $H_e^{3'}$, $H_e^{4'}$, $H_e^{5'}$), 1.54 (br s, 1H, $H_a^{4'}$), 1.72 (m, 2H; $H_e^{2'}$, $H_e^{6'}$), 1.95 (m, 2H; $H_a^{2'}$, $H_a^{6'}$), 3.30 (m, 1H; $H^{1'}$), 3.55 (m, 3H; H^2, H^3, H^5), 3.69 (m, 3H; H^4, H^6), 4.38 (d, 1H; H_β^1; J = 7.4 Hz), 4.97 (d, 1H; H_α^1; J = 3.2 Hz).

Isolation of cyclohexyl glucoside from enzyme reactions

The cyclohexanol phase of the enzyme reaction was separated and extracted repetitively with water yielding an aqueous solution of glucose and cyclohexyl glucoside. After evaporation of the water under reduced pressure at 40°C, the residue was extracted with dichloromethane. Evaporation of the dichloromethane yielded pure β-cyclohexyl glucoside.

CI-MS: 263 (MH$^+$), 163, 145.

^{1}H-NMR (200 MHz, CDCl$_3$): δ 1.26 (m, 5H; $H_a^{3'}$, $H_a^{5'}$, $H_e^{3'}$, $H_e^{4'}$, $H_e^{5'}$), 1.54 (s, 1H, $H_a^{4'}$), 1.72 (m, 2H; $H_e^{2'}$, $H_e^{6'}$), 1.92 (m, 2H; $H_a^{2'}$, $H_a^{6'}$), 3.34 (m, 2H; $H^{1'}$, H^5), 3.55 (m, 3H; H^2, H^3, H^4), 3.81 (br s, 2H; H^6), 4.38 (d, 1H; H_β^1; J = 7 Hz).

RESULTS AND DISCUSSION

The influence of glucose on the storage stability of almond β–glucosidase

Enzyme source dependency

Almond β-glucosidase is abundantly available and not expensive. Firstly, experiments concerning the activity of almond β-glucosidase from various sources were carried out. Two different extracts from almond meal and two commercially available purified enzymes were used. The activity measurements were carried out without an aglycon in the reaction medium.

[#] a = axial, e = equatorial

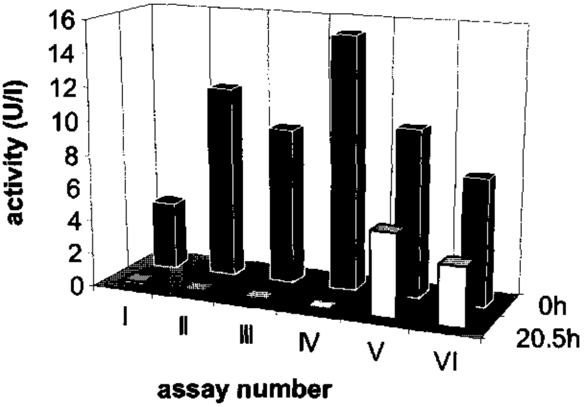

Figure 2: Activity of β-glucosidase from different sources at t=0 and after 20.5 h of incubation. I almond meal extract, sodium acetate/acetic acid buffer; pH 5.9, II almond meal extract, citrate/phosphate buffer, pH 6.0; III purified enzyme from Boehringer in 1 ml citrate/phosphate buffer, pH 6.0; IV purified enzyme from Sigma in 1 ml citrate/phosphate buffer, pH 6.0; V extract II, saturated with glucose; VI purified enzyme IV, saturated with glucose

As shown in figure 2, the activity of all enzyme preparations decreased drastically with time. There seemed to be no difference in activity decrease between extracted and purified enzymes and also different buffers seemed to be of no influence on the activity decrease. It is known that polyols stabilise enzymes (especially enzymes of plant origin) and that enzymes are stabilised by the presence of their substrate. Therefore, experiments were performed in saturated glucose solutions. Indeed, the activity was retained partly in the glucose saturated solutions. Little difference was observed between the almond meal extract and the purified enzyme.

The fact that the inactivation rate of enzymes is reduced at high substrate concentrations is generally known, but in the case of β-glucosidases few analytical data are available. Only the stabilising effect of glucose during lyophilisation is described earlier in a study where β-glucosidase from *Fusarium oxysporum* was lyophilised from an aqueous glucose solution.[14] A glucose concentration of 0.5 M had a positive effect on the enzyme storage stability in a 30 hour incubation.

Optimum glucose concentration

The optimum reaction temperature and pH were determined before the optimisation of the medium was investigated (data not shown). As a result of these studies the temperature was set at 50°C and the pH at 6.0 in all incubations.

The effects of the glucose concentration in homogeneous mixtures[3, 20, 21] or in two-phase systems[22], using the same enzyme source, were described in the literature. All studies focus on the increase of product yield by optimising the glucose concentration at a fixed incubation time. Therefore, no results on the influence of the glucose concentration on the stability of the enzyme itself have been described. To investigate in more detail the effect of glucose on the activity of almond β-glucosidase, the residual activity was monitored at five different glucose concentrations.

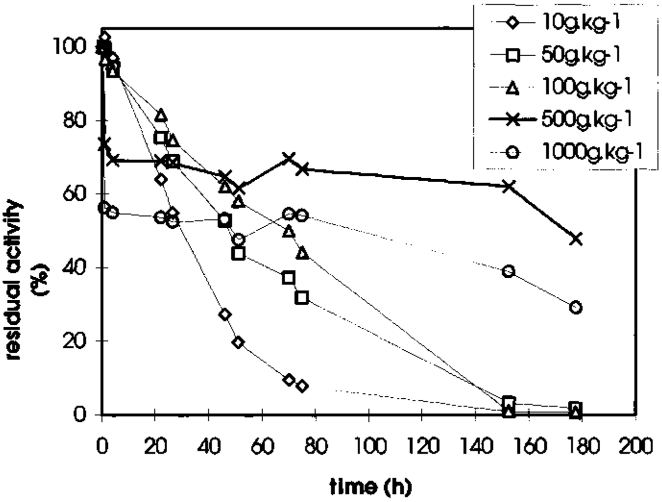

Figure 3: Effect of the glucose concentration on the enzyme stability.

Figure 3 clearly shows that with a low glucose concentration the activity of almond β–glucosidase decreased faster than with a high glucose concentration. To compare the enzyme performance, the apparent productivity was calculated. The apparent productivity is defined as the total amount of pNP produced during the measurement, divided by the initial amount of enzyme added. The apparent productivity of the enzyme in a 10 $g.kg^{-1}$ solution was about 4 mmol $pNP.U^{-1}$, while its apparent productivity in the 500 $g.kg^{-1}$ solution was over 22 mmol $pNP.U^{-1}$ (figure 4).

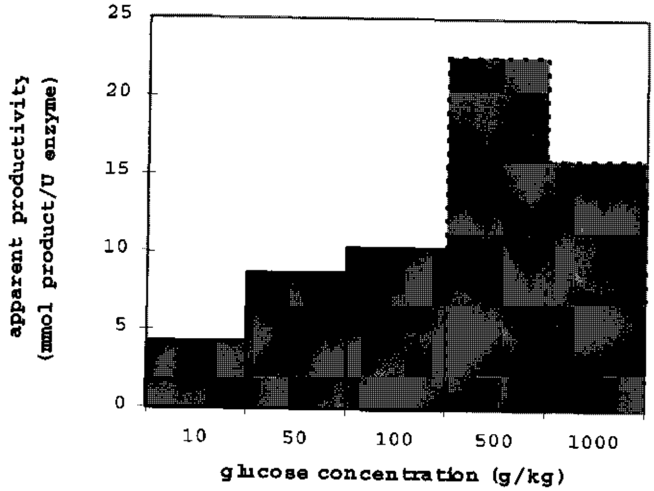

Figure 4: Apparent productivity of the enzyme at different glucose concentrations.

The areas under the lines in figure 3 were calculated up to 170 hours. Therefore, the results at 500 and 1000 $g.kg^{-1}$ glucose are in fact higher than shown in figure 4 (represented by the dashed frames). Based on the trend shown in figure 3, it may be assumed that the inactivation rate for 1000 $g.kg^{-1}$ is higher than for 500 $g.kg^{-1}$. To ensure good stability and high activity of the enzyme, the glucose concentration should be around 500 $g.kg^{-1}$. The fact that glucose concentrations higher than 500 $g.kg^{-1}$ have a detrimental effect on the enzyme stability may be due to the decreased water activity, since less water is available to hydrate the enzyme.

The initial deactivation of the enzyme in the experiments with high glucose concentrations was much faster than in the experiments with low glucose concentration. However, this was most probably not due to enzyme inactivation but caused by enzyme inhibition by glucose during the activity measurement. By adding a sample to the UV-cell, the glucose concentration in the cell increased from 0 to 111 mM at the highest glucose concentration used. This inhibitory effect of glucose on pNP-glucoside hydrolysis has been described in an earlier study on almond β–glucosidase.[23]

Reversibility

To determine whether the enzyme deactivation is reversible, glucose was added to the solution with the lowest glucose concentration (10 $g.kg^{-1}$) at the time when the residual activity had dropped to almost zero. No effect on the residual activity was measured. Thus, the deactivation is irreversible.

Operational stability of β-glucosidase

Glucosylation

A wide range of alcohols has been glucosylated using almond β-glucosidase.[3, 5, 7, 8, 24] However, only one cyclic secondary alcohol has been glucosylated.[5] Therefore, we have chosen cyclohexanol as a substrate to determine the effect of glucose on the enzyme activity during turnover, at 'optimised' water phase conditions.

Influence of the water concentration

At first the optimum volume ratio between the water phase and the organic phase was determined. The water content in this kind of enzyme reactions is important. The water activity can decrease through absorption of water by the organic phase. Moreover, water is involved in the enzymatic reaction (equation (2)), which means that an excess of water will shift the reaction equilibrium towards hydrolysis. In our experiments, the overall water concentration in the reaction mixture was varied, keeping the glucose to water ratio constant. The organic phase consisted of pure cyclohexanol.

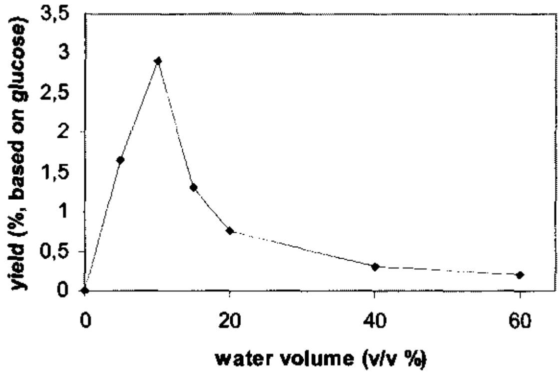

Figure 5: Maximum glucoside yield as a function of the relative volume of the water phase.

The optimum water phase/organic phase ratio in the reaction mixture with cyclohexanol was 10% (see figure 5). It is clear that a minimum amount of water must be present for the enzyme to be able to work. Without water, no glucoside production was observed. With water volumes above 10% the product yield rapidly decreased. This can be explained by regarding the amount of water which is present around the catalyst. At high organic phase volumes (i.e. low water phase volumes), more water is absorbed by the organic phase and thus less water is available for the catalyst, which leads to low productivity. Reactions with a low organic phase volume have a relative high water activity, which will result in an equilibrium shift towards hydrolysis. A similar effect was observed in the glucosylation of octanol.[24]

Influence of the glucose concentration on the glucosylation reaction

As mentioned above, the glucose concentration is of great importance for the stability of the enzyme and thus for the glucoside yield. Therefore, a comparison between a reaction with the optimal glucose concentration and one with a low glucose concentration was made.

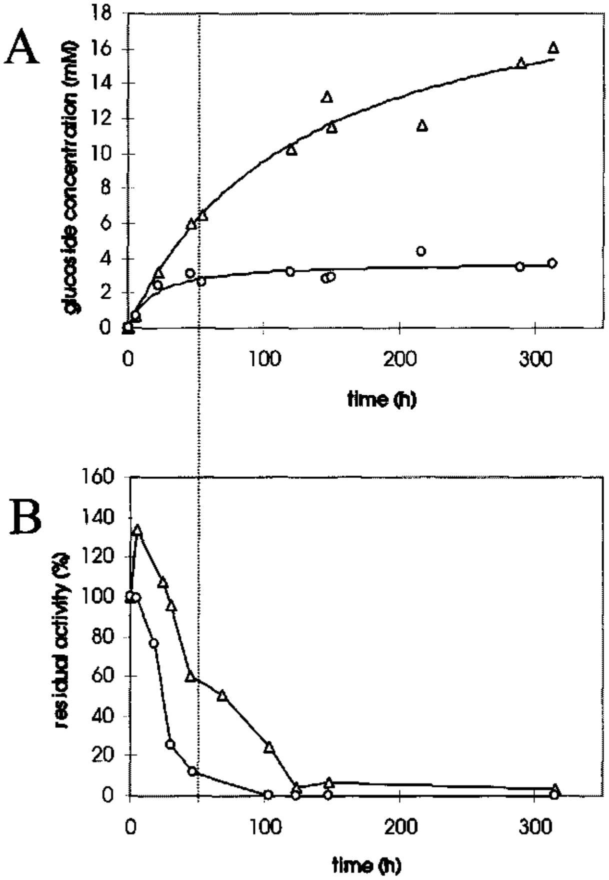

Figure 6 :A. Glucoside yield (mM) at two glucose concentrations in time. o: 100 g.kg^{-1} glucose, Δ: 500 g.kg^{-1} glucose. Points are measured, lines are fitted. **B.** Residual enzyme activity (% of the initial activity) during the glucosylation reactions at two glucose concentrations in time.

As can be seen in figure 6, the product yield in the reaction with 100 g.kg^{-1} glucose hardly increased after 60 hours of reaction time (vertical dashed line in both pictures). HPLC measurements showed that still considerable amounts of glucose were available. At the same time the enzyme activity was about 10% of the initial activity. In the reaction with 500 g.kg^{-1} glucose, however, an activity of 60% of the initial activity remained and glucoside yield was strongly increased in comparison with the 100 g.kg^{-1}

reaction. The glucoside yield in the reaction with 500 $g.kg^{-1}$ glucose was nearly five times higher than in the 100 $g.kg^{-1}$ reaction.

It could be argued that the reaction in the 100 $g.kg^{-1}$ system stopped after 60 hours due to reaching thermodynamic equilibrium. In that case, addition of extra glucose at this point should shift the equilibrium and result in an increased yield. We tested this and found the glucoside yield to be unchanged after addition of glucose. Therefore, it was concluded that the lower yield was not the result of an equilibrium shift to the hydrolysis side but that it was solely caused by enzyme inactivation.

It is also clear that addition of an organic phase had an effect on the stability of the enzyme. The productivity of the enzyme was considerably lower in a two phase system than in a 500 $g.kg^{-1}$ glucose solution without organic phase. This may be due to the partition of glucose over the two phases but also to the hygroscopicity of cyclohexanol, which diminishes the water concentration that is essential for hydration of the enzyme.

Partitioning of glucose could also be the reason for the higher residual activity measured in the first 30 hours of incubation compared with the initial activity (figure 6B). In the beginning of the incubation, the glucose concentration in the water phase will decrease because of the partitioning of glucose over the two phases. A lower glucose concentration added to the UV cell in an activity measurement will result in a lower inhibition of the hydrolysis reaction by glucose and thus give a higher residual activity. Apparently, the strong deactivation of the enzyme in the reaction with a low initial glucose concentration overruled this effect. A similar effect, although in an experiment without an organic phase, was observed in a glucosylation reaction of glucobioses .[25]

Kinetics

Based on the reaction mechanism we expected a ping-pong bi-bi mechanism. Therefore, the initial velocity of the reaction was measured at different substrate concentrations in order to determine K_M and V_{max}. A constant volume of aqueous phase (containing 500 $g.kg^{-1}$ glucose) and an organic phase consisting of varying concentrations of cyclohexanol in *t*-amyl alcohol was used as reaction system.

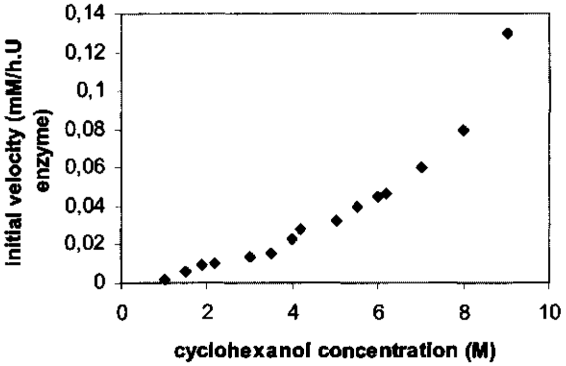

Figure 7: Initial velocity versus cyclohexyl concentration (the glucose concentration is constant).

The observed measurements did not correlate with the expected kinetics. One reason for this effect could be the inhibition of the enzyme by the organic co-solvent or by the substrate. The results of the measurements could be explained, if the co-solvent would be a stronger inhibitor than the substrate. This was tested in a hydrolysis reaction of pNP-glucoside where *t*-amyl alcohol and cyclohexanol were added to the assays. Both cyclohexanol and *t*-amyl alcohol did inhibit the hydrolysis reaction, but cyclohexanol was a slightly stronger inhibitor than *t*-amyl alcohol. Another explanation for the observed kinetics could be cooperativity, however, the already mentioned inhibition of both substrates excludes this factor.

Apparently, the partitioning of glucose, glucoside and water over the two phases creates a very complex system in which it is difficult to draw conclusions based on kinetic measurements. Kinetic measurements performed with ethyl acetate as a co-solvent resulted in the same picture as figure 7.

CONCLUSIONS

Glucose has a strong stabilising effect on almond β–glucosidase. The presence of glucose slows down the irreversible denaturation of the enzyme. The productivity of the enzyme under storage conditions increases from 4 mmol pNP.U^{-1} in pure buffer to over 22 mmol pNP.U^{-1} at the optimum glucose concentration of 500 g.kg^{-1}.

Experiments aiming at the glucosylation of the secondary alcohol cyclohexanol showed that glucose has a dual effect on the product yield. Literature shows that an increased glucose concentration leads to decreased water activity, causing a shift of the thermodynamic reaction equilibrium towards glucosylation. In addition, our experiments show that the operational stability of the enzyme increases in the presence of a high glucose concentration. Therefore, the glucose concentration has to be optimised when performing an almond β-glucosidase-mediated glucosylation reaction. The construction of large-scale reactors for glucosylation reactions would strongly benefit if both the optimum concentrations of the reactants and the enzymatic parameters (K_M, V_{max}) would be known. However, the latter appeared to be very difficult to determine in the complex two-phase system that was used. Furthermore, the obtained yields based on the aglycon are still low.

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CHAPTER 3.2

OPTIMIZATION OF PRODUCTION AND DOWNSTREAM PROCESSING OF THE ALMOND β-GLUCOSIDASE MEDIATED GLUCOSYLATION OF GLYCEROL*

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ABSTRACT

Glycosides of glycerol are useful starting materials for the synthesis of non-ionic biodegradable surfactants. These surfactants can play a substantial role as emulsifiers in foodstuffs which makes a 'food-grade' and economically feasible synthesis industrially interesting. This paper describes the synthesis of glyceryl glucoside from glycerol and glucose with almond β-glucosidase as the catalyst. A mixture of (2*R*)-1-, (2*S*)-1-, and 2-glyceryl-β-D-glucopyranoside in a molar ratio of 15:15:2 was obtained, with a yield of 54% (0.45 mmol.g^{-1}). The influence of the enzyme stability, the water concentration and the water activity on the glucoside yield were determined. No clear influence of the water activity could be observed, since it remained near unity during these experiments. However, a decreasing water concentration had a positive effect on the glucoside yield. A molar fraction based equilibrium constant of 2.4 ± 0.6 was found. With this equilibrium constant and the mass balances, the glucoside yield could be calculated for all possible combinations of initial substrate and water fractions in the reaction mixture. This straightforward model gives a good prediction of the measured glucoside yield, according to a parity plot. The model is used to optimize the glucoside yield while minimizing one of the substrates concentrations at equilibrium, in order to facilitate downstream processing. A fivefold reduction of the equilibrium molar fraction of glucose was possible with only a two times lower glucoside yield. Optimization to a minimum equilibrium molar fraction of glycerol was found not be possible without seriously compromising the glucoside yield.

INTRODUCTION

Acylated glyceryl glycosides are non-ionic surfactants which can be used in cosmetics and foodstuffs since they are biodegradable and food-grade.[1, 2] Glyceryl glycoside is an intermediate in the synthesis of these compounds, and several papers have been dedicated to its preparation. Glyceryl glycosides can be produced chemically using the Koenigs-Knorr reaction or variations thereof.[2-4] Overall product yields vary from 58 to 70%, based on protected substrates. However, due to the use of toxic reagents and solvents, the chemical synthesis of glyceryl glycosides is not applicable in food industry. Therefore, an enzymatic synthesis without the mentioned drawbacks is beneficial for application of glyceryl glycosides in food industry. A major drawback of the enzymatic synthesis in comparison to the chemical synthesis, though, is the downstream processing of the product from a mixture of highly similar polar reactants; glucose, glycerol and water.

Enzymatic glycosylation can be carried out with glycosyl transferases or with glycosidases. Glycosyl transferases are the enzymes responsible for the synthesis of glycosides in nature. Glycosidases catalyze, under physiological conditions, the hydrolysis of glycosidic bonds.[5] Due to the ample availability and low costs of the latter and the fact that they do not need expensive cofactors or activated substrates, glycosidases are preferred for industrial application. Depending on the glycosyl donor, the glycosylation reaction of glycerol by glycosidases can be kinetically controlled (transglycosylation) or thermodynamically controlled (direct glycosylation). In the transglycosylation reaction, higher yields can be reached in a shorter period of time than in the direct glycosylation due to an 'overshoot' of the equilibrium in the first reaction.[6] During batch transglycosylation, the glycoside yield is at a maximum, when the velocity of glycosylation and hydrolysis are equal. At this point the reaction has to be terminated, because thermodynamic control will take over and the glycoside yield will decrease. Literature examples of kinetically controlled transglycosylation reactions of glycerol are given in table 1.

Since it is not known whether these reactions were terminated at the maximum glucoside yield, a fair comparison of the product yields is not possible. Noteworthy, however, are the yields of 100% based on phenyl-β-D-galactopyranoside and phenyl-β-D-glucopyranoside by both Woudenberg-van Oosterom *et al.* and Trincone *et al.*

Table 1: Literature data on transglycosylation reactions of glycerol

Ref	Enzyme	Glycosyl donor	Products
7	*A.oryzae* β-galactosidase *K.lactis* β-galactosidase	Aryl-β-D-galactopyranosides	(2*R*)-1-, (2*S*)-1-, 2-glyceryl-β-D-galactopyranoside[a]
8	*E.coli* β-galactosidase	Lactose	1-, 2-glyceryl-β-D-galactopyranoside[b]
	P.citrinum endo-β-1,4-galactanase	Galactotetraose	1-, 2-glyceryl-β-D-galactopyranoside, glyceryl galactobioside[c]
9	*S.solfataricus* β-glucosidase	Phenyl-β-D-glucopyranoside	(2*R*)-1-, (2*S*)-1-, 2-glyceryl-β-D-glucopyranoside[d]
10	*K.lactis* β-galactosidase	Lactose	1-, 2-glyceryl-β-D-galactopyranoside, galactobioside[e]

[a]Excess of 1-glyceryl-β-D-galactopyranoside. [b]Ratio 9:1. [c]Ratio 1:5:?. [d]Ratio 45:45:10. [e]Ratio 88:10:2

A drawback of the transglycosylation reaction is the use of, sometimes expensive, glycosyl donors. Furthermore, the formation of by-products from the glycosyl donors (the alcohol- or non-reactive sugar part) makes it difficult to purify the product. Therefore, the direct glycosylation is favorable if a high yield can be achieved. In the case of direct glycosylation the sugar donor is a monosaccharide. Up to now, direct glycosylation of glycerol has only been performed under rather unusual reaction conditions (table 2).

Table 2: Literature data on the direct glycosylation of glycerol

Ref	Enzyme	Glycosyl donor	Products	Yield (%, based on glycosyl donor)
11[a]	almond β-glucosidase	Glucose	1-glyceryl-β-D-glucopyranoside	17
7[b]	*A.oryzae* and *K.lactis* β-galactosidase	Galactose	1-glyceryl-β-D-galactopyranoside	34

[a]Supersaturated glucose solutions in the presence of 'plasticizers'. [b]Reaction in a water-free system.

As can be seen in table 2 the only product is the glycoside on the 1-position of glycerol. No specifications were given about the enantioselectivity of the enzyme. The use of extra components like plasticizers, or the need for enzyme stabilization because of the absence of water, could obstruct the industrial application of the glycosylation reaction. Summarizing, for application of the glycosylation of glycerol in food industry, a reaction has to be developed with 'food-grade' reaction conditions and high yields, based on low-cost enzymes and substrates.

In this section the glucosylation of glycerol with almond β-glucosidase under mild reaction conditions is studied. The equilibrium constant was calculated using a set of equilibrium measurements. This equilibrium constant enables the determination of the initial substrates concentrations for an optimum glucoside yield. The equilibrium concentrations of the remaining substrates can be minimized, which will facilitate the design of a suitable downstream process.

MATERIALS AND METHODS

Chemicals

Almond β-glucosidase (E.C.3.2.1.21) was obtained from Sigma (5.6 U.mg^{-1}). Glucose, sodium phosphate and citric acid were obtained from Sigma. Glycerol was obtained from Acros; for all other chemicals see chapter 3.1.

Equipment

Incubations were carried out in thermostated New Brunswick Scientific G24 or Innova 4080 incubator shakers. Concentrations were measured with an HPLC system, which was composed of a Gynkotek pump and of a Gynkotek autosampler, using a Spherisorb S5-amino column (150 x 4.6mm). The samples were eluted with acetonitrile/water (1 ml.min^{-1}, 90/10 v/v %) and detected with a Sedex or an Alltech Evaporative Light Scattering Detector. Enzyme activity measurements were carried out using a Perkin Elmer Lambda 18 UV/Vis spectrophotometer. NMR measurements were carried out on a Bruker AC 400. Mass spectra were measured with a Finnigan Mat 95 mass spectrometer. Water activity was measured with a Rotronic Hygroscop DT.

Enzymatic glucosylation

The enzymatic reaction was carried out in a well-capped 20 ml vial containing 6 g of a mixture of different ratios of glucose, glycerol and water. The water fraction consisted of a citrate/phosphate buffer pH 6.0 (0.16 M) with an almond β-glucosidase activity varying from 0.4 U.ml^{-1} to 30 U.ml^{-1} buffer solution. The reaction was started by the addition of glycerol. The single-phase system was shaken at approximately 280 rpm at 50 °C. At regular time intervals samples of 50 μl were taken from the reaction mixture and diluted with 1 ml of methanol for HPLC analysis. Product concentrations were calculated with external standards of enzymatically produced and purified glyceryl glucoside.

Preparative glucosylation of glycerol

The enzymatic reaction was carried out as described above. After reaching equilibrium (54% analytical yield based on glucose) the reaction was terminated and the product was purified with column chromatography (Silica 60; butanol/acetic acid/ether/water: 45/30/15/5). Freeze-drying was used to remove the eluent. A white, hygroscopic product was obtained. The product consisted of a mixture of (2R)-1-, (2S)-1-, and 2-glyceryl-β-D-glucopyranoside in a ratio of 15:15:2.

Rf (Silica 60; butanol/acetic acid/ether/water: 45/30/15/5): 0.46

FAB-MS: 277 $(M+Na)^+$, 255 $(M+H)^+$, 223, 207, 169, 133, 115, 93, 75, 57

FD-MS: 293 $(M+K)^+$, 277 $(M+Na)^+$, 255 $(M+H)^+$

^{1}H-NMR (400 MHz, D_2O, reference acetone-d_6): δ 3.27 (m, 4H, H_2, H_3, H_4, H_5), 3.64 (m, 7H, $H_{1'}$, $H_{2'}$, $H_{3'}$, H_6), 4.32 (d, J=7.9 Hz, 1H, (2R)-1 H_1), 4.33 (d, J=7.9 Hz, 1H, (2S)-1 H_1), 4.45 (d, J=7.9 Hz, 1H, 2- H_1)

^{13}C-NMR (400 MHz, D_2O, reference acetone-d_6): δ 61.1 (C_6), 62.9 ($C_{3'}$), 70.0 (C_4), 70.8 and 71.0 ($C_{2'}$), 71.3 and 71.6 ($C_{1'}$), 73.5 (C_2), 76.0 (C_3), 76.3 (C_5), 102.9 and 103.1 (C_1)

Enzyme stability measurements

The enzymatic reaction was carried out as described above. After addition of glycerol a sample of 50 μl was taken for activity measurement (initial activity). After the reaction had reached equilibrium another sample of 50 μl was taken for activity measurement (remaining activity). The activity of β-glucosidase was measured as described in chapter 3.1.

Water activity measurement

The complete reaction mixture (6 g) was added to the measurement cell. After equilibration for 2 h at 50°C the water activity was determined.

MATHEMATICAL MODEL

The equilibrium constant of the glucosylation reaction is defined by the following equation:

$$K_{eq} = \frac{\gamma_{gg}\gamma_{w}}{\gamma_{glu}\gamma_{gly}}\frac{X_{gg}X_{w}}{X_{glu}X_{gly}} = \Gamma\frac{X_{gg}X_{w}}{X_{glu}X_{gly}} = \Gamma K'_{eq} \qquad (1)$$

Where K_{eq} is the overall equilibrium constant, K'_{eq} is the apparent equilibrium constant, γ_{gg}, γ_{w}, γ_{glu} and γ_{gly} are the activity coefficients of glyceryl glucoside, water, glucose and glycerol, respectively, and X_{gg}, X_{w}, X_{glu} and X_{gly} are the molar fractions of glyceryl glucoside, water, glucose and glycerol at equilibrium, respectively.

In our case, K'_{eq} appears to be constant ($d\Gamma/dX_i = 0$). Hence, molar fractions can be used to estimate K'_{eq} ($= X_{gg}X_{w}/X_{glu}X_{gly}$).

Equation 1 can be re-written to initial molar fractions of glucose, glycerol and water, and the molar fraction of glyceryl glucoside at the equilibrium (equation 2).

$$K'_{eq} = \frac{X_{gg}(X_{w,ini} + X_{gg})}{(X_{glu,ini} - X_{gg})(X_{gly,ini} - X_{gg})} \qquad (2)$$

Where $X_{w,ini}$, $X_{glu,ini}$ and $X_{gly,ini}$ are the initial molar fractions of water, glucose and glycerol, respectively.

For a given K'_{eq} this yields equation 3:

has one axial OH-group, whereas glucose has none. Studies on a related enzyme from *Agrobacterium* has shown that substrate recognition in β-glucosidases mainly depends on the presence of the 6-hydroxyl group.[14] In our system, L-rhamnose is indeed not accepted as a sugar donor or as a sugar acceptor; nor does it show any inhibitory properties (results not shown). Besides reducing the molar fraction of water, addition of rhamnose to our system will possibly affect the water activity, which is an often used optimization parameter in glucosylation reactions.[15-18] The influence of the molar fraction of water on the glucoside yield is presented in figure 2.

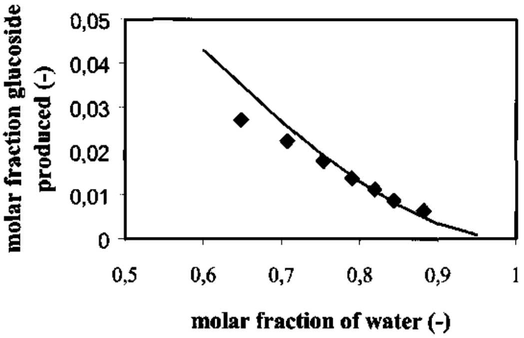

Figure 2: Production of the molar fraction of glyceryl glucoside at different initial molar fractions of water with an initial molar ratio of glycerol/glucose of 3.9:1. Rhamnose was added as an inert component to reduce the initial molar fraction of water. Dots: experimental data, line: prediction based on equation 3.

Figure 2 clearly shows that a lower initial molar fraction of water in the mixture results in a higher molar fraction of produced glucoside. The water activity was measured and turned out to approximate 1 in all reaction mixtures. Hence, addition of L-rhamnose does not influence the water activity but only reduces the water concentration.

Although the water activity remains essentially constant, the equilibrium shifts. This must be due to a change of the activities of the other components. Unfortunately, we were unable to measure the activities of all components in the reaction mixture. However, in our case K'_{eq} appeared to be constant resulting in $K'_{eq} = 2.4 \pm 0.6$. This is in agreement with the equilibrium constant of 1.9 which was found for the glucosylation of C_6- to C_{10} *n*-alkyl alcohols given by Panintrarux.[19] Based on this K'_{eq}-value, the equilibrium molar fractions of glyceryl glucoside were calculated and compared with the experimentally obtained molar fractions (figure 3).

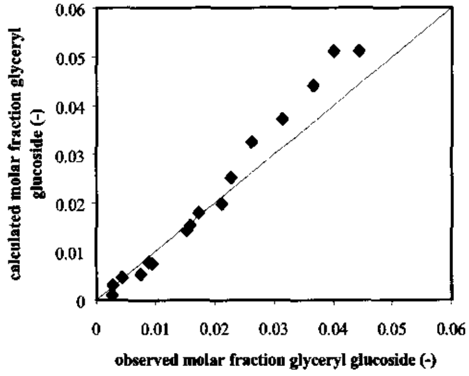

Figure 3: Parity plot of the molar fraction of glyceryl glucoside. Dots: calculated molar fraction of glyceryl glucoside according to equation 3 versus the experimentally obtained molar fraction.

As can be seen in figure 3, a good correlation between the calculated values and the observed values is obtained. Although elementary, the model predicts the equilibrium concentration of glyceryl glucoside at various conditions reasonably well. Furthermore, figure 3 shows that molar fractions can be used to estimate the apparent equilibrium constant.

Optimization of the glyceryl glucoside yield

Based on equation 3 and the measured apparent equilibrium constant, the glucoside production for *all* combinations of initial substrates and water fractions can be calculated. The results are given in figure 4.

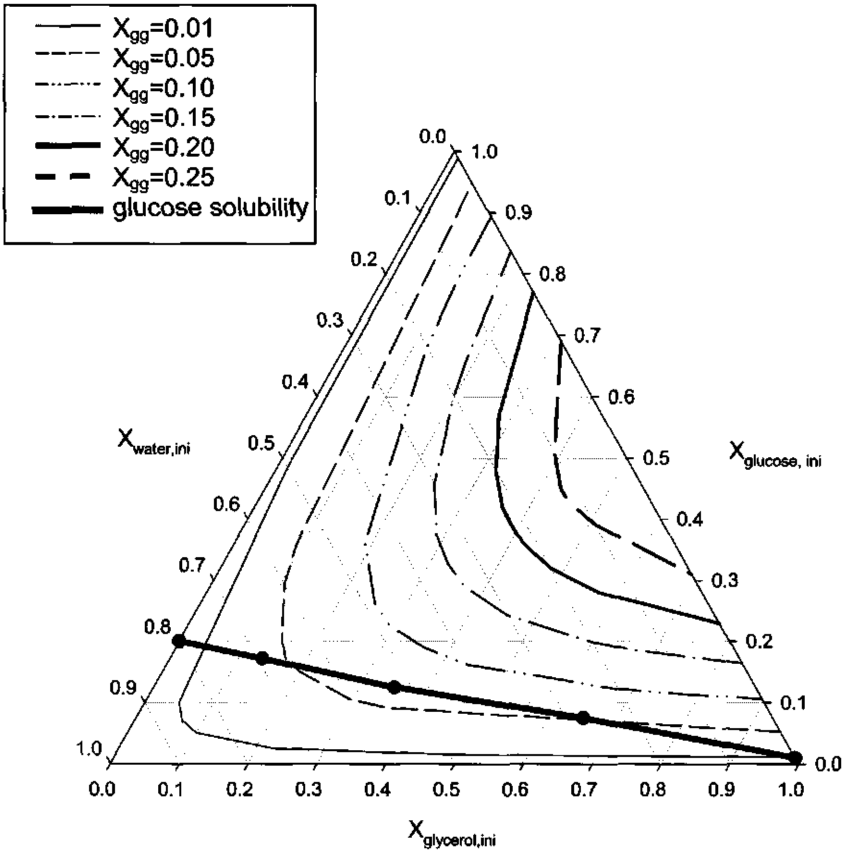

Figure 4: Ternary equilibrium diagram for a mixture of glucose, glycerol and 0.16 M citrate/phosphate buffer pH 6.0. The lines represent the glyceryl glucoside fraction at the equilibrium of the reaction calculated with equation 3 (see also legend) at the given initial molar fractions of glucose, glycerol and water. The bold line represents the maximum glucose solubility in the reaction mixture at 50°C.

At constant glucose concentration, the glucoside production increases with a decreasing initial water fraction. Based on figure 4, the optimum conditions for a maximum glucoside yield would be at initial glucose and glycerol fractions of 0.5, without any water present. However, at these reaction conditions, glucose does not completely dissolve in the reaction mixture. The limit of physically possible reaction conditions, according to maximum glucose solubility, is given as a bold line in figure 4. This borderline represents the maximum glucoside production as a function of X_w and X_{gly} at the measured maximum glucose solubility in the reaction mixture at 50°C (about 2.5 g glucose per g glycerol/water mixture). Based on this practical barrier the maximum accessible glucoside molar fraction is 0.072 (1.05 mmol.g^{-1} reaction mixture). This molar fraction can be reached at the initial molar fractions of 0.12, 0.41 and 0.47 of glucose, glycerol and water, respectively. A reaction performed with these optimum initial molar fractions yielded a concentration of glyceryl glucoside of 1.01 mmol.g^{-1}, again confirming the validity of the model. Hence, using the proposed model could improve the already obtained product yield twice.

The activity of the enzyme in the reaction mixtures near the optimum initial reaction conditions was measured at the start of the reaction and after the reaction had reached equilibrium (>100 h). The average remaining β-glucosidase activity is high (86 ± 5.6%). Apparently, the different ratios of substrates and water in the mixtures near the optimum reaction conditions do not affect the enzyme stability.

Designing optimal downstream processing conditions

The major drawback of the enzymatic glucosylation of glycerol versus the chemical synthesis is its difficult downstream processing. The most promising method, both in laboratory and in industry, seems to be a two-step adsorption process.[13] After filtration of the immobilized enzyme the mixture is applied on an Amberlite IRA-900 ion-exchange column to remove the mono- and disaccharides. In the second step, the glycoside is separated from glycerol by adsorption on a Norit ROX 0.8 column. Through minimizing one of the substrate concentrations at the equilibrium, while providing a high glucoside yield, one adsorption step may be omitted. With the equilibrium constant and the mass balances we can estimate the optimum initial molar fractions of glycerol, glucose and water to meet these conditions.

Adsorption on the ion-exchange column could be redundant if the glucose concentration at the equilibrium would be (close to) zero. According to our calculations, an equilibrium molar fraction of glucose of 0.001 is possible if initial molar fractions of glucose, glycerol and water of 0.022, 0.89 and 0.088 are used, respectively. If we interpolate these values in figure 4 a molar fraction of glyceryl glucoside of 0.021 can be expected. However, it was not possible to reach this yield in practice, because the enzyme appeared to be not active at these low molar fractions of water. The water activity in this mixture was measured to be 0.13. The minimum water activity for β-glycosidases from various sources in glycosylation reactions of C_3- to C_8 alcohols is 0.4 to 0.8.[15-18] In our case, the water activity of reaction mixtures where the enzyme still had some activity, and a measurable glucoside yield was found, appeared to be >0.17. It is tempting to ascribe the enzyme deactivation solely to this low water activity, but it was already shown that deactivation of lipase in a glycerol-water system was caused by the high glycerol concentration and not by the water activity.[20]

Thus, in addition to the first practical barrier as a result of the glucose solubility, a second practical barrier due to enzyme inactivation exists (see figure 5).

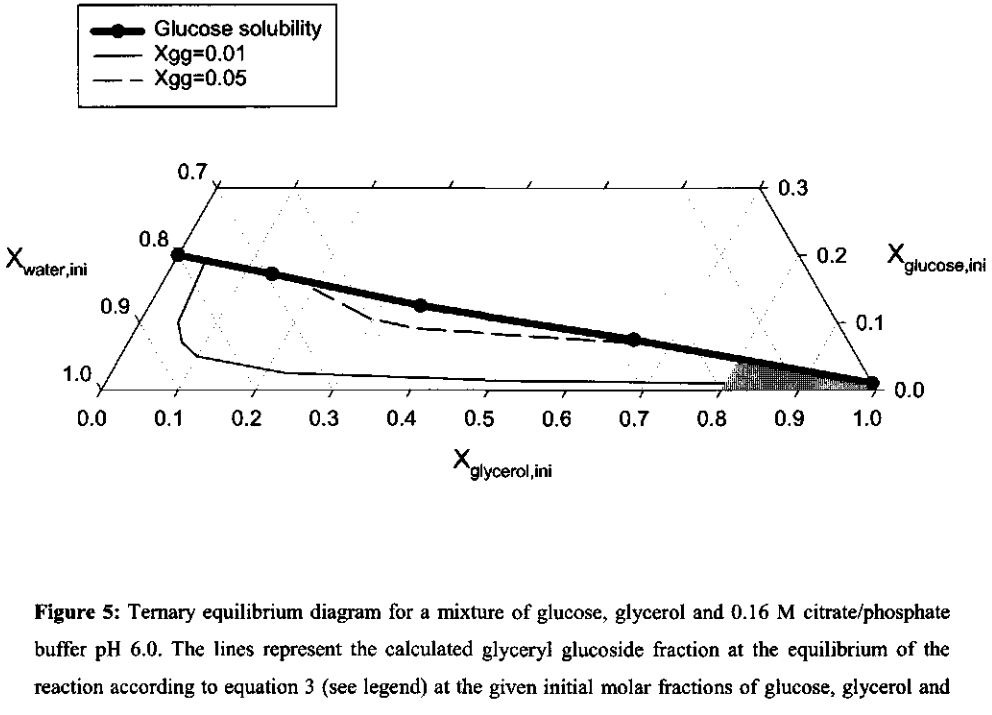

Figure 5: Ternary equilibrium diagram for a mixture of glucose, glycerol and 0.16 M citrate/phosphate buffer pH 6.0. The lines represent the calculated glyceryl glucoside fraction at the equilibrium of the reaction according to equation 3 (see legend) at the given initial molar fractions of glucose, glycerol and water. The glucoside molar fractions are calculated up to the practical barriers due to maximum glucose solubility in the reaction mixture (bold black line) and enzyme inactivation (shaded area).

Taking into account these practical limits, the reaction providing the lowest glucose molar fraction at the equilibrium should have initial molar fractions of glucose and water where the initial molar fraction of glycerol is 0.79. The maximum obtainable glucoside molar fraction at this $X_{glycerol,ini}$ is 0.038, taking into account the maximum solubility of glucose. The initial molar fractions for glucose and water are 0.042 and 0.168, respectively. The equilibrium molar fraction of glucose in this reaction can be calculated and will be 0.004. The product yield is about two times lower than the highest possible yield, but the product will be contaminated by only 10% of glucose. If the reaction is optimized to the highest glucoside yield possible, without regarding the remaining fractions of glycerol or glucose, this contamination will be about 55%. The same strategy can be used for the optimization of the reaction towards a low equilibrium molar fraction of glycerol. However, with glycerol it appears impossible to find much lower equilibrium molar fractions of glycerol without suffering largely in glucoside yield.

CONCLUSIONS

The optimization of the reaction conditions for the enzymatic glucosylation of glycerol was investigated. The maximum obtained yield is higher than the yield obtained in enzymatic reactions under 'unusual' reaction conditions and is comparable with the yield in organic synthesis. A lower initial molar fraction of water resulted in a higher fraction of glucoside. This effect could be ascribed to a shift of the equilibrium of the reaction.

The apparent equilibrium constant of the reaction was found to be 2.4±0.6. With this constant a simple but accurate model for the description of the equilibrium concentrations of all reactants could be defined. Due to the limited glucose solubility in the reaction mixture a practical barrier was found, which resulted in a maximum accessible glucoside molar fraction of 0.071 (1.05 mmol.g^{-1}). This was experimentally confirmed.

The model could serve as a guideline for downstream processing. An optimal combination of substrate concentrations could be found, such that the equilibrium composition was more suitable for downstream processing. This optimum was somewhat altered due to a second limitation resulting from enzyme deactivation at high glycerol concentrations. With this taken into account, a glucoside molar fraction of 0.038 was calculated to give an equilibrium molar fraction of glucose of only 0.004. This yield is about two times lower than the maximum attainable, but the product will contain only 10% glucose, which is a fivefold reduction. Minimization of the

CHAPTER 4

THE DEVELOPMENT OF A BIOREACTOR FOR THE ENZYMATIC PRODUCTION OF GLUCOSIDES

CHAPTER 4.1

THE INTEGRATED ENZYMATIC PRODUCTION AND DOWNSTREAM PROCESSING OF HEXYL GLUCOSIDE*

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* This chapter was submitted: B.M. de Roode, J. van Beek, M.C.R. Franssen, A. van der Padt and R. Boom (2001).

ABSTRACT

A bioreactor was designed based on the optimal reaction conditions for the β-glucosidase mediated glucosylation of hexanol. Due to fast deactivation of the enzyme in a CSTR, a spray column reactor was used, providing a low shear environment for the enzyme. This resulted in an enzyme half life time of almost 30 days. The glucoside production in the reactor was 2.5 $g.l^{-1}$, the initial production rate was 2.24 $mg.U^{-1}.h^{-1}$. The two phases were separated with a flat sheet polypropylene membrane, which was pretreated using block copolymers to prevent breakthrough of water. Indeed, no breakthrough of water was observed during 41 days of continuous operating.

In-line adsorption was used to semi-continuously remove the produced glucoside. Ten different adsorbents were tested in equilibrium adsorption experiments. Based on these results, alumina was chosen for in-line adsorption. Five alumina columns were used in the same reactor batch resulting in an average glucoside adsorption of 11.15 $mg.g^{-1}$. The maximum glucoside adsorption in the full process appeared to be much lower than in the equilibrium experiments. This decrease could be attributed to the presence of water in the column influent. Furthermore, glucose displaces the glucoside after the maximum glucoside adsorption has been reached. Based on these results and the desorption characteristics, a regeneration regime for the column is proposed. Straightforward calculations with this reactor system were performed for the production of 1 kg of hexyl glucoside.

INTRODUCTION

Glucosylation

β-Glucosidases catalyze the hydrolysis of glucosides and the glucosylation of various aglycons.[1, 2] Furthermore, β-glucosidases are cheap and abundant, which makes them suitable for industrial application. The hydrolysis reaction has potential relevance in the wine-making industry,[3, 4] while products from the glucosylation reaction may find their use in the food[5] and surfactant[6] industry. A major drawback of the glucosylation reaction catalyzed by β-glucosidase is the unfavorable equilibrium position that results in a low product yield. However, improvement of the product yield is possible by 'tuning' the reaction conditions.[7-12] Using bioreactors that enable *in situ* downstream processing of the reaction mixture can provide further improvement of the product yield. The use of such a system integrates the push of the equilibrium to the product side (by maintaining high substrate concentrations), and the pull of the equilibrium through a continuous removal of the product. The bioreactor choice for glucosidase mediated reactions in the literature is mainly dependent on the physical state of the enzyme. Most studies focus on the immobilization of β-glucosidase and, therefore, packed-bed[13-17] and fluidized bed[13] reactors are well represented. In one case a stirred tank reactor is used with an immobilized enzyme.[18] Stirred tank reactors are further used predominantly with free enzymes.[19-21]

With the exclusion of one reactor,[18] all these reactors were build in a dead-end configuration and not much attention is given to the downstream processing of the product. In the present paper we will introduce a reactor system in which the combined push-pull strategy for the β-glucosidase mediated glucosylation of hexanol is utilized.

Bioreactor system selection

First, a reactor will be designed based on the optimum reaction conditions. The reactor should operate at optimum reaction conditions as described previously.[22] Among others, these imply a glucose concentration of 500 g per kg of buffer solution, minimizing inactivation of the enzyme, and an aglycon phase/buffer phase volume ratio of 9. Since the enzyme is relatively stable and cheap there is no need for immobilization. Therefore, two types of two-phase reactors come into focus, a CSTR or a spray column reactor. We have chosen for the spray column reactor.

To achieve in-line downstream processing an adsorption column was used. In the literature preparative purification of glucosides from various matrices was mainly carried out with column chromatography on silica.[10, 11, 23, 24] Unfortunately, no quantitative data were reported in these studies. Zang and coworkers did a comparative study on the adsorption of dodecyl maltoside in water on graphite, alumina, silica, titania and hematite.[25] The highest adsorption of 0.26 $mg.g^{-1}$was accomplished on alumina. Alumina was also successfully used for the separation of hexyl glucoside from hexanol with a maximum adsorption of 8.3 $mg.g^{-1}$.[18] Other promising adsorbents are: a) molecular sieve, which is used to separate fructose from a mixture of sugars from water[26], b) active carbon, used to separate mono-, di- and trisaccharides from water[27], and c) XAD-2, used for glucoside isolation from apples.[28]

Therefore, we will test silica, alumina, active carbon, XAD-2, XAD-4 and molecular sieve for their suitability in our system. This list will be completed with a) starch, which is likely to show a good interaction with polar components in an apolar environment, and that could make desorption of the glucoside from the column unnecessary since it can be used as a food additive itself, and b) polyamide, which is used as a HPLC column for glucoside separation. All these adsorbents will be tested in equilibrium adsorption experiments.

Finally, the best adsorbent will be used in an in-line adsorption column in the reactor system for the continuous production of hexyl glucoside (see figure 1).

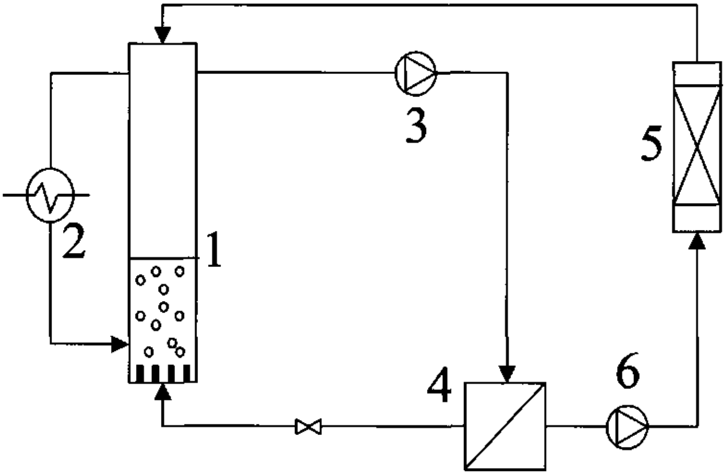

Figure 1: Schematic presentation of the reactor system for the production and downstream processing of hexyl glucoside. 1) Spray column reactor, 2) Water bath (50°C), 3) Gear pump, 4) Membrane module, 5) Adsorption column, 6) Plunger pump.

Since the aqueous phase contains the enzyme, this phase should not be allowed to enter the adsorption column, to prevent adsorption of the enzyme to the adsorbent. This is achieved by introducing a hydrophobic microfiltration membrane between the spray column reactor and the adsorption column. The membrane allows permeation of the aglycon phase, which contains the glucoside, but retains the aqueous phase with the enzyme. An additional benefit is the retention of excess water and glucose, which adsorb competitively with the glucoside, which lowers the adsorption yield. To prevent the breakthrough of water the membrane was pretreated according to the method of Schroën.[29]

MATERIALS AND METHODS

Chemicals

Almond β-glucosidase (E.C.3.2.1.21) was obtained from Sigma (5.6 U.mg^{-1}). Glucose, hexanol, hexadecane, sodium phosphate and citric acid were obtained from Sigma; *p*-nitrophenol-β-D-glucoside was from Aldrich; ethanol (96%) was from Nedalco, and acetonitrile (HPLC-grade) was from Lab-Scan. The block copolymer F108 (based on one polypropylene oxide block and two polyethylene oxide blocks) was a gift from ICI. Nylon syringe filters (pore size: 0.45 μm) were obtained from Alltech.

Adsorbents

Alumina (Brockman I, particle size: 50 - 200 μm) was obtained from Baker. Amberlite XAD-2 and XAD-4 (particle size: 20 - 60 mesh) were purchased from BDH. Activated Carbon (Darco KB-B, particle size: ≤ 100 mesh) was obtained from Aldrich, and polyamide 6S (particle size: ≤ 160 μm) was from Riedel-de Haën. Potato starch, Silica 60 (particle size: 0.06 - 0.2 mm) and molecular sieves (particle size: 2 mm, pore sizes: 0.3 nm and 1 nm) were obtained from Merck. To obtain a molecular sieve with a smaller particle size, the particles with 1 nm pores were crushed and sieved to a particle size of 125 - 250 μm.

Equipment

The HPLC system used has been described in chapter 3.1. Enzyme activity measurements were carried out using a Perkin Elmer Lambda 18 UV/Vis spectrophotometer. Membrane separation was performed with an Accurel® flat sheet polypropylene membrane from Akzo Nobel (55 x 130 mm, maximum pore size: 0.58 μm). The membrane was used in a flat sheet membrane module built in-house. Omnifit glass columns (50 mm x 10 mm i.d. and 250 mm x 10 mm i.d) with an adjustable plunger were used to prepare the adsorption column.

Bioreactor

The enzymatic reaction was carried out in a bioreactor as described in figure 1. The reactor system consisted of a double walled glass spray column (1) (i.d.: 30 mm, column length: 210 mm) which was thermostated with a water bath (2) at 50 °C. The continuous phase was brought into the bottom of the column with a Verder 2040 gear pump (3) at a velocity of 1 $ml.s^{-1}$ via a stainless steel nozzle with 12 tips (equally divided over the nozzle area, each 10 mm long, 1 mm i.d.). The continuous phase was led over the membrane module (4). The volumetric flow through the membrane was 2.38 $ml.s^{-1}.m^{-2}.bar^{-1}$. The permeate was led to an adsorption column (5) with a Gilson 303 plunger pump (6). The column effluent was recycled to the spray column.

Adsorption experiments

Before usage, all adsorbents were dried at 120 °C for at least 1 h. To a 4 ml vial, 10 to 200 mg adsorbent and 1 ml hexyl glucoside in hexanol (concentration range from 0.1 to 5.0 $g.l^{-1}$) was added. The mixture was brought under reduced pressure at room temperature for at least 1 h. After restoring atmospheric pressure, the vial was capped and shaken at room temperature for at least 16 h at approximately 350 rpm. After shaking, the mixture was filtered over a nylon filter to remove the adsorbent. Samples of 50 μl were taken from the hexanol phase and were diluted with 1 ml of methanol for HPLC analysis. For the adsorption experiments with wetted hexanol and the reaction mixture the same procedure was followed with wetted hexanol containing 3.44 $g.l^{-1}$ hexyl glucoside and collected effluent streams from different reactor experiments, respectively. This effluent consisted of hexanol, saturated with buffer, containing 4.9 $g.l^{-1}$ hexyl glucoside and 1.0 $g.l^{-1}$ glucose.

Membrane preparation

The membrane was pre-wetted by rinsing with hexadecane for 30 minutes; hexadecane permeated through the membrane. Next, the membrane was rinsed for 15 minutes with an emulsion of 1:2 v/v hexadecane in water, containing 6 (g block copolymer).l^{-1} of emulsion. Both retentate and permeate (the permeate consisted only of hexadecane) were recycled to the emulsion. Subsequently, the membrane was rinsed with water, which did not permeate through the membrane. Finally, the membrane was rinsed with a hexanol/buffer emulsion. Only hexanol permeated through the membrane. In all rinsing steps, the pump was operated at a capacity of 1.67 $ml.s^{-1}$.

In-line adsorption and off-line desorption experiments

To 1.6 g of dried adsorbent, 10 ml of hexanol saturated with buffer was added. The mixture was brought under reduced pressure at room temperature for at least 1 h. After restoring atmospheric pressure, the mixture was added to the column. An adjustable plunger was used to avoid a dead volume in the packed column. The void volume was determined gravimetically. The column was connected into the process stream and operated in the up flow mode. The effluent of the column was fed back to the spray column reactor. On specific time intervals samples were taken from the effluent of the column for HPLC analysis. After the effluent concentration of hexyl glucoside equaled the influent concentration of hexyl glucoside the column was removed from the process stream and purged with air for 30 min. to remove the void of hexanol. Subsequently, the column was eluted at room temperature for 30 min. with a desorption eluent (water or ethanol). The concentrations of glucose and glucoside in the effluent were measured. The column was desorbed until the effluent contained no hexyl glucoside or glucose. The amounts of desorbed glucoside and glucose were compared with the adsorbed amounts of glucoside and glucose, respectively.

Continuous production and adsorption of hexyl glucoside in the bioreactor

The spray column was filled with 180 ml of buffer saturated hexanol (continuous phase). Hexanol was saturated with a citrate/phosphate buffer pH 6.0 (0.16 M) at 50 °C. The reaction was started by the addition of 20 ml water phase (stationary phase) at the top of the spray column. The water phase consisted of a citrate/phosphate buffer pH 6.0 (0.16 M) with an almond β-glucosidase activity of 0.2 U.(ml buffer solution)$^{-1}$. Five adsorption columns, operated in an up-flow mode, were successively used in the same reactor batch. The effluent was monitored and the adsorption column was removed as soon as $C_{glucoside,effluent}$ equaled $C_{glucoside,influent}$.

Enzyme stability measurements

The enzymatic reaction was carried out as described above. Samples were taken from the water phase in the bottom section of the spray column. The activity of β-glucosidase was measured as described previously.[22]

MATHEMATICAL MODELS

Data from the adsorption experiments were fitted to four different adsorption models (see table 1), where q is the adsorption, q_{max} is the maximum adsorption and k is the adsorption constant.

Table 1: Adsorption models to which the experimental data was fitted.

Model	q (mg.g^{-1})		c_e (g.l^{-1})	
Langmuir	$\frac{q_{\max} k c_e}{1 + k c_e}$	(1)	$\frac{B + \sqrt{B^2 - 4kc_i}}{2k}$	(5)
			with: $B = kc_i - kmq_{max} - 1$	
Sorption	kc_e	(2)	$\frac{c_i}{km + 1}$	(6)
Saturation	$q_{\max}$	(3)	$c_i - mq_{max}$	(7)
			$c_i \geq mq_{max} \rightarrow c_e = c_i - mq_{max}$	
			$c_i \leq mq_{max} \rightarrow c_e = 0$	
No adsorption	0	(4)	c_i	(8)

In the adsorption experiments the initial concentration (c_i) and the mass of the adsorbents (m) were known and the equilibrium concentration (c_e) was measured. Therefore, the models were re-written to yield these three parameters explicitly.

Criteria for choice of the best model

The best fit of the models to the data was determined by comparing the lower models 6 to 8 with the extended model 5 with the use of F-statistics to the *lack-of-fit* test. The variance of the lack of fit and the variance of the extended model were compared as follows:

$$f = \frac{(RSS_{low} - RSS_{ext})/(v_{low} - v_{ext})}{(RSS_{ext}/v_{ext})} \qquad (-) \qquad (9)$$

Were RSS_{low} and RSS_{ext} are the sum of squares of the residuals of the lower and the extended model, respectively, and v_{low} and v_{ext} are the degrees of freedom of the lower and the extended model, respectively ($v = n - p$, where n is the number of datapoints and p is the number of parameters). If f was smaller than the tabulated value for $F^{v_{low}-v_{ext}}_{v_{ext}}$ the lower model was considered adequate since the additional parameter was not statistically significant. If more than one lower model was satisfactory the best fit of these models was selected based on the lowest number of parameters (Ockams razor). In case of an equal number of parameters the model with lowest RSS was considered most adequate.

Calculation of confidence intervals

The confidence intervals for the estimated parameters of models 5-7 were calculated using the estimated standard error of the parameters s:

$$c.i. = st_{0.95,v} \qquad (10)$$

where $t_{0.95,v}$ is the upper 95% point of the t-distribution with v degrees of freedom. The square of the standard error is corresponding to the variance multiplied by the diagonal element of the p x p covariance matrix of the parameters:

$$s^2 = \left(\frac{RSS}{v}\right)(J'J)^{-1} \qquad (11)$$

where J is the n x p Jacobian matrix which represents the derivative of the nonlinear functions with respect to the parameters. In the case of the linear model 7, the Jacobian equals X, thus making the estimation of confidence intervals straightforward.

A Monte Carlo method was used to calculate the confidence interval of q. The adsorption at a given c_e of 2.5 g.l^{-1} was calculated using the estimated q_{max} and k and equations 1 and 2. A new set of q_{max} and k was calculated using s_{qmax} and s_k, and the normal distribution. With this set q was calculated. After repeating these last two steps many times (>2000), the confidence interval for q was obtained from the ordered list by eliminating the upper and lower 2.5 % of the simulations, thereby giving the 95% confidence intervals.

RESULTS AND DISCUSSION

Bioreactor choice.

Initially, a CSTR with an in-line removal step of the product was chosen. Vigorous stirring was necessary to maintain a high hexanol/buffer surface area. This resulted in a rapid loss of enzyme activity (no residual activity after 40h). It is well known that enzyme activity decreases rapidly at an air-liquid interface under intensive stirring conditions.[30] Indeed, a batch experiment without air in the reaction vessel showed that the half life time of the enzyme could be increased to almost 3 days. However, the rate of deactivation was still unacceptable. Not only the air-liquid interface but also the vigorous stirring in combination with the high polar/apolar interfacial area may be the reason for this fast deactivation. A solution could be immobilization of the enzyme or the construction of a reactor without the mentioned drawbacks. Operational stability measurements in a spray column reactor showed that the enzyme is very stable in this shearless environment (half life time of almost 30 days). Therefore, a spray column reactor was used. With the spray column reactor it was possible to produce 2.5 $g.l^{-1}$ of hexyl glucoside. The initial production rate was 2.24 $mg.U^{-1}.h^{-1}$.

Membrane development

The membrane acts as a barrier to prevent the aqueous phase from entering the adsorption column. To achieve this, a hydrophobic membrane material was chosen. Experiments with a polypropylene microfiltration membrane showed a breakthrough of the water phase after 12 h. Therefore, the membrane was treated with a PEO-PPO-PEO block co-polymer after which the membrane remained non-permeable for water for at least 41 days. Hexyl glucoside permeated through the membrane without problems (see figure 2).

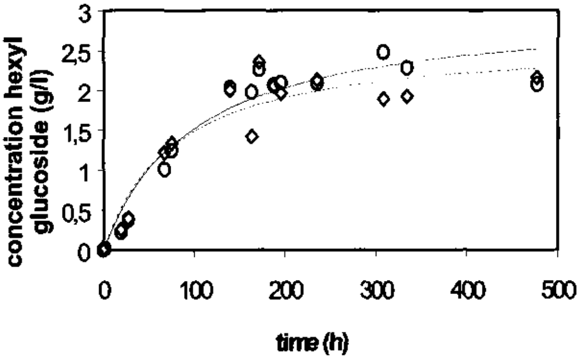

Figure 2: Hexyl glucoside concentration in the retentate-(○) and permeate(◊) phase during the enzymatic production of hexyl glucoside in the bioreactor. Lines: Fit based on Michaelis-Menten kinetics.

The maximum solubility of water in hexanol at room temperature is around 2 w/w%. This will permeate through the membrane.

Adsorbents selection

Based on the best fit of the four models to the experimental data the adsorption was calculated for an initial glucoside concentration of 2.5 $g.l^{-1}$ (based on the concentration which was reached in the membrane selection experiments). The results are given in table 2.

Table 2: Maximum adsorption (q) and adsorption constant (k) for the adsorption of hexyl glucoside in hexanol on 10 different adsorbents. The parameters were calculated according to the presented model. The adsorption is also given at the initial glucoside concentration of 2.5 $g.l^{-1}$.

Adsorbents	q_{max} ($mg.g^{-1}$)	k	model	q at $c_i = 2.5$ $g.l^{-1}$ ($mg.g^{-1}$)
Alumina	129.46 ± 14.76	2.14 ± 0.17	1	109.07 ± 4.23
Mol.sieve prep	98.80 ± 12.50	-	3	98.80 ± 12.50
Mol.sieve 1.0	88.06 ± 4.45	1.20 ± 0.04	1	66.04 ± 5.19
Silica	-	5.26 ± 0.01	2	13.15 ± 0.68
Active carbon	14.54 ± 0.07	2.14 ± 0.94	1	12.25 ± 0.42
Starch	-	3.64 ± 0.02	2	9.10 ± 0.10
Mol.sieve 0.3	-	-	4	0
Polyamide	-	-	4	0
Xad-2	-	-	4	0
Xad-4	-	-	4	0

From the results it is evident that alumina was the best adsorbent with a maximum adsorption of almost 130 $mg.g^{-1}$ and an adsorption of almost 110 $mg.g^{-1}$ at the given initial glucoside concentration. Silica, although frequently used in glucoside purification, does not exhibit a high adsorption capacity for hexyl glucoside under these conditions (13.15 $mg.g^{-1}$). Starch and polyamide were found unsuitable for glucoside adsorption as well (9.10 $mg.g^{-1}$ and no measurable adsorption, respectively). The difference between the molecular sieves with different pore sizes is most probably due to the fact that hexyl glucoside is a relatively large molecule (ca. 1.3 x 0.5 nm) in comparison to the pores. Molecular sieve 1.0 was not directly suitable as an adsorbent since the size of the spheres (average diameter: 2 mm) induces channeling in our lab-scale column. Therefore, the spheres were crushed and sieved to obtain an adsorbent with better column preparation properties (table entry: 'mol.sieve prep'). The crushed molecular sieve had a better adsorption performance than the unprepared molecular sieve (an adsorption of 98.80 over 66.04 $mg.g^{-1}$).

Both alumina and crushed molecular sieve appear to be the best candidates for an in-line application. However, as already discussed, the permeate contained water. After equilibrium adsorption tests with hexyl glucoside in wetted hexanol a larger decrease in glucoside adsorption appeared on molecular sieve than on alumina. Therefore, alumina was further used as an adsorbent for the in-line adsorption column.

Continuous production and adsorption of hexyl glucoside

Breakthrough curves were measured using dry alumina and alumina wetted with buffer saturated hexanol. Almost no difference in the maximum adsorption was observed. Therefore, dry alumina was used for in-line adsorption. A series of five columns was used to remove hexyl glucoside from the same reactor batch. The columns were operated with different amounts of dry alumina and with different flows (see table 3).

Table 3: Maximum adsorption (in mg.g^{-1}) and number of transfer units (between brackets, estimated with the van Deemter equation and the bed height) for five columns operated with different amounts of dry alumina and different flows in the same reactor batch.

Amount of dry alumina (g)	1.69	5.00	20.00
Flow (ml.min^{-1})			
0.1	10.27 (0.1)	-	-
0.2	-	16.90 (0.3)	-
0.5	-	-	8.33 (1.8)
1.0	-	11.80 (1.4)	8.43 (3.5)

The highest adsorption was reached with a column load of 5 g dry alumina (adsorption of 16.90 and 11.80 mg.g^{-1}), while it was quite constant with an increasing number of transfer units. Based on these results, optimum adsorption conditions appear to be a combination of a high flow with a low number of transfer units. The average adsorption was 11.15 mg hexyl glucoside per gram of alumina, which is in agreement with the literature.[18]

The maximum adsorption in the in-line column experiments was much lower than the maximum adsorption in the equilibrium experiments (an average of 11.15 compared to 129.47 mg.g^{-1}, respectively). This was caused by the fact that the permeate in the column experiments also contains water and glucose, which were absent in the equilibrium experiments. Therefore, equilibrium experiments were performed with hexyl glucoside in wetted hexanol and hexyl glucoside in a reactor mixture. The maximum adsorption in these cases was 33.36 $mg.g^{-1}$ and 31.86 $mg.g^{-1}$, respectively, which is more in accordance with the column experiments. Apparently, the presence of water and glucose in the influent strongly decreased the adsorption of the glucoside. The effect of glucose on glucoside adsorption appeared to be small, since the values differ only marginally. However, desorption experiments showed that the column contained no glucoside but only glucose after continuous adsorption for 19h. Apparently, glucose displaced the glucoside after the maximum glucoside adsorption is reached. This was confirmed experimentally by monitoring the glucoside concentration in the effluent in time (see figure 3).

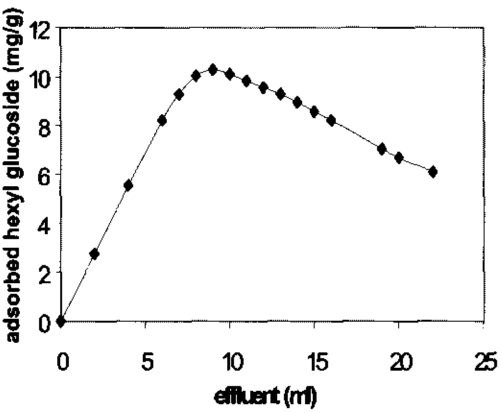

Figure 3: Breakthrough curve of hexyl glucoside on an alumina column in time. $C_{influent, glucoside} = 2.34\ g.l^{-1}$, $C_{influent, glucose} = 0.5\ g.l^{-1}$, $m_{alumina} = 1.69\ g$, flow = 0.1 $ml.min^{-1}$

Based on the breakthrough curve in figure 3 it is clear that the column has to be replaced as soon as the maximum adsorption has reached (i.e. when $C_{glucoside,\ effluent}$ equals $C_{glucoside,\ influent}$).

Desorption

For a semi-continuous operation of the reactor system a suitable operating regime for the adsorption column has to be determined. After product adsorption the column has to be desorbed with an appropriate solvent. The possible use of the product in foods limits the choice of the desorption fluids to those that are considered food-grade. Therefore, only water and ethanol were chosen. Since reuse of the column is preferable, the column should be as 'clean' as possible after the desorption step. A last point, which has to be taken into consideration, is the adsorption of glucose that will contaminate the product when the column is desorbed. The results of the desorption experiments are given in table 4.

Table 4: Glucoside and glucose yields after desorption of an alumina column with water or ethanol as desorption eluent.

Desorption eluent	Glucoside yield (% of adsorbed)	Glucose yield (% of adsorbed)
Water	74	100
Ethanol	100	0

As can be read from the table, water desorbed only 74% of the product, but did remove all the glucose from the column. This is most probably due to the lower solubility in water of the rather apolar glucoside compared to glucose. When ethanol was used as the desorption eluens, 100% of the glucoside desorbed. In this case glucose, which is less soluble in ethanol at room temperature, did not desorb. Thus, ethanol is a very attractive regenerant, since it also can be easily removed from the glucoside by evaporation.

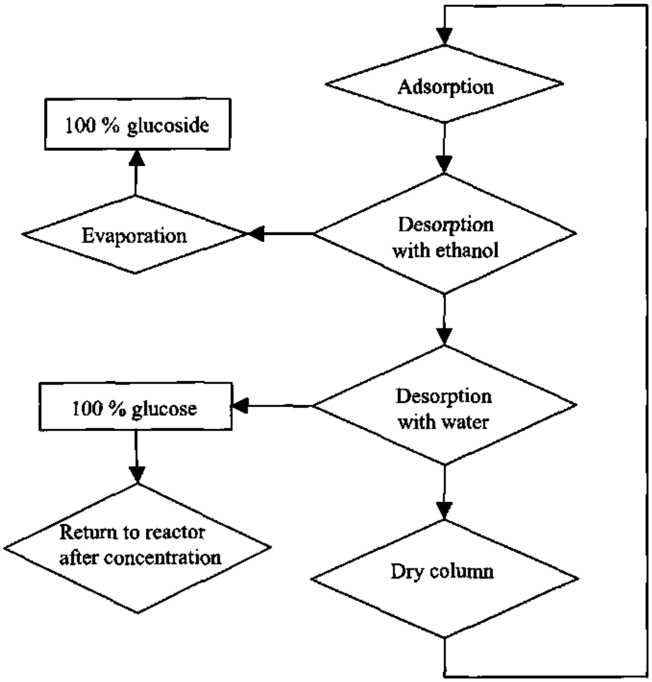

Figure 4: Flow chart of the operating regime for the reuse of the column in the reaction system.

To obtain a pure product and an 'empty' column which can be reused in the process the operating regime should be as given schematically in the flow chart (figure 4).

Process design

If we aim at a production of 1 kg of hexyl glucoside a straightforward process set up can be estimated. Even at a high production rate, a typical reaction time is in order of magnitude of hours, while the characteristic diffusion time is in order of magnitude of seconds. Therefore, a small reactor volume can be used since mass transfer limitations are not expected. Based on the initial production rate of 2.24 $mg.U^{-1}.h^{-1}$ and first order deactivation for the enzyme, a relation between the initial amount of enzyme needed and production time can be made (see figure 5):

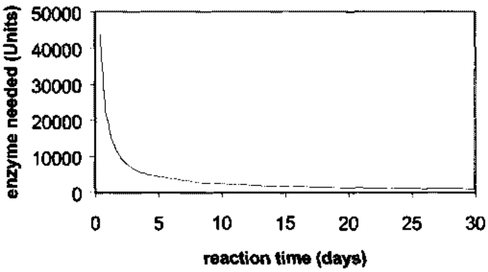

Figure 5: Required amount of enzyme (adjusted for first order deactivation) and corresponding production time for 1 kg hexyl glucoside.

As shown in figure 5, the required amount of glucoside can be produced in half a day with an initial amount of 38000 units of almond β-glucosidase. The remaining enzyme activity after this time period will be higher than 98%. Although the enzyme is relatively cheap, the catalyst is not used very efficiently in this case. If the enzyme is used until the half-life time (24 days), the initial amount of enzyme required is 1200 units. The amount of alumina that has to be regenerated to yield 1 kg of pure glucoside is 90 kg.

CONCLUSIONS

A bioreactor for the semi-continuous production of hexyl glucoside has been developed. The enzyme proved to be very stable in the low shear environment of a spray column reactor. The half-life time of the enzyme was almost 30 days, whereas the enzyme is totally deactivated in 40 h in a CSTR. The high deactivation rate in a CSTR may be caused by vigorous stirring in combination with the presence of an air-liquid interface and a large polar/apolar liquid interface.

To prevent migration of the aqueous phase containing the enzyme to the downstream process a pretreated polypropylene microfiltration membrane was used as a barrier between the spray column reactor and the adsorption column. After treatment with a block-copolymer the membrane proved to be non-permeable for water for at least 41 days, while the glucoside permeated easily.

Based on equilibrium adsorption, alumina was chosen as adsorbent for in-line adsorption. Based on an initial hexyl glucoside concentration of 2.5 $g.l^{-1}$, the maximum adsorption was found to be almost 110 $mg.g^{-1}$. This maximum adsorption could not be achieved in an in-line column. The presence of water in the column influent proved to be the major reason for this decrease in maximum adsorption.

Based on these results, an operating regime for the column change was given. It was found to be possible to obtain a pure product and a completely regenerated column by successive treatment with ethanol and water and a final drying step.

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ABSTRACT

Geraniol plays an important role in the fragrance and flavor industry. The corresponding glucoside has interesting properties as a 'slow release' aroma compound. Therefore, the enzymatic production and downstream processing of geranyl glucoside were investigated. Geranyl glucoside was produced in a spray column reactor with an initial production rate of 0.58 $mg.U^{-1}.h^{-1}$. A pretreated hydrophobic microfiltration membrane was used to prevent migration of the aqueous, enzyme containing, phase to the downstream process. No retention of the glucoside, which accumulated in the geraniol phase, was found. Based on examples from the literature, four downstream processes were tested on their viability for this system.

Extraction with water and foaming were not suitable to recover geranyl glucoside from geraniol. In the first case, the glucoside selectivity for the geraniol phase was found to be high, which made extraction with water unsuccessful. In the second case it was possible to obtain a stable foam, but significant enrichment of the foam with glucoside did not occur.

Adsorption on alumina and distillation under reduced pressure were applied successfully and tested in-line with the bioreactor. A maximum glucoside adsorption of 7.86 $mg.g^{-1}$ was achieved on alumina. After desorption and evaporation of the extractant the pure glucoside was obtained quantitatively. A pure product could not be obtained after distillation due to the fact that a small amount of glucose was present in the permeate as well, which accumulated in the bottom fraction.

It was shown that with this reactor system a production of 1 kg of geranyl glucoside in 2 days is possible using an initial amount of 50,000 units of enzyme.

INTRODUCTION

Glucosylation

Geraniol plays an important role in fragrance and flavor industry because its odor is typical for the fragrance of roses. As a result, geraniol is a constituent of 43% of the fine fragrances found on the market.[1] In many natural sources, like grape,[2] clove,[3] apples,[4] pepper,[5] and tea,[6] the non-odorous and non-volatile glucoside form of geraniol is more abundant than free geraniol. In these cases, geraniol is released only when needed in the metabolism of the plant. This 'controlled-release' of a fragrance has interesting potential in industry. The slow hydrolysis of glucosides, sometimes accompanied by a suitable enzyme, results in the delayed release of flavors and fragrances.[7, 8] Synthesis of glucosides can be performed chemically[9, 10] or enzymatically. For instance, β-glucosidases catalyze the glucosylation of a variety of aglycons.[11-13] Furthermore, β-glucosidases are cheap and abundant, which makes them suitable for industrial applications. A major drawback of the glucosylation reaction catalyzed by β-glucosidase is the unfavorable equilibrium position that results in a low product yield. However, improvement of the product yield is possible by 'tuning' the reaction conditions,[14-20] or using a bioreactor system that enables in-line downstream processing of the reaction mixture, thereby removing the product.

Bioreactor setup

Geranyl glucoside was produced enzymatically in a bioreactor, as was described previously for the production of hexyl glucoside (see figure 1).[21]

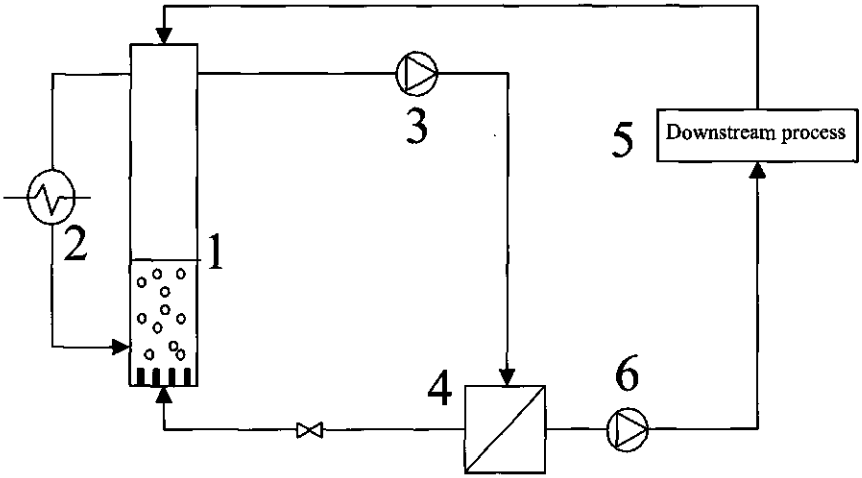

Figure 1: Schematic presentation of the bioreactor with in-line downstream processing. 1) Spray column reactor, 2) Water bath (50°C), 3) Gear pump, 4) Membrane module, 5) Downstream process, 6) Plunger pump

The aqueous, enzyme containing, phase should not enter the downstream process. This was achieved by introducing a hydrophobic microfiltration membrane between the spray column reactor and the downstream process. The membrane allowed permeation of the aglycon phase, which contained the glucoside, but retained the disperse aqueous phase with the enzyme. An additional benefit was the retention of excess water and glucose, which could interfere with the downstream process. To prevent the breakthrough of water the membrane was pretreated according to Schroën's method.[22]

In principle, four different methods for downstream processing can be envisaged:

1) extraction with water, 2) selective adsorption, 3) foaming, 4) distillation.

Extraction with water was successfully used for the isolation of chemically produced C_8 to C_{12} alkyl glucosides.[23] Adsorption on alumina was already proven to be successful for the downstream processing of hexyl glucoside.[21]

Glucosides from hydrophobic aglycons (C_{12}-C_{22} alkyl chains) are applied industrially as foaming agent,[24] so this property could also be used as a downstream process. Foams are dispersions of gas in a relatively small amount of liquid.[25, 26] A surface-active agent (*e.g.* a glucoside) is necessary to initiate foaming in a liquid. Typical applications of foam formation as useful industrial unit operations are foam fractionation, ion flotation and froth flotation of minerals. These applications are based on the enrichment of the foam phase due to drainage of the liquid from the foam. In our case, enrichment of the glucoside in the foam can be achieved if the foam is sufficiently drained and if the surface excess concentration is high enough.

Finally, distillation comes into focus, because of the large difference in boiling point between the glucoside and the aglycon. Distillation was used for the downstream processing of chemically produced glucoside mixtures from C_8 to C_{16}-alcohols,[27, 28] and C_{12} to C_{18}-alcohols.[29] In all cases, glycerol was used to enhance the distillation of the alcohols, due to the extraction of the glucosides from the alcohol phase to the glycerol phase. In addition, glycerol prevented the glucosides to crystallize during the distillation and, thus, provided a liquid product stream.

In this paper, we applied these downstream processes to obtain geranyl glucoside. All methods are evaluated based on their glucoside removal capacity. If possible, the processes were used in-line with the bioreactor.

MATERIALS AND METHODS

Chemicals

Almond β-glucosidase (E.C.3.2.1.21) was obtained from Sigma (5.6 $U.mg^{-1}$). Glucose, geraniol, hexadecane, glycerol, sodium phosphate and citric acid were obtained from Sigma; *p*-nitrophenol-β-D-glucoside was from Aldrich; Acetonitrile (HPLC-grade) was from Lab-Scan. The block copolymer F108 (based on one polypropylene oxide block and two polyethylene oxide blocks) was a gift from ICI.

Adsorbent

Alumina (Brockman I, particle size: 50 - 200 μm) was obtained from Baker. Before usage, alumina was dried at 120 °C for at least 1 h.

Equipment

The HPLC system used has been described in chapter 4.1. Samples of 50 μl were taken from the geraniol phase and were diluted with 1 ml of methanol. These samples were injected into the HPLC and eluted with a gradient of acetonitrile/water at a flow of 1 $ml.min^{-1}$. After 4 min of 85% acetonitrile and 15 % water, water increased to 30% and acetonitrile decreased to 70% in 1 min. The eluent was kept at isocratic conditions for the next 5 minutes, after which the gradient returned to the initial settings in 1 min and was kept at these conditions for the next 6 min. Concentrations were calculated with external standards of hexyl glucoside, since geranyl and hexyl glucoside have the same responsfactors on the Evaporative Light Scattering Detector (ELSD). Membrane separation was performed with an Accurel® flat sheet polypropylene membrane from Akzo Nobel (55 x 130 mm, maximum pore size: 0.58 μm). The membrane was used in a flat sheet membrane module built in-house. An Omnifit glass column (250 mm x 10 mm i.d) with an adjustable plunger was used to prepare the adsorption column.

Membrane preparation

The membrane was pre-wetted by rinsing with hexadecane for 30 minutes; hexadecane permeated through the membrane. Next, the membrane was rinsed for 15 minutes with an emulsion of 1:2 v/v hexadecane in water, containing 6 (g block copolymer).l^{-1} of emulsion. Both retentate and permeate (the permeate consisted only of hexadecane) were recycled to the emulsion. Subsequently, the membrane was rinsed with water, which did not permeate through the membrane. Finally, the membrane was rinsed with a geraniol/buffer emulsion. Only geraniol permeated through the membrane. In all rinsing steps, the pump was operated at a capacity of 1.67 $ml.s^{-1}$.

Bioreactor

The enzymatic reaction was carried out in a bioreactor as described in figure 1. The reactor system consisted of a double walled glass spray column (1) (i.d.: 30 mm, column length: 210 mm) which was thermostated with a water bath (2) at 50 °C. The continuous phase was brought into the bottom of the column with a Verder 2040 gear pump (3) at a velocity of 1 $ml.s^{-1}$ via a stainless steel nozzle with 12 tips (equally divided over the nozzle area, each 10 mm long, 1 mm i.d.). The continuous phase was led over the membrane module (4). The volumetric flow through the membrane was 2.38 $ml.s^{-1}.m^{-2}.bar^{-1}$.

In case of adsorption, the permeate was led to an adsorption column (5) with a Gilson 303 plunger pump (6). The column effluent was recycled to the spray column. In case of distillation, the distillate was pumped back to the spray column with the plunger pump.

Continuous production of geranyl glucoside in the bioreactor

The spray column was filled with 270 ml of geraniol (continuous phase). Geraniol had been saturated with a citrate/phosphate buffer pH 6.0 (0.16 M) at 50 °C. The reaction was started by the addition of 30 ml water phase (stationary phase) at the top of the spray column. The water phase consisted of a citrate/phosphate buffer pH 6.0 (0.16 M) with an almond β-glucosidase activity of 0.3 $U.ml^{-1}$ buffer solution.

Downstream processing

Off-line extraction

Geraniol from a reactor batch, containing 1.4 $g.l^{-1}$ geranyl glucoside, was shaken at room temperature with 10, 30, 60, and 90 v/v % of water. After phase separation, samples were taken from both the organic and the aqueous phase and analyzed with HPLC.

Off-line foaming

The foaming ability of the glucoside was tested by aerating 25 ml and 50 ml of geraniol from a reactor batch, containing 1.4 $g.l^{-1}$ geranyl glucoside, in a 190 mm high cylinder (i.d. 21 mm) and a 410 mm high cylinder (i.d. 26 mm), respectively. Air was led into the organic phase via a sparger with a flow of 77 ml.min^{-1}. In both cases the air flow was regulated in such a way that the highest stable foam layer possible was obtained. Foam reaching the top of the cylinder was skimmed off, sampled and measured on HPLC. If the foam did not reach the top of the cylinder the upper 5 mm of the foam was collected.

In-line adsorption

In case of adsorption, the permeate was led to the adsorption column. The column was filled with 16.5 g dried alumina. Two adsorption columns operated in an up-flow mode, and were successively used in the same reactor batch. The effluent was monitored and the adsorption column was removed as soon as $C_{glucoside,effluent}$ equaled $C_{glucoside,influent}$. The column was purged with air for 30 min to remove geraniol between the adsorbents particles. Subsequently, the column was eluted at room temperature for 30 min with ethanol. The eluent was collected and evaporated, yielding pure geranyl glucoside. Regeneration of the column was possible after elution with water and a drying step.

In-line distillation

In case of distillation, the permeate was led via a restriction (1) in a three-necked flask filled with 50 ml of glycerol (2), which was heated to 140 °C at 40 mbar (see figure 2).

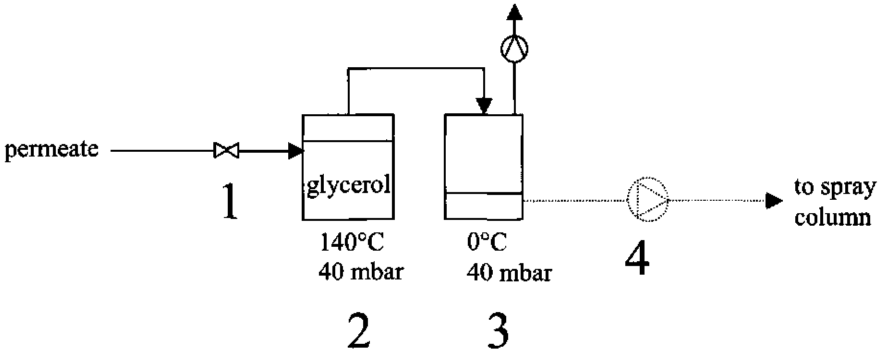

Figure 2: Schematic presentation of the in-line distillation. 1) Restriction, 2) Distillation vessel, 3) Condensation vessel, 4) Plunger pump

The permeate flow was reduced to ca. 1 ml.min^{-1}. The vapor phase was led via a PTFE-tube to a condensation vessel (3). After condensation of 20 ml geraniol, atmospheric

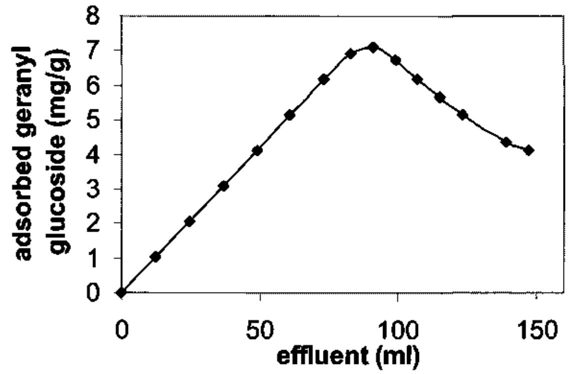

Figure 4: Breakthrough curve of geranyl glucoside on an alumina column in time. $C_{\text{influent, glucoside}} = 1.41$ $g.l^{-1}$, $C_{\text{influent, glucose}} = 0.1$ $g.l^{-1}$, $m_{\text{alumina}} = 16.5$ g, flow = 0.5 $ml.min^{-1}$. After ca. 90 ml elution, the glucoside was displaced by glucose in the feed.

After the maximum glucoside adsorption is reached, glucose displaces the glucoside. Therefore, the column has to be replaced by a fresh one as soon as this maximum adsorption is reached. Total desorption of geranyl glucoside and a completely regenerated column were possible after successive treatment of the column with ethanol and water, followed by a drying step. With this adsorption setup a glucoside yield of almost 130 mg per downstream process cycle was achieved.

Distillation

Batch-wise distillation under reduced pressure was applied in-line as well. Pure geraniol was obtained in the condensation vessel at a flow rate of 1 $ml.min^{-1}$. After restoring atmospheric pressure, geraniol was fed back to the reactor. Glycerol, which contained 30 mg of geranyl glucoside, remained in the distillation vessel. Although not confirmed experimentally, glycerol can be reused until the maximum solubility of geranyl glucoside in glycerol is reached. In previous studies, the maximum molar fraction of glucose in glycerol at 50°C was measured to be 0.02.[20] Although geranyl glucoside is less polar than glucose it is safe to assume as a lower estimate a similar maximum solubility of geranyl glucoside in glycerol at the distillation temperature. Thus, a glucoside yield of 4.0 g per downstream process cycle was calculated. Besides geranyl glucoside and glucose, also degradation products from geraniol accumulated in the distillation vessel. Fortunately, the degradation products were not miscible with glycerol and geranyl glucoside did not migrate to this phase. Therefore, this phase could easily be removed from the distillation vessel. Removing glucose, however, is hardly possible.

Process design

If we aim at a production of 1 kg of geranyl glucoside, a straightforward process set up can be estimated. Mass transfer limitation was not expected, since the absolute production rate is low. Even at a high production rate, the characteristic time of reaction is in the order of magnitude of hours, while the characteristic time of diffusion is in the order of magnitude of seconds. Therefore, the reactor volume can be chosen as low as possible. Based on the initial production rate of 0.58 $mg.U^{-1}.h^{-1}$ and first order deactivation of the enzyme, the relation between the initial amount of enzyme and the production time was estimated (see figure 5).

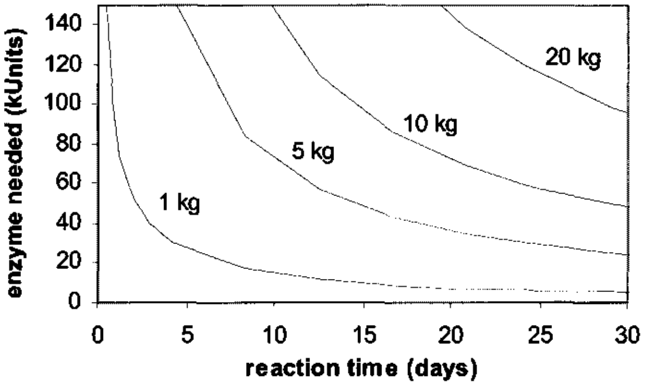

Figure 5: Required amount of enzyme (adjusted for first order deactivation) and corresponding production time for the production of 1 to 20 kg of geranyl glucoside.

If the enzyme is used until its half-life of 2 days, then a production of 1 kg geranyl glucoside is possible using 50,000 units of enzyme. With the same initial amount of enzyme it is possible to produce 5 kg of geranyl glucoside in 17 days. A higher production of glucoside is possible, but is limited by the solubility of the required initial amount of enzyme in the water phase.

Since the capacity of the downstream processes was chosen arbitrarily, the yield per DSP cycle is not the right criterion for selecting the best downstream process. In contrast to adsorption, distillation appeared to be more efficient in removing both the product and the by-products, hence in this case pure geraniol was fed back to the reactor. However, in case of adsorption, the pure glucoside was obtained, which was not the case for distillation. Cost evaluation is not sensible due to the small scale of operation of the processes. Therefore, the choice of a downstream process should be made solely based on the required product purity. The required quality of the product is mainly dependent on customer demands. If the product is used in a formulation (which is most likely for a 'slow release' aroma compound), distillation can be used. If absolute purity is required adsorption should be used as a downstream process.

CONCLUSIONS

The downstream processing of enzymatically produced geranyl glucoside was investigated. Geranyl glucoside was produced in a two-phase spray column reactor with an initial production rate of 0.58 $mg.U^{-1}.h^{-1}$. The geraniol phase, in which the glucoside accumulated, was separated from the reaction mixture using a custom pretreated hydrophobic microfiltration membrane. Four types of downstream processes were applied to isolate the glucoside from the geraniol phase: back-extraction with water, foaming, adsorption on alumina and distillation under reduced pressure.

Both off-line back-extraction and off-line foaming did not result in enrichment of the glucoside. Apparently, the glucoside affinity for geraniol was high, since it was impossible to extract the glucoside with water. Therefore, this method was not further used. It was possible to obtain a stable foam by aerating the process stream. However, drainage of geraniol from the foam was insufficient or the surface excess concentration of the glucoside was not sufficiently high to yield a glucoside enriched foam layer.

Adsorption on alumina was successfully applied in-line. The maximum adsorption was 7.86 $mg.g^{-1}$ which was in accordance to previous studies. It was possible to obtain a pure product and a completely regenerated column by successive treatment with ethanol and water and a final drying step. Finally, distillation under reduced pressure was found to be a suitable alternative in-line downstream process. In this case, a pure product could not be obtained, since glycerol was added to enhance the distillation of geraniol and to prevent the glucosides from crystallization during the process. Furthermore, besides the glucoside a small amount of glucose was present in the permeate, and this remained in the distillation vessel as well.

With straightforward calculations it was shown that 1 kg of geranyl glucoside could be produced in 2 days using an initial amount of 50,000 units of enzyme.

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CHAPTER 5

ADDITIONAL RESULTS AND GENERAL DISCUSSION

INTRODUCTION

The obtained results described in chapters 2-4 of this thesis can be summarized as follows:

1 - The success of the enzymatic glucosylation of aglycons was explained and predicted by calculating the charge of the reactive oxygen atom of the aglycon.

2 - The optimum reaction conditions for the almond β-glucosidase mediated glucosylation of primary and secondary alcohols, with glucose as sugar donor, were determined and applied successfully.

3 - Glyceryl glucoside was synthesized in a high yield, but the product purification was more complicated than that of glucosides from apolar aglycons due to the presence of components with highly similar (solubility) properties in the reaction mixture.

4 - In case of the glucosylation of apolar aglycons, a high production of glucosides was possible in a reactor system consisting of a spray-column reactor, a phase separation step with a hydrophobic membrane and a suitable downstream process.

5 - Adsorption on alumina and distillation under reduced pressure were found to be the best downstream processes out of four investigated process principles.

Based on these results, the overall conclusion can be drawn that the objectives of the project were achieved. However, still some questions remain unanswered:

1 - How stable are the produced glucosides with respect to a particular (food-) application?

2 - What are the possible alternatives for almond β-glucosidase?

3 - How can glyceryl glucoside be isolated from the reaction mixture?

4 - Can the four downstream processes that were used for geranyl glucoside be applied for hexyl glucoside? And what is the scope of these downstream processes?

5 - Is the presented reactor system industrially feasible?

In this chapter answers to these questions will be given. Results from glucoside stability experiments will be presented and the implications of these results for a (food) application will be discussed. Furthermore, alternative enzymes for the glycosylation reaction will be reviewed. Preliminary results with β-glucosidase from *Pyrococcus furiosus* will be shown as an example of a potentially interesting alternative glucosidase. In addition, attempts to isolate hexyl glucoside and glyceryl glucoside that were not shown in the previous chapters will be discussed in this chapter. Finally, the industrial viability of the bioreactor system and the two successfully applied downstream processes will be presented by calculations of the minimum equipment requirements.

GLUCOSIDE STABILITY

As discussed in the introduction of this thesis, the applications of glucosides are numerous. In most cases it is important that the glucoside is stable in various environments. A study to the spontaneous hydrolysis of glucosides revealed a half-life of methyl glucoside of 4.7 million years at 25°C and pH 7.4.[1] At 150°C and the same pH this half-life was reduced to 1 year. Of course, the stability of the glucoside is for an important part dependent on the aglycon and its ability to act as a leaving group. For example, the half-life of 2,4-dinitrophenyl-β-galactopyranoside at 25°C and pH 6.5 was 40 h.[2] Glucosides based on fragrances are of interest for a 'controlled release' aroma application and, therefore, the rate of hydrolysis should be susceptible to change, *e.g.* by changing the temperature or the pH. Stability experiments with hexyl glucoside as a model compound for these 'controlled release' glucosides, showed that this glucoside was very stable as well. No decrease in glucoside concentration was found under the following conditions (see table 1).

Table 1: Conditions during which no hydrolysis of hexyl glucoside was found

Solvent	Temperature (°C)	Time	pH
Hexanol	4	At least 150 days	-
"	20	"	-
Water	4	"	7
"	20	"	7
"	100	At least 15 min.	7
"	100	"	6
"	100	"	5
"	100	"	4
"	100	"	3
"	100	"	2
"	100	"	1
"	100	"	0

After 3 h in boiling water at pH 0 the glucoside was hydrolyzed completely. However, it is hard to imagine these severe conditions in an application for a 'controlled release' aroma compound. Therefore, formulating the glucoside together with a glucoside hydrolase may be necessary.

ENZYME CHOICE

Alternative enzymes

The initial objective of the project was to develop an enzyme catalyzed reaction in which an aglycon was coupled to a sugar. The choice for almond β-glucosidase was made based on the ample literature on its glucosylation reaction, the availability of the enzyme and its ability to work with "cost-effective" sugar donors (see table 2). It is interesting to estimate whether other enzymes could be used for the same purpose. The selection of alternative enzymes, therefore, should be based on the same criteria.

Table 2: Market prices of sugar donors (week ending dec. 15, 2000) that are available in bulk quantities[3]

Sugar	Price (US$.kg^{-1})
Starch	0.14-0.30*
Lactose	0.49
Glucose	0.56
Sucrose	0.79
Fructose	0.86
Cellulose	5.18

* *based on different starch sources (http://home3.inet.tele.dk/starch/isi/market/market.htm)*

As shown in table 2, the market prices of the sugars are in the same range except for the price of cellulose. If the price of the sugar donor is taken as the only criterion, the processes that use starch or lactose are the most competitive. The enzymes that accept the five cheapest sugar donors are summarized in table 3.

Table 3: Enzymes that can use fructose, glucose, sucrose, lactose or starch as substrate, with published examples of glycosylation reactions.[4] ●: enzyme accepts this sugar donor, +: commercially available, -: very expensive or not commercially available.

EC number	Enzyme	Lactose	Glucose	Starch	Sucrose	Fructose	Availability	Literature
2.4.1.4	Amylosucrase				●	●	-	5-7
2.4.1.5	Dextransucrase				●	●	+	8, 9
2.4.1.9	Inulosucrase		●		●		-	10, 11
2.4.1.10	Levansucrase		●		●		-	12, 13
2.4.1.19	Cyclomaltodextrin glucanotransferase			●			-	14
2.4.1.99	Sucrose 1F-fructosyltransferase		●		●		-	15
3.2.1.1	α-Amylase			●			+	16-18
3.2.1.2	β-Amylase			●			+	19[a]
3.2.1.3	Glucan 1,4-α-glucosidase (glucoamylase)		●	●			+	20
3.2.1.10	Oligo-1,6-glucosidase (isomaltase)			●			+	21[b]
3.2.1.20	α-Glucosidase		●	●			+	22-25[c]
3.2.1.23	β-Galactosidase	●					+	25-31[c]
3.2.1.26	β-Fructofuranosidase (invertase)				●	●	+	28
3.2.1.41	α-Dextrine endo-1,6-α-glucosidase (pullulanase)			●			+	32
3.2.1.48	Sucrose α-glucosidase (sucrase)				●		-	33[b]
3.2.1.58	Glucan-1,3-β-glucosidase		●	●			-	34
3.2.1.70	Glucan-1,6-α-glucosidase (glucodextranase)			●			-	35[b]
3.2.1.74	Glucan-1,4-β-glucosidase		●		●		-	36
3.2.1.108	Lactase	●					-	37[b]

[a]review, [b]only hydrolysis, no literature on glycosylation available, [c]more literature is available

It is clear that many enzymes can perform the glycosylation reaction with cheap sugar donors. Based on the other criteria (enzyme and literature availability), four enzymes from this list remained as possible alternatives for almond β-glucosidase, namely: α- and β-amylase, α-glucosidase and β-galactosidase. The extensive literature on bioreactor applications of α-amylase[38-42] and β-galactosidase[43-53] (although all related to the hydrolysis reaction) made these two enzymes the best possible alternatives for the reaction described in this thesis. A drawback when using these enzymes might be the fact that they only accept lactose or polysaccharides as cheap sugar donor. Consequently, in the case of α-amylase, a mixture of mono-, di- and higher glucosylated glucosides will be formed, which all have different characteristics in a downstream process and in an eventual application. When β-galactosidase is used, the sugar donor, although cheap, will not be used very efficiently since glucose remains as a non-reacted side product. However, in a study to the glycosylation of butanol with lactose using both β-glucosidase and β-galactosidase simultaneously, it was shown that this drawback could easily be overcome.[54] Noteworthy is the available literature on bioreactor applications of glucoamylase (EC 3.2.1.3) for the hydrolysis of glucosides and polysaccharides.[55-57] Although literature on the glycosylation reaction for this enzyme is scarce, the principle of microscopic reversibility makes this enzyme a possible likely alternative as well.

Alternative sources of β-glucosidases

Sources of β-glucosidases can be found in every domain of the phylogenetic tree.[58] Since the biological function of the enzyme in these organisms is more or less similar, they require the same reaction conditions in glucosylation reactions. Enzymes from extremophiles, however, operate at conditions (like temperature, pH, pressure, etc.) which are different from their 'normal' counterparts,[59, 60] and are therefore a useful extension of the enzyme toolbox.

Pyrococcus furiosus is a hyperthermophilic microorganism (optimal growth temperature 80-110°C) that belongs to the domain of the Archaea. β-Glucosidase from *P. furiosus* was purified, characterized,[61] and successfully cloned in *E. coli*.[62] The enzyme characteristics (activity, stability and kinetic parameters) were comparable with the wild type enzyme, and the mechanism of the enzyme was found to be highly similar to other β-glucosidases from thermo- and mesophilic sources.[63, 64] However, it has already been shown that the thermophilic enzyme has a different specificity for aglycons than mesophilic β-glucosidases. Tertiary alcohols, which are normally not glucosylated by β-glucosidases, can successfully be coupled to glucose via transglucosylation of cellobiose by the thermophilic enzyme.[65]

Preliminary results from glucosylation experiments with cloned β-glucosidase from *P. furiosus* in our lab demonstrate that this enzyme is capable of glucosylating hexanol.[66] In contrast to the literature,[65] glucose was successfully used as sugar donor. Moreover, the *P. furiosus* enzyme showed a higher initial activity than the enzyme from almonds under the same reaction conditions. Although in the literature the *P. furiosus* enzyme showed lower aglycon specificity than other β-glucosidases, it was found to be impossible to glucosylate eugenol, thus substantiating the results from chapter 2. Ironically, the optimum reaction temperature for this hyperthermophilic enzyme appeared to be 50°C. Higher temperatures induced an increased deactivation rate due to Maillard reactions. However, the ability of the enzyme to work at a high temperature, resulting in a higher initial production rate, demonstrates its promising possibilities. Unfortunately, industrial application at this moment is still hampered by the current limited availability of this enzyme.

DOWNSTREAM PROCESSING

Downstream processing of a two-phase system

As was described in chapter 4.2, four different methods have been tested for the downstream processing of geranyl glucoside. Adsorption and distillation were found to be most suitable for this compound, while back-extraction and foaming appeared to be not suitable. In the case of hexyl glucoside, although not described in chapter 4.1, back-extraction and foaming have also been investigated as possible downstream processes. Extraction with different ratios of water/hexanol resulted in a maximum extraction of 34 % (mol/mol_{total}) of the glucoside from the hexanol phase at a water/hexanol ratio of 2.5. Furthermore, the partition coefficient appeared to be low (4.28 ± 0.86 ($(M_{hexanol})/(M_{water})$)).

Foaming was also tested for the downstream processing of hexyl glucoside from hexanol, but foaming was not observed. The poor results could be due to a number of factors. First, the concentration of the glucoside could be too low to form a stable foam. However, as was shown in chapter 4.2, foaming of geranyl glucoside in geraniol was possible with an almost threefold lower glucoside concentration. Secondly, the hydrophobic part of the glucoside could be too short to yield a glucoside with good surfactant properties. In the literature, glucosides from C_{12} to C_{22}-alkanols are reported to have surfactant properties.[67] This indicates that the relatively short alkyl chain of hexyl glucoside could be the cause of the bad foaming properties.

In conclusion, adsorption and distillation appear to be the most indiscriminate downstream processes for this reaction system. However, when using very apolar aglycons, a lower maximum adsorption on alumina might be expected due to the increased solubility of the glucoside in the aglycon. When using distillation, the difference in boiling point between glycerol and the aglycon has to be higher than 10°C, to prevent glycerol from evaporating.

Downstream processing of glyceryl glucoside

Some experiments towards the downstream processing of glyceryl glucoside from a one-phase mixture were carried out as well. As shown in chapter 3.2, the main focus of this part of the project was to achieve an equilibrium reaction mixture, which contained almost pure glucoside as a result of smart tuning of the initial substrate concentrations, thus facilitating the downstream process. Unfortunately, obtaining a pure glucoside in the equilibrium reaction mixture appeared to be impossible, although it was feasible to reduce the glucose concentration after reaching equilibrium considerably. Table 4 gives an overview of tested methods for downstream processing of glyceryl glucoside from a glucose/glycerol mixture in water.

Table 4: Tested downstream processes for the purification of glycerol glucoside. *Italics*: downstream process did not yield a glucoside

1st DSP	Products	2nd DSP	Products
Chromatography on silica	Glucoside in eluent	Freeze drying	Glucoside
Chromatography on Amberlite IRA 900 (OH^-)	Glucoside, glycerol (retention of glucose)	Chromatography on Norit® (retention of Glucoside)	Glucoside[68]
Reaction with 1M NaOH	Glucoside, glycerol, reaction products derived from glucose	*Chromatography on Norit® (retention of Glucoside)*	-
Evaporation of equilibrium mixture	Glucoside, glycerol	*Cooling (crystallization of glucoside)*	-

As can be seen, all methods require two successive downstream processes, and only in the first two methods glyceryl glucoside could be obtained. However, subsequent chromatography and freeze-drying is laborious and industrial application is far-fetched. Successive chromatography on an ion exchanger and Norit® is more suitable for an industrial scale process. The reaction with NaOH was based on the fact that glucose, in contrast to the glucoside, can be converted to lactic acid under strong basic conditions. After reaction for 3.5 h at 60°C, a mixture of glucose fragments, glucoside and glycerol was obtained. It was impossible to obtain the glucoside from this mixture with column chromatography on Norit®. Finally, crystallization of the glucoside seemed to be an attractive method. First, the glucose concentration was reduced considerably in the equilibrium mixture by tuning the initial substrate concentrations. Secondly, the excess of water was evaporated after which the mixture was stored at -16°C. Unfortunately, no crystallization of the glucoside occurred within 24 h (no seeding attempted).

Based on these results, manipulating the equilibrium mixture contents as demonstrated in chapter 3.2, seems the best option for further research to the downstream processing of glyceryl glucoside.

INDUSTRIAL APPLICABILITY

In general, the industrial feasibility of a process is demonstrated by cost estimations of the process on an industrial scale. As was already shown in chapter 4.2, a cost estimation of our reactor system is difficult since the scale of the process is small. Equipment costs will be low and the consumption of electricity, steam and water will only be a very small fraction of the utilities that are already available in most process plants. Furthermore, the reactor can be used to produce different glucosides and, therefore, the prices of raw materials vary. For example, bulk prices of linear medium chain alcohols are about 0.5 US\$.kg^{-1}, while prices of fragrances vary to ca. 20 US\$.kg^{-1}. In addition, the amount of necessary enzyme varies with each different aglycon. However, a list of minimum equipment requirements can give a better insight in the industrial viability of the process. Therefore, calculations were done for the reactor and the two downstream processes for the production of 1 kg of geranyl glucoside.[69] Constants that were used for the different process streams are given in table 5, and piping requirements are given in table 6.

Table 5: Constants used for the different streams in the process

	Geraniol	Water	Steam	Ethanol	Glycerol	Air
M (g.mol^{-1})	154	18	-	46	92	15
ρ (kg.m^{-3})	889.4	998	$0.6^{(100°C)}$	789.3	1260	1.3
η.(mPa.s)	9.5	1.0	$12.5.10^{-3}$ (100°C)	1.2	1500	$17.1\ 10^{-3}$
H_{evap} (kJ.kg^{-1})	$326^{(230°C)*}$	-	$2257^{(100°C)}$ $2745^{(150°C)}$	$843^{(78.5°C)}$	-	-
Cp (kJ.kg^{-1}.°C^{-1})	$1.62^{(20°C)}$ $1.76^{(50°C)}$ $2.20^{(140°C)}$	$4.20^{(20°C)}$	-	-	$1.24^{(20°C)}$ $1.57^{(140°)}$	-

*based on value for 1-decanol at 230°C

Table 6: Piping requirements and pressure drop for the different process streams

	Geraniol	Water	Steam	Ethanol	Glycerol	Air
$d_{pipe,opt}$ (mm)[a]	5	12	-	12	5	-
ΔP_{pipe} (kPa)[b]	36.6	13.5	-	13.2	111.2	-

[a] calculated for stainless steel with $d_{pipe,opt} = 226G^{0.5}\rho^{-0.35}$, with G = volumetric flow in $m^3.s^{-1}$.

[b] $\Delta P = 4.07 \cdot 10^{10} \phi^{1.84} \eta^{0.16} \rho^{-1} d^{-4.84}$, with $d = d_{pipe,opt}$ in mm.

Bioreactor setup

With respect to the reactor from chapter 4, some minor adjustments should be made. Fresh geraniol from the downstream process is fed back at the bottom of the reactor after heating. The retentate is fed back at the top of the reactor (see figure 1).

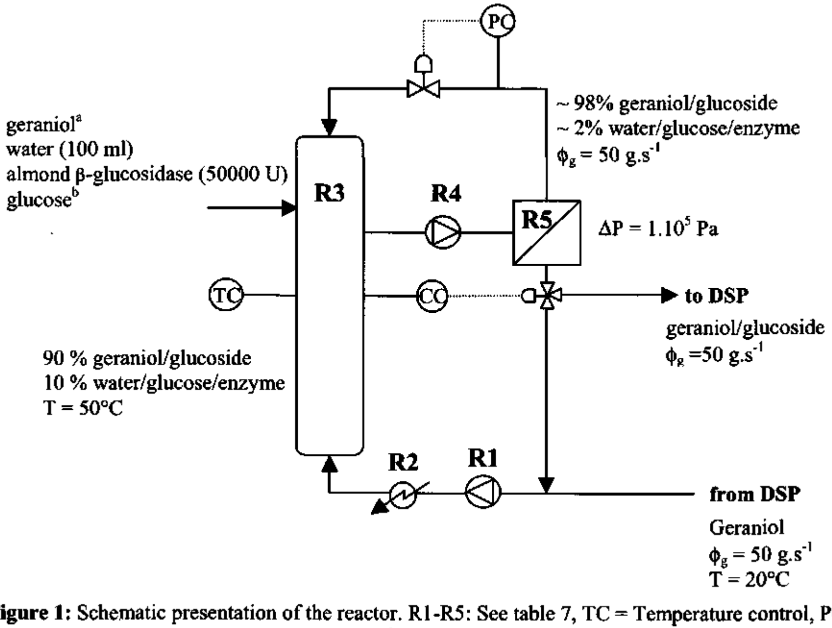

Figure 1: Schematic presentation of the reactor. R1-R5: See table 7, TC = Temperature control, PC = Pressure control, CC = Concentration control. [a]900 ml initially, and fed at an average rate of 11.4 ml.h^{-1} for 2 days, [b]50 g initially, and fed at an average rate of 10.8 g.h^{-1} for 2 days.

The initial amounts of substrates and enzyme were based on the results from chapter 4.2. For the production of 1 kg of geranyl glucoside in 2 days, 50000 units of enzyme is needed (corrected for enzyme deactivation). Both glucose and geraniol are fed to ensure the optimum reaction conditions as found in chapter 3.1 (organic phase/water phase ratio of 9, and a glucose concentration of 500 g per kg water). The pressure difference over the membrane is based on the measured volumetric flow over the membrane of 2.38 $ml.s^{-1}.m^{-2}.bar^{-1}$ (see chapter 4.1). With the given membrane area, a desired permeate flow rate of 50 $g.s^{-1}$ is realized with a pressure difference of 1 bar (=1.10^5 Pa). This could be realized with a pressure-controlled valve in the retentate stream. The permeate stream is regulated with a concentration controlled valve, and is only led to the DSP when a sufficient glucoside concentration is reached. The minimum equipment requirements for the reactor are given in table 7.

Table 7: Minimum equipment requirements for the spray column reactor/membrane separation

No	Equipment	Minimum requirements
R1	Gear pump[a]	Power: 10 W In case of distillation[b]: 25 W
R2	Electrical heater	Power: 250 W
R3	Reactor column	Glass, isolated, h = 0.5 m, d = 0.05 m Stainless steel nozzle with 24 tips, each 1 cm long, 1 mm diameter.
R4	Gear pump[a]	Power: 25 W
R5	Membrane unit	Stainless steel Pre-treated hydrophobic membrane, A = $0.72.10^{-2}$ m^2

[a] $Power = \frac{\Delta PG}{Eff}100$, with G = volumetric flow in $m^3.s^{-1}$, and Eff = pump efficiency from data plots (35% for gear pumps at these capacities)

[b] based on the increased pressure differential as a result of the distillation under reduced pressure

Adsorption column setup

For the adsorption as downstream process it was shown experimentally that the optimum adsorption conditions were a high flow combined with a minimum number of transfer units (see chapter 4.1). Therefore, relatively small columns should be used without lowering the required total amount of alumina. This is achieved by switching between two columns. When the first column is used for adsorption, the other one is regenerated (see figure 2).

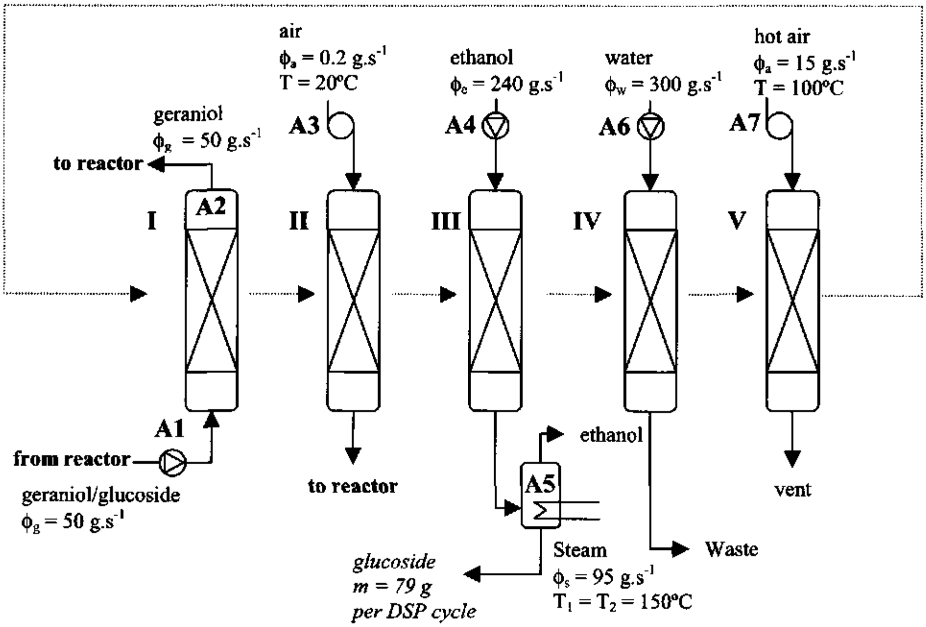

Figure 2: Schematic presentation of the adsorption step.

I. Adsorption until $C_{glucoside,\ effluent} = C_{glucoside,\ influent}$ (16 min), II. Purging of geraniol (5 min), III. Desorption of glucoside with ethanol (2.5 min), IV. Desorption of glucose with water (2.5 min), V. Drying of alumina with hot air (5 min), 1 minute spare time to switch the columns, A1-A7: see table 8.

For adsorption of 1 kg of geranyl glucoside, 130 kg of regenerated alumina is required (the maximum adsorption of geranyl glucoside on alumina was 7.86 mg.g^{-1}). Based on a column with 10 kg of alumina, the columns have to be switched 12 times. The geraniol flow from the reactor is set to 50 g.s^{-1} (~56 ml.s^{-1}), which results in a glucoside flow rate of 84 mg.s^{-1} at an equilibrium concentration of 1.5 g.l^{-1} (see chapter 4.2). Therefore, $C_{glucoside,\ effluent} = C_{glucoside,\ influent}$ will be reached in 16 minutes. Thus, the regeneration has to be completed in 16 minutes as well. For each regeneration step 4 column volumes of regenerant (air, ethanol, water, and hot air) are used (see table 8).

Table 8: Minimum equipment requirements for the adsorption step

No	Equipment	Minimum requirements	Additional data
A1	Gear pump[a]	Power: 8 W	-
A2	Adsorption column (2x)	Glass, h: 1 m, d: 0.12 m	$\Delta P_{packing}$ = 500 Pa
A3	Centrifugal pump[b]	Power: 95 W	Eff = 80%, Z=1, R= 8.314, n=1.23, P_1=14.10^2, P_2=1.10^5
A4	Gear pump[a]	Power: 15 W	-
A5	Evaporator	A^c = 2.9 m^2	U = 1000
A6	Gear pump[a]	Power: 15 W	-
A7	Centrifugal pump[b]	Power: 9.5 kW	See 3

[a] see table 7, footnote a

[b] $Power = \frac{-W\phi}{Eff}100$, with W = work produced by gas. $-W = Z\frac{RT}{M}\frac{n}{n-1}\left[\left(\frac{P_2}{P_1}\right)^{(n-1)/n}-1\right]$

[c] $A = \frac{Q}{U\Delta T_m}$ with U = overall heat transfer coefficient from data plots

Distillation setup

For distillation under reduced pressure as downstream process, the permeate stream is reduced via a flow controlled valve to prevent low pressure in the reactor. Glycerol is circulated continuously until the maximum glucoside concentration is reached. Then, the effluent valve is opened and subsequently the glycerol influent valve is opened. Fresh glycerol enters the system without pumping, due to the low pressure in the system (see figure 3).

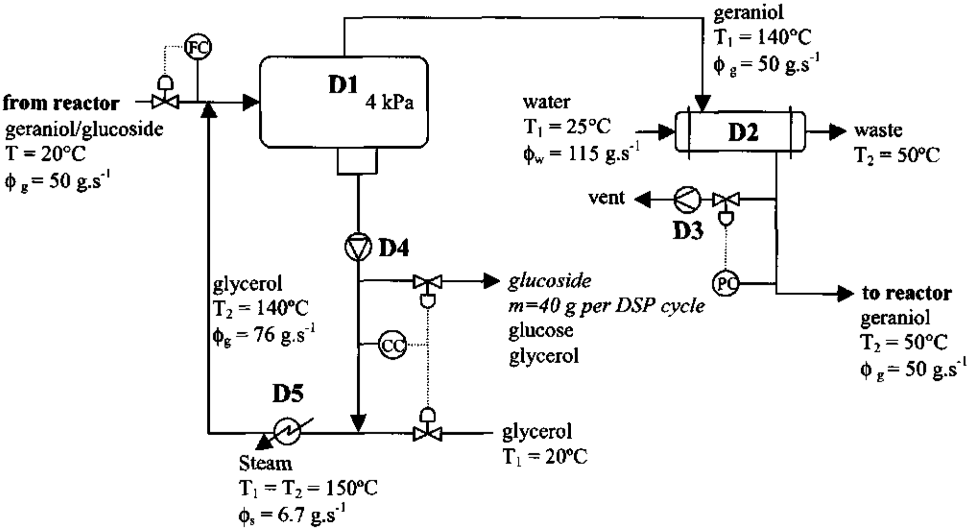

Figure 3: Schematic presentation of the distillation. CC = Concentration control, FC = Flow control, PC = Pressure control. D1-D5: see table 9.

The distillate (geraniol) is fed back to the reactor at the required reaction temperature of 50°C, making pre-heating of the reactor influent unnecessary. The heat exchanger (figure 3, no. D5) is dimensioned for heating glycerol from 20 to 140°C. After heating, the glycerol stream temperature will not drop considerably, and at this point the steam mass flow has to be reduced. The glucoside amount given in figure 3 was estimated with data from chapters 3.2 and 4.2. The maximum molar fraction of glucose in glycerol was 0.02, and this maximum was assumed for glucoside as well. Together with a measured product composition of 93% glucoside and 7% glucose, and a chosen glycerol volume of 0.5 l, each batch will contain 40.2 g of glucoside. Hence, about 25 batches of glycerol are necessary to yield 1 kg of geranyl glucoside. Although not shown in this setup, the degradation products of geraniol have to be separated from these batches. Minimum requirements for the distillation step are given in table 9.

Table 9: Minimum equipment requirements for the distillation step

No	Equipment	Minimum requirements	Additional data
D1	Flash distillation	Glass, $V = 1\ m^3$	-
D2	Condenser	$A^a = 1.55\ m^2$	$U=300$, $F_t=0.5$
D3	Vacuum pump[b]	Power: 175 W	Eff = 80%, $Z=1$, $R= 8.314$, $n=1.23$, $P_1=4.10^3$, $P_2=1.10^5$
D4	Gear pump[c]	Power: 40 W	-
D5	Heat exchanger	$A^b = 1.26\ m^2$	$U=500$

[a] $A = \dfrac{Q}{U\Delta T_m}$

[b] see table 8, footnote b.

[c] see table 7, footnote a.

Based on these calculations it can be concluded that the total reactor system is viable. Equipment requirements are moderate as are the utility requirements. The requirements for the two downstream processes do not differ much, and therefore a selection is mainly dependent on the required product purity.

If a pure glucoside is desired, adsorption is preferred. When distillation is used, the product stream will contain 6.0% (w/w) glucoside, 0.5% glucose and 93.5% glycerol.

CONCLUSIONS

The major objective of this project was to design a production process that combines the optimum reaction conditions for the enzymatic glucosylation with a selective removal of the desired product. The results from chapter 4, together with the viability study from this chapter demonstrate that this objective was completed satisfactorily. Moreover, calculations show that a high space time yield can be achieved without a excessive waste production. Furthermore, the ability of the system to produce different glucosides demonstrates the process flexibility. Therefore, commercial application of the reactor system is feasible even when no 'food-grade' status or 'natural' production is required for an end product. With respect to the enzyme choice, almond β-glucosidase appeared to be the best enzyme compared to literature examples for this specific reaction. Although alternative enzymes and enzyme sources show promising possibilities, their commercial application is hampered because of the limited availability of the enzymes and/or the use of sugar donors that induce a more difficult downstream processing or an inefficient use of the sugar donor.

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SUMMARY

Glucosides are of commercial interest for the industry in general and for the pharmaceutical and food industry in particular. Chemical preparation of glycosides is not applicable in the food industry, and therefore an enzyme-catalyzed reaction would be an alternative. However, until now the low yield in the enzymatic reaction prevents the production of glycosides on a commercial scale. Therefore, high yields should be established by a combination of optimum reaction conditions and a continuous removal of the product. The objectives of the research described in this thesis were to develop a glucosylation reaction mediated by almond β-glucosidase, together with a reactor that integrates the enzymatic production and downstream processing of glucosides.

The enzyme appeared to be specific for aliphatic aglycons. It appeared impossible to glucosylate phenolic aglycons. This different reactivity was investigated, explained and described in chapter 2. The successful enzymatic glucosylation of an aglycon appeared to be mainly dependent on the nucleophilicity of the aglycon. Although their chemical glucosylation was facile, phenolic aglycons were not nucleophilic enough to be glucosylated enzymatically. By using PM3 and AM1 semi-empirical methods, the magnitude of this nucleophilicity was calculated and was found to correlate with the charge on the reacting atom of the aglycon. Based on this trend, the aglycons were divided in reacting and non-reacting, which allowed a prediction of their reactivity in the glucosylation reaction.

Based on this knowledge, the optimum reaction conditions were determined and described in chapter 3.1. The highest yield and enzyme half-life in the glucosylation reaction of cyclohexanol was found at a glucose concentration of 500 g per kg of buffer solution, an organic phase/buffer phase ratio of 9:1 and a temperature of 50°C.

In chapter 3.2, the production of glyceryl glucoside with almond β-glucosidase was described. In this case, downstream processing of the glucoside from a mixture of compounds with highly similar (solubility) properties was complex. Therefore, efforts were made to optimize the composition of the mixture at the equilibrium to facilitate downstream processing. Using the molar fraction based equilibrium constant and the mass balances, the glucoside yield was calculated for all possible combinations of initial substrate and water fractions in the reaction mixture. This was used to optimize the glucoside yield while minimizing one of the substrate concentrations at equilibrium. A

fivefold reduction of the equilibrium molar fraction of glucose was possible with only a twofold lower glucoside yield. Optimization to a minimum equilibrium molar fraction of glycerol was found to be impossible without seriously compromising the glucoside yield.

The development of a bioreactor with an integrated downstream process was described in chapter 4. In chapter 4.1, the glucosylation of hexanol in a two-phase system in a spray column reactor was described. A hexyl glucoside production of 2.5 $g.l^{-1}$, and an initial production rate of 2.24 $mg.U^{-1}.h^{-1}$ was achieved. The two phases were separated with a flat sheet polypropylene membrane, which was pretreated using block copolymers to prevent breakthrough of water. In-line adsorption was used to semi-continuously remove the produced glucoside. From equilibrium adsorption experiments with ten different adsorbents, alumina was chosen for in-line adsorption. Although the maximum glucoside adsorption in the full process appeared to be much lower than in the equilibrium experiments, an average glucoside adsorption of 11.15 $mg.g^{-1}$ was achieved. Alumina was regenerated, yielding a pure glucoside and a reusable column.

The same bioreactor was used for the production of geranyl glucoside, which is described in chapter 4.2. Geranyl glucoside was produced with an initial production rate of 0.58 $mg.U^{-1}.h^{-1}$. Based on examples from the literature, four downstream processes were tested on their viability for this system. Both extraction with water and foaming were not suitable to recover geranyl glucoside from geraniol. Adsorption on alumina and destillation under reduced pressure were successfully applied and tested in-line with the bioreactor. A maximum glucoside adsorption of 7.86 $mg.g^{-1}$ was achieved on alumina. After desorption, the pure glucoside was obtained quantitatively. A pure product could not be obtained after destillation due to the fact that a small amount of glucose was present in the permeate as well.

Finally, in chapter 5, the results from the previous chapters were evaluated and placed into perspective. Furthermore, additional results that were not discussed in the previous chapters were presented. The results from glucoside stability experiments were presented and the implications of these results for an application were discussed. It appeared that glucosides are very stable under extreme conditions with respect to a food application. Therefore, formulating the glucoside together with a glucoside hydrolase might be necessary. Furthermore, alternative enzymes for the glycosylation reaction were reviewed. α-Amylase and β-galactosidase appeared to be possible attractive alternatives, although in the first case a mixture of glucosides is produced, while in the second case the sugar donor is not used very efficiently. Preliminary results with β-glucosidase from *Pyrococcus furiosus* were shown as an example of a potentially interesting alternative glucosidase source. The results were promising, but unfortunately the enzyme is not commercially available yet. In addition, attempts to downstream process hexyl glucoside and glyceryl glucoside that were not shown in the previous chapters were discussed in this chapter. Finally, the industrial viability of the bioreactor system and two successfully applied downstream processes were presented by calculations of the minimum equipment requirements. It was shown that a high space time yield can be achieved with a minimum of requirements and without a high waste production.

SAMENVATTING

Glucosiden zijn commercieel interessant voor de industrie in het algemeen en de farmaceutische- en voedingsmiddelenindustrie in het bijzonder. De chemische bereiding van glucosiden is in dit laatste geval niet toepasbaar en dan vormt een enzymatische methode een alternatief. Echter, als gevolg van de lage productopbrengst bij de enzymatische methode is een commerciële toepassing tot op heden nog niet mogelijk. Een hoge opbrengst zou verwezenlijkt kunnen worden door een combinatie van de optimale reactie-omstandigheden en een continue verwijdering van het product. In het onderzoek dat in dit proefschrift beschreven is, zijn de optimale reactie-omstandigheden voor de glucosideringsreactie met β-glucosidase onderzocht en is er een bioreactor ontwikkeld die de enzymatische productie en opwerking van glucosiden integreert.

Het enzym bleek specifiek alifatische aglyconen te glucosideren. Het bleek onmogelijk om fenolen te glucosideren. Dit verschil in reactiviteit is onderzocht en verklaard in hoofdstuk 2. Het succes van glucosidering bleek voornamelijk af te hangen van de nucleofiliciteit van het aglycon. Ondanks dat de chemische glucosidering vrij eenvoudig is, kunnen fenolische aglyconen niet enzymatisch geglucosideerd worden vanwege hun te lage nucleofiliciteit. Met behulp van PM3- en AM1- semi-empirische rekenmethoden kon de grootte van de nucleofiliciteit bepaald worden. Deze bleek te correleren met de lading op het reactieve zuurstofatoom van het aglycon. Hierdoor konden de aglyconen ingedeeld worden in reagerende en niet reagerende aglyconen en kon de reactiviteit van β-glucosidase ten opzichte van aglyconen voorspeld worden.

Met behulp van deze kennis werden de optimale reactie-omstandigheden onderzocht, vastgesteld en beschreven in hoofdstuk 3.1. De hoogste enzym halfwaardetijd en productopbrengst tijdens de glucosidering van cyclohexanol werd gevonden met een glucose concentratie van 500 g per kg buffer oplossing, een organische fase/buffer fase verhouding van 9:1 en bij een temperatuur van 50°C.

Daarnaast is in hoofdstuk 3.2 de productie van glycerylglucoside met β-glucosidase beschreven. Hierbij ontstaat een mengsel van stoffen met sterk op elkaar lijkende eigenschappen, zodat de opwerking van het glucoside lastig is. Daarom is er gepoogd om het evenwichtsmengsel zo te optimaliseren dat de opwerking eenvoudiger werd. Met behulp van de, op molaire fracties gebaseerde, evenwichtsconstante en massabalansen, kon de glucoside opbrengst berekend worden voor iedere fysisch mogelijke combinatie van initiële substraat- en waterfracties in het mengsel. Dit gegeven is gebruikt om de glucoside opbrengst tijdens evenwicht te verhogen en tegelijkertijd de substraatconcentraties te minimaliseren. Het bleek mogelijk om de molfractie glucose bij evenwicht met een factor vijf te verminderen met slechts een twee keer zo lage glucoside opbrengst. Minimalisatie van de molfractie glycerol bleek gepaard te gaan met een drastische verlaging in de glucoside opbrengst.

De ontwikkeling van de bioreactor met een geïntegreerde opwerkingsstap is beschreven in hoofdstuk 4. In hoofdstuk 4.1. is hexanol in een twee-fasen systeem geglucosideerd in een sproeikolom met een productie van 2.5 $g.l^{-1}$ en een initiële productiesnelheid van 2.24 $mg.U^{-1}.h^{-1}$. De twee fasen werden gescheiden met behulp van een hydrofoob polypropyleen membraan dat was voorbehandeld met block copolymeren om doorbraak van water te voorkomen. Adsorptie werd in-line toegepast als opwerkingsstap. Uit evenwichtsadsorptie experimenten met tien verschillende adsorbentia is aluminiumoxide als beste naar voren gekomen. Alhoewel de adsorptie in het proces veel lager was dan in de evenwichtsexperimenten was het mogelijk om 11.15 $mg.g^{-1}$ glucoside te adsorberen op aluminiumoxide. Na elutie met ethanol kon het glucoside puur verkregen worden. De kolom kon hierna geregenereerd worden door elutie met water en een droogstap.

Dezelfde bioreactor werd gebruikt in hoofdstuk 4.2 voor de productie van geranyl glucoside. Deze stof kon geproduceerd worden met een initiële productiesnelheid van 0.58 $mg.U^{-1}.h^{-1}$. Op basis van literatuurvoorbeelden werden vier opwerkingsstappen getest op hun bruikbaarheid voor dit systeem. Extractie met water en schuimvorming bleken beide niet geschikt te zijn als opwerkingsstap. Adsorptie op aluminiumoxide en vacuümdestillatie daarentegen werden wel succesvol toegepast in het reactorsysteem. De maximum adsorptie op aluminiumoxide was 7.86 $mg.g^{-1}$. Na desorptie kon het glucoside kwantitatief verkregen worden. Het bleek niet mogelijk om een zuiver product te krijgen met vacuümdestillatie als gevolg van de aanwezigheid van een kleine hoeveelheid glucose in de permeaatstroom.

Tenslotte worden in hoofdstuk 5 de resultaten van de vorige hoofdstukken geëvalueerd. Bovendien worden nieuwe resultaten besproken die niet aan bod zijn gekomen in de vorige hoofdstukken. De stabiliteit van glucosiden in het algemeen wordt besproken. Gebleken is dat de glucosiden zeer stabiel zijn onder (voor voedingsmiddelen) extreme omstandigheden, waardoor het nodig kan zijn om glucosidasen toe te voegen in de formulering van het uiteindelijke product. Verder wordt een overzicht gegeven van alternatieve enzymen voor de glycosideringsreactie. Zowel α-amylase en β-galactosidase kwamen naar voren als potentiële alternatieven. Echter, in het eerste geval onstaat er een mengsel van verschillende glucosiden, terwijl in het tweede geval de suikerdonor niet erg efficiënt gebruikt wordt. Tevens worden voorlopige resultaten besproken van de glucosidering met een hyperthermofiel β-glucosidase uit *Pyrococcus furiosus*. De resultaten zijn veelbelovend, maar helaas is dit enzym nog niet commercieel verkrijgbaar. Daarnaast worden de pogingen om hexyl- en glycerylglucoside op te werken, die niet in de voorgaande hoofdstukken zijn behandeld, in dit hoofdstuk besproken. Tenslotte worden de minimale benodigdheden berekend voor het in dit proefschrift beschreven proces op industriële schaal. Hieruit bleek dat de reactor zeer goed toegepast kan worden om op grote schaal glucosiden te produceren met een minimale afvalproductie en minimale benodigdheden.

SAMENVATTING IN NORMAAL NEDERLANDS

Veel geur- en smaakstoffen zijn vluchtig en verdampen daarom snel. Op zich is dat prettig, want daardoor zijn ze gemakkelijk te ruiken of te proeven. Als je die geur- en smaakstoffen wilt gebruiken in diepvriesproducten die bijvoorbeeld thuis afgebakken moeten worden, dan kan dit verdampen echter voor problemen zorgen. Als fabrikant van die producten moet je namelijk heel goed weten hoe snel een bepaalde stof verdampt (en dus verdwijnt uit je product) om ervoor te zorgen dat de klant nog steeds een smaakvol product krijgt. Een methode is bijvoorbeeld bij het maken van de producten iets teveel van de geur- en smaakstof toe te voegen om zo voor de verdamping te corrigeren. Dat is duur (je moet meer toevoegen dan je eigenlijk wilt) en het kan ook wel eens mis gaan (het product wordt sneller gekocht en gegeten dan je had verwacht). Het zou dus handig zijn als je op de een of andere manier die geur- en smaakstof zou kunnen vastzetten tot het moment dat het product gebakken wordt en dan pas verdampt. Op die manier kun je dus precies de juiste hoeveelheid geur- en smaakstof toevoegen. Goedkoper voor jou als producent en je weet zeker dat de smaak van je product precies is zoals je het bedoeld had.

Scheikundig gezien is dat vastzetten heel goed mogelijk. Het is bijvoorbeeld mogelijk om de geur/smaakstof te koppelen aan suiker (deze aan suiker gekoppelde stoffen worden 'glycosiden' genoemd). Onder de juiste omstandigheden (een beetje vocht, hoge temperatuur) kun je die koppeling weer breken en de geur/smaakstof komt vrij. Het suiker blijft achter in je product in een zo lage hoeveelheid dat je dat niet meer proeft. Het probleem is alleen dat voor de scheikundige bereidingswijze van die glycosiden gebruik gemaakt wordt van allerlei giftige stoffen. Die wil je niet terugvinden in je eindproduct en wettelijk gezien is het dan ook verboden om stoffen in voedingsmiddelen te gebruiken die volgens dit soort giftige methoden zijn gemaakt. Om nu toch glycosiden te kunnen maken heb ik een enzym gebruikt. Het enzym dat ik gebruikt heb heet Bèta-glucosidase en komt uit amandelen. Het voordeel van dit enzym is dat het dezelfde koppeling kan maken met alleen suiker, de geur/smaakstof en water om z'n werk te doen. Allemaal niet-giftige grondstoffen en dus wel geschikt om een glycoside te maken dat gebruikt kan worden in voedingsmiddelen.

Er was echter nog wel een probleem. In de natuur verbreekt het enzym juist de koppeling die ik wil maken. Ik heb de omstandigheden zo moeten aanpassen (heel veel suiker en geurstof gebruiken, een heel klein beetje water en een temperatuur van

50°C) om het enzym precies het omgekeerde te laten doen dan waar hij eigenlijk voor gemaakt is. Het vinden van die juiste omstandigheden en laten zien wat het enzym allemaal wel en niet kon heb ik beschreven in de hoofdstukken 2 en 3.

En nog ging het niet helemaal zoals ik graag zou willen. Zelfs onder de beste omstandigheden maakte het enzym eigenlijk maar een heel klein beetje van die glycosiden. Ik gebruikte een bepaalde hoeveelheid water, suiker en geurstof en op een gegeven moment kon het enzym niet meer glycoside maken dan er op dat moment gemaakt was. Op dat moment was nog niet alle suiker en geurstof verbruikt, maar het enzym kon alleen maar verder met glycoside maken als ik de hoeveelheid glycoside zou verlagen. Ik zou het potje waar alles in zit leeg moeten maken, het gevormde glycoside er uit moeten halen en alles weer terug doen in het potje zodat het enzym weer aan het werk kon. Dat is op zich niet zo'n efficiënte manier en daarom heb ik gebruik gemaakt van wat techniek. Om het geheel efficiënter te maken heb ik er een continu systeem van gemaakt, d.w.z. ik heb het enzym met veel suiker in wat water gedaan en daar heb ik de geurstof continu doorheen laten borrelen. Op zich was er nog niet veel veranderd aan de beginsituatie, want ook nu maakte het enzym maar een bepaalde hoeveelheid glycoside. Maar omdat ik mijn geurstof nu rondpompte kon ik het naar een ander vat pompen waar ik het glycoside er continu uit haalde. Dat kon bijvoorbeeld door de geurstof met glycoside langs een fijn soort zand te laten stromen die specifiek het glycoside uit mijn geurstof haalde. De 'schone' geurstof kon ik weer terugpompen naar het enzym en het glycoside kon ik met alcohol van het zand afspoelen. Dit continu proces heb ik beschreven in hoofdstuk 4.1. Het einddoel was echter nog niet in zicht. De experimenten in hoofdstuk 4.1 heb ik namelijk uitgevoerd met een modelstof. De geurstof zelf is vrij duur en dus heb ik eerst gekeken of het principe wel werkte met een stof die wel op de geurstof leek, maar veel goedkoper was. Daarom heb ik het allemaal nog eens herhaald, maar nu met de echte geurstof. Daarbij heb ik ook nog een paar andere mogelijkheden bekeken om het glycoside uit de geurstof te halen. De enige methode die, behalve het langs fijn zand stromen, ook succesvol bleek was het koken van het geurstof/glycoside mengsel. Hierbij verdampte de geurstof (die ik daarna wel weer opving en terugpompte naar het enzym) en bleef het glycoside over. Dat heb ik beschreven in hoofdstuk 4.2.

Tenslotte laat ik in hoofdstuk 5 zien dat er nog een groot aantal andere enzymen zijn die deze reactie ook kunnen uitvoeren. Daarbij laat ik zien dat de reactor met de zuiveringsstap, zoals ik die in de hoofstukken 4.1 en 4.2 beschreven heb, heel goed industrieel toegepast zou kunnen worden.

LIST OF SYMBOLS

Chapter 2

B = quantummechanical resonance energy	$kcal.mol^{-1}$
c_{elec} = orbital coefficient of the reacting atom in the LUMO	-
c_{nuc} = orbital coefficient of the reacting atom in the HOMO	-
E_{HOMO} = orbital energy of the Highest Occupied Molecular Orbital	eV
E_{LUMO} = orbital energy of the Lowest Unoccupied Molecular Orbital	eV
ΔE_{def} = total deformation energy	$kcal.mol^{-1}$
$\Delta E^{\ddagger}$ = activation energy	$kcal.mol^{-1}$
Q_{elec} = charge on reacting atom of the electrophile	-
Q_{nuc} = charge on reacting atom of the nucelophile	-

Chapter 3.2

K_{eq} = overall equilibrium constant	-
K'_{eq} = apparent equilibrium constant	-
γ_{gg} = activity coefficient of glyceryl glucoside	-
γ_{glu} = activity coefficient of glucose	-
γ_{gly} = activity coefficient of glycerol	-
γ_{w} = activity coefficient of water	-
X_{gg} = molar fraction of glyceryl glucoside at equilibrium	-
X_{glu} = molar fraction of glucose at equilibrium	-
$X_{glucose,ini}$ = initial molar fraction of glucose	-
X_{gly} = molar fraction of glycerol at equilibrium	-
$X_{glycerol,ini}$ = initial molar fraction of glycerol	-
X_{w} = molar fraction of water at equilibrium	-
$X_{water,ini}$ = initial molar fraction of water	-

Chapter 4.1

c_e = equilibrium concentration	$g.l^{-1}$
c_i = initial concentration	$g.l^{-1}$
k = adsorption constant	-
m = mass	g

n = number of datapoints	-
ν_{ext} = degrees of freedom of the extended model	-
ν_{low} = degrees of freedom of the lower model	-
p = number of parameters	-
q = adsorption	$mg.g^{-1}$
q_{max} = maximum adsorption	$mg.g^{-1}$
RSS_{ext} = residual sum of squares of the extended model	-
RSS_{low} = residual sum of squares of the lower model	-
s_{qmax} = standard error of q_{max}	-
s_k = standard error of k	-

Chapter 5

A = area	m^2
Cp = heat capacity	$J.g^{-1}.^{o}C^{-1}$
d = diameter	m
$d_{pipe,opt}$ = optimum pipe diameter	mm
Eff = pump efficiency	%
F_t = friction factor	-
ϕ_a = air mass flow	$g.s^{-1}$
ϕ_e = ethanol mass flow	$g.s^{-1}$
ϕ_g = geraniol mass flow	$g.s^{-1}$
ϕ_s = steam mass flow	$g.s^{-1}$
ϕ_w = water mass flow	$g.s^{-1}$
G = volumetric flow	$m^3.s^{-1}$
h = height	m
H_{evap} = evaporation enthalpy	$J.g^{-1}$
η = viscosity	Pa.s
m = mass	g
M = molecular mass	$g.mol^{-1}$
P_1 = initial pressure	Pa
P_2 = final pressure	Pa
ΔP = pressure difference	Pa

ΔP_{pipe} = pressure drop induced by pipe	Pa
$\Delta P_{packing}$ = pressure drop induced by packing	Pa
R = universal gas constant	$J.K^{-1}.mol^{-1}$
ρ = density	$kg.m^{-3}$
t_1 = inlet tube-side temperature	°C
t_2 = outlet tube-side temperature	°C
T = temperature	°C
T_1 = inlet shell-side temperature	°C
T_2 = outlet shell-side temperature	°C
ΔT_m = mean temperature difference	°C
U = overall heat transfer coefficient	$W.m^{-2}.°C^{-1}$
W = work	$J.s^{-1}$
Z = compressibility factor	-

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SOURCE OF IMAGES

All images on the chapter title pages are from the *Splendor Solis* and were copied from the Alchemy Website (http://www.levity.com/alchemy/home.html). The *Splendor Solis* is one of the most beautiful of illuminated alchemical manuscripts. The earliest version, considered to be that now in the Kupferstichkabinett in the Prussian State Museum in Berlin, is dated 1532-35, and was made in the form of a medieval manuscript and illuminated on vellum, with decorative borders like a book of hours, beautifully painted and heightened with gold. The work itself consists of a sequence of 22 elaborate images, set in ornamental borders and niches. The symbolic process shows the classical alchemical death and rebirth of the king. Although the style of the *Splendor Solis* illuminations suggest an earlier date, they are quite clearly of the 16th century. The *Splendor Solis* was associated with the legendary Salomon Trismosin, allegedly the teacher of Paracelsus.

De figuren op de titelpagina's van de hoofdstukken zijn uit de *Splendor Solis* en zijn gekopieerd van de 'Alchemy Website' (http://www.levity.com/alchemy/home.html). De *Splendor Solis* is één van de mooiste, bekende alchemistische manuscripten. De vroegste versie, waarschijnlijk het exemplaar in het Kupferstichkabinett in het Pruisisch Staats Museum in Berlijn, is uit 1532-35. Het op perkament geschreven boek is gemaakt in de vorm van een middeleeuws manuscript met decoratieve kaders als in een getijdenboek, met prachtig geschilderde voorstellingen, verluchtigd met goud.

De inhoud van het boek bestaat uit een reeks van 22 gedetailleerde afbeeldingen in rijkelijk versierde kaders. De afbeeldingen zijn een weergave van de klassieke alchemistische dood en wedergeboorte van de koning. Alhoewel de stijl van de afbeeldingen van de *Splendor Solis* een vroegere datum doet vermoeden, zijn ze duidelijk uit de 16e eeuw. De *Splendor Solis* wordt toegeschreven aan de legendarische Salomon Trismosin, de vermoedelijke leermeester van Paracelsus.

CURRICULUM VITAE

Rotterdam! Geboortestad van Pietje Bell, Kruimeltje, Ketelbinkie en sinds 20 januari 1967 ook van Bartholomeus Mattheus (Matthew) de Roode. Na het achtereenvolgens behalen van het MAVO en HAVO diploma aan de Libanon Scholengemeenschap in Rotterdam, startte hij in 1986 met de HLO-APLI opleiding aan het van 't Hoff-instituut te Rotterdam. Dezelfde opleiding werd aan de Hogeschool Rotterdam e.o. in 1990 in Delft afgesloten met een stage bij TNO-Maatschappelijke Technologie te Delft. In datzelfde jaar startte hij via een doorstroomprogramma de studie Scheikunde aan de Universiteit van Amsterdam. De onderzoeksstage werd verricht bij prof. van Leeuwen van de vakgroep Homogene Katalyse, de scriptie werd geschreven bij prof. Krishna van de vakgroep Chemische Reactorkunde. In 1992 werd de studie met de specialisatie Chemische Technologie afgesloten.

Van 1993 tot 1995 werkte hij als chemisch analist bij Technisch Laboratorium Rotterdam en Ets. Gordinne et Cie., beiden te Rotterdam, om vanaf medio 1995 de functie van Hoofd Laboratorium te vervullen bij Verstraeten Verbrugge Watco te Rotterdam-Botlek.

De drang naar onderzoek begon te knagen, want in januari 1997 startte hij met een promotie-onderzoek aan de vakgroepen Proceskunde en Organische Chemie van Wageningen Universiteit. De inhoud van dit onderzoek staat beschreven in dit proefschrift.

De Muze

Die nacht kwam de muze mij bezoeken.
Ik zag haar staan en sprong op uit mijn bed.
Al rennend wreef ik de slaap uit mijn ogen.
Fluister, fluister, fluister, fluister, fluister.
Ja, even wachten, de computer staat nog niet aan!
De muze boog zich over mij.
Haar licht scheen op mijn toetsenbord.
Het gonsde in mijn hoofd.
De tijd stond stil, mijn vingers niet.
Rikketik, fluister, fluister, rikketik.
Rikketik, BWAAAAAAAMMM!
Ik keek naar links, de muze ook.
Mijn vrouw stond in haar nachtjapon.
Het schuim stond op haar lippen.
"Wat is hier aan de hand?"
"En wie is dat mokkel????"
-"D-d-dat is de muze, schat."
Haar licht scheen nu niet zo fel.
Mijn vrouw haar ogen schoten vuur.
Haar vuisten schoten uit.
De muze vluchtte via het bovenraam.
Haar oog werd al blauw.
Mijn vrouw kwam dreigend op mij af.
Het werd een lange nacht.
Ik heb de muze nooit meer teruggezien.

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