A RATIONAL APPROACH TO BIOTRANSFORMATION PROCESS DESIGN: CHEMO-ENZYMATIC SYNTHESIS OF N-ACETYL-D-NEURAMINIC ACID

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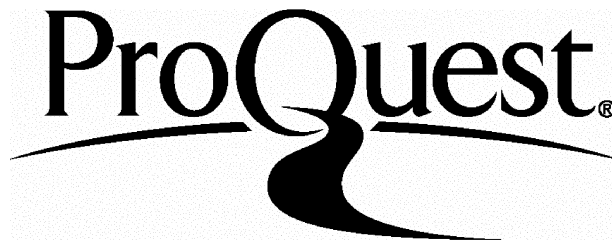University College London

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Calef

        Dilegua o notte!...Tramontate o stelle !...

              All' alba vincero' !...

Giacomo Puccini, Turandot, act III.

ACKNOWLEDGEMENTS

ABSTRACT

This thesis describes a rational approach to process design for biotransformation processes. The approach enabled process flow-sheet decisions to be made based on structured scientific evidence and provided key data affecting design choices. The two step synthesis of N-acetyl-D-neuraminic acid (Neu5Ac) was taken as a model for process design. Neu5Ac was synthesised starting from N-acetyl-D-glucosamine (GlcNAc) and pyruvate (Pyr) in two steps. The first was the base-catalysed epimerisation of GlcNAc to N-acetyl-D-mannosamine (ManNAc). The second step was a biotransformation, carried out by the addition of pyruvate to ManNAc to yield Neu5Ac in equilibrium. The latter reaction was catalysed by the *E. coli* Neu5Ac aldolase.

The first phase to the structured approach to biotransformation process design was based on characterisation. A first group of experiments investigated the components and reaction properties and this set of data indicated advantageous conditions on which to focus subsequent characterisation. The second set of data concerned the interactions between reactants, product and biocatalyst at the conditions selected above. Reaction kinetics at high substrate concentrations has determined that pyruvate was strongly inhibitory on initial rates of reaction and ManNAc was found inhibitory above 750 mM. The experimental data were then used to evaluate and select different process options, based on design rationale and engineering heuristics. As a result of this approach, process operating windows have been generated, defining possible operating regions for subsequent scale-up. Conventional reactor designs and novel integrated layouts such as *in situ* product removal were then experimentally assessed. In addition, the option of integration of the epimerisation with the biotransformation (at alkaline pH) has been evaluated. The reactor options were then compared for product, enzyme and downstream separation limiting process scenarios. The methodology of this rational approach to process design was then discussed for the whole class of aqueous phase equilibrium controlled biotransformations.

This design approach has advanced in two ways as the result of the findings in this thesis. The characterisation step has been divided further in two sets of experiments, so that unfeasible options were quickly ruled out, accelerating the design procedure. In addition, economic considerations in the form of different processing scenarios have been introduced in the identification of design constraints.

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ABBREVIATIONS

ATP	Adenosine triphosphate
BSA	Bovine serum albumin
BSTR	Batch stirred tank reactor
bv	Bed volume
CSTR	Continuous stirred tank reactor
DDW	Double distilled water
DSP	Downstream processing
EtOH	Ethanol
FBSTR	Fed-batch stirred tank reactor
FUA	Frontal uptake analysis
GlcNAc	N-acetyl-D-glucosamine
^{1}H NMR	Proton nuclear magnetic resonance
HPLC	High performance liquid chromatography
ISPR	*In-situ* product removal
KDa	Kilodalton
K_{eq}	Equilibrium constant
LDH	Lactate dehydrogenase
ManNAc	N-acetyl-D-mannosamine
meq	Milliequivalent
NADH	αNicotinamide dinucleotide, reduced form
Neu5Ac	N-acetyl-D-neuraminic acid
OD	Optical Density
PEP	Phosphoenolpyruvate
PFR	Plug flow reactor
Pyr	Pyruvate
rpm	Revolutions per minute
STR	Stirred tank reactor
Tris	Tris[hydroxymethyl]aminoethane
USP	Upstream processing
UV	Ultra-violet

NOMENCLATURE

ΔA	-	Decrease in adsorbance during reaction
$[A]_O$	mol / L	Pyruvate concentration at time = 0
$[B]_O$	mol / L	ManNAc concentration at time = 0
Amaxh	μmol / min / mg	Maximal specific activity, synthesis
Amaxr	μmol / min / mg	Maximal specific activity, cleavage
E	Kg	Total enzyme in reactor
[E]	g / L	Neu5Ac aldolase concentration
K	s^{-1}	Rate constant
k_a	h^{-1}	Kinetic constant of GlcNAc epimerisation
ka_{app}	h^{-1}	Approximate k_a
k_b	h^{-1}	Kinetic constant of ManNAc epimerisation
KiA	mol / L	Inhibition constant for Pyruvate
KiB	mol / L	Inhibition constant for ManNAc
Km	mol / L	Michaelis Menten constant
KmA	mol / L	Michaelis-Menten constant, Pyruvate
KmB	mol / L	Michaelis-Menten constant, ManNAc
KmP	mol / L	Michaelis-Menten constant, Neu5Ac
L_c	cm	Cuvette length
[ManNAc], [B]	mol / L	ManNAc concentration
[Neu5Ac], [P]	mol / L	Neu5Ac concentration
[Pyr], [A]	mol / L	Pyruvate concentration
Q	L / s	Volumetric reactants flow rate
$R_{P/X}$	-	Ratio of product per unit biocatalyst
S_0	mol / L	Initial substrate concentration
$S_a(t)$	g / g / d	Specific activity as a function of time
T	s	Batch reaction time
t_c	d	Operational life of the catalyst
t_i	min	Incubation time

V	L	Reaction volume
V_C	mL	Column volume
V_F	mL	Frontal volume
V_{sampl}	mL	Sample volume
V_{tot}	mL	Total cuvette volume
Vmax	$\mu mol / min / mg$	Maximal reaction rate
X	-	Fractional conversion of reactant to product or product / substrate yield
ε	$cm^2 mol^{-1}$	Extinction coefficient for NADH
ρ_c	g / mL	Resin density

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INTRODUCTION

1.1 PREFACE

In this chapter, an overview of biotransformation process design is presented together with a review of the synthesis of N-acetyl-D-neuraminic acid (Neu5Ac). In the first part, enzyme biotransformations are introduced and biotransformation reactor design is reviewed along with an overview of the selection of unit operation sequences in biochemical processes. The concepts of expert system and operating window are introduced and examined as tools for bioprocess design. In the second part, the chemo-enzymatic formation of Neu5Ac is taken as a model in process design for chemical and enzymatic reactions and aqueous equilibrium controlled biotransformations. The properties of reaction components and the production process for the synthesis of Neu5Ac are analysed in order to provide the data framework for the following experimental work (Chapter 2). The aims of this thesis are then presented for both the general approach to biotransformation process design and the chemo-enzymatic synthesis of Neu5Ac.

1.2 PROCESS DESIGN FOR BIOTRANSFORMATIONS

1.2.1 BIOTRANSFORMATIONS

1.2.1.1 GENERAL OVERVIEW

Biotransformation or biocatalysis has been developing as the technology in which a selective enzymatic transformation of a substrate yields a defined product. It is often the case of a single step in a synthesis composed of chemical reactions (Poppe and Novak, 1992). Interest in biotransformations has expanded as biochemical methods have proved to be a complementary technology to other traditional methods of synthetic organic chemistry

(Faber, 1992, Lilly, 1992). In biocatalysis, either cells or cell free enzymes can be employed, in an array of solutions that varies according to the type, the form of the catalyst and the environment of the reaction, as shown in Table 1.1. Various forms of the catalyst can be utilised to achieve the same desired transformation. Extensive research has been conducted in the field of cell and enzyme immobilisation. This is usually justified for increased operational stability (Wang et al., 1979, Faber, 1992), catalyst retention, ease of recovery and re-use (Lilly, 1992), even if diffusion limitations (Wang et al., 1979), reduced activity per unit volume (Pollack et al., 1980) and alterations on pH and kinetic behaviour (Katchalski et al., 1976) could take place. The review of rationale, techniques (Faber, 1992) and reaction engineering parameters (Bucholz, 1982) in catalyst immobilisation is, however, beyond the scope of this work. Enzymatic multistep synthesis, energy or cofactor regeneration problems and unstable enzymes may dictate the choice of a whole cell for the biotransformation (Dervakos et al. 1989). However, enzymes can be preferred to cells in case of a single reaction step and when they are not membrane associated, can be produced in large amounts and the activity is sufficiently stable (Lilly, 1992).

1.2.1.2 ENZYME CATALYSED BIOTRANSFORMATIONS

Enzymes are the most widespread form of biochemical catalysts (Stryer, 1995). Conditions of enzyme reactions are generally narrow: neutral pH range and room temperature (Faber, 1992). Enzymes are highly selective and have high reaction specificity (Wong and Whitesides, 1994). These characteristics are particularly employed for chiral resolution of molecules in asymmetric synthesis with high regio-selectivity (Poppe and Novak, 1992, Stinson, 1995). Reaction conditions can influence the enzyme catalysed process. Enzyme activity is dependent on factors such as compound concentration, pH, temperature, and ionic strength (Faber, 1992; Poppe and Novak, 1992; Wong and Whitesides, 1994). (In addition, enzymes are fully bio-degradable and therefore represent little environmental hazard, Faber, 1992). These features make enzymes useful catalysts in steps that are difficult to achieve by chemical methods (Lilly, 1992; Katchalski-Katzir, 1993). Process disadvantages are also observed using enzyme catalysed reactions. Aqueous media are usually the preferred environment of enzymes. However, a number of enzymes can operate in non-aqueous media (Halling, 1996). Difficulties arise with the use of insoluble or labile compounds in aqueous conditions. Solutions to this problem include the use of organic solvents, reverse micelles and catalyst immobilisation (Woodley and Lilly, 1994). Catalyst immobilisation and reuse

Table 1.1: **Types of biocatalysts** (after Poppe and Novak, 1992).

Biocatalyst	Form	Environment
Whole Cell		
growing cultures	free cells	aqueous phase
resting cells	microcapsules	aqueous phase-organic cosolvent
treated or modified cells	microemulsions	two phase system
	immobilised cells	cell preparations in organic phase
Cell Free Preparation		
cell free extracts	free form	aqueous phase
purified enzymes	microcapsules	aqueous phase-organic cosolvent
treated or modified enzymes	microemulsions	two phases system
multienzyme systems	immobilised form	water restricted organic solvent

is common on industrial scale (Katchalski-Katzir, 1993; Yamada, 1996). The stability of some enzymes may be low. Several techniques are already available to stabilise the catalyst: these include immobilisation, removal or deactivation of proteases and chemical or genetic modification (Faber, 1992; Poppe and Novak, 1992; Wong and Whitesides, 1994). Hydrolytic enzymes account for half of all biotransformations as high availability, stability and absence of cofactors make them easy to use (Faber, 1992). Lyases, which perform a subtraction or addition to a carbon double bond, transferases and isomerases represent only a small proportion of the biocatalysts for industrial biotransformations (Margolin, 1993; van der Werf *et al.*, 1994). Aldolases and transketolases from the lyase class have been employed in the synthesis of carbohydrate compounds (Faber and Frassen, 1993). The characteristics of Neu5Ac aldolase (E.C. 4.1.3.3) that catalyses the synthesis of N-acetyl-D-neuraminic acid from N-acetyl-D-mannosamine (ManNAc) and pyruvate (Pyr) are reviewed in section 1.3.3.

1.2.2 BIOREACTORS

1.2.2.1 GENERAL OVERVIEW

The choice of a suitable reactor is essential for process design. Wang *et al.* (1979), van't Riet and Tramper (1991) and Lilly (1992) reviewed the fundamental issues involved in design and operation of biochemical reactors. The difference between reactors arises from the mode of operation (batch, fed batch, continuous) and the reactors fluid flow pattern (well mixed, plug flow and reactors with approximation to plug flow, such as tubular, spiral, porous sheet and multistage continuous systems). In production scale, loss of productivity can be due to loss of enzyme from the reactor, poor mass transfer, loss of enzyme activity and / or loss of product (Wang *et al.*, 1979). In this section, a brief review of reactor configurations, kinetics and design strategy for biotransformations is presented.

1.2.2.2 BIOREACTOR CONFIGURATIONS

The stirred tank reactor (STR) is usually a cylindrical vessel with the height equal or greater than two diameters. It is equipped with one or more stirrers for agitation and aeration, baffles and air sparger (van't Riet and Tramper, 1991). Mixing produces homogeneity and mass transfer is achieved as a function of stirring power; hence viscous fluids can be processed. This bioreactor is widely used in biotransformations from laboratory up to full production scale (van't Riet and Tramper, 1991). The packed bed is a reactor consisting of a

tubular pipe packed with the biocatalyst particles. It can be operated up flow or down flow in continuous operations. The kinetic behaviour of the ideal plug flow reactor (PFR) assumes no radial flow and is equivalent to a series of continuous STR (van't Riet and Tramper, 1991). In the enzyme membrane reactor the catalyst is in soluble form and is retained in the reaction vessel by a suitable membrane, usually ultra-filtration (Kula and Wandrey, 1987; Kragl et al., 1993 b). Two types of membrane reactors are usually employed: a flat membrane reactor (usually on small scale) and, in production, reactors with ultra-filtration membranes in hollow fibre module forms. This kind of reactor can be used either in a continuous or in a batch type operation.

1.2.2.3 BIOREACTOR KINETICS

A mass balance analysis can be derived for an ideal continuous stirred tank reactor (CSTR), a batch stirred tank reactor (BSTR) and a plug flow reactor (PFR) and applied to the whole range of reactors behaving under the same kinetics. Reactor equations can be included in the irreversible Michaelis-Menten kinetics of an enzyme reaction to give Equations 1.1-1.3 (Wang et al., 1979, Lilly, 1992).

For a BSTR:

$$X \, S_0 + Km * \ln (1 \, / \, 1\text{-}X) = KET \, / \, V \qquad (1.1)$$

For a PFR:

$$X \, S_0 + Km * \ln (1 \, / \, 1\text{-}X) = KE \, / \, Q \qquad (1.2)$$

For a CSTR:

$$X \, S_0 + Km * (X \, / \, 1\text{-}X) = KE \, / \, Q \qquad (1.3)$$

Where:

X		Fractional conversion of reactant to product.
S_0	mol / L	Initial substrate concentration.
Km	mol / L	Michaelis Menten constant.
K	s^{-1}	Rate constant.
E	Kg	Total enzyme in reactor.
T	s	Batch reaction time.
V	L	Reaction volume.
Q	L / s	Volumetric reactants flow rate.

At the beginning of the reaction, if S_0 is larger than Km, the reaction is zero order and $X \, S_0$ becomes the dominant term of the equations. When Km is larger than S_0, the reaction

becomes first order and the second term in the kinetic equation become dominant. Under these conditions, the amount of enzyme required in the CSTR to carry out a 99% conversion is about twenty times as much as that required in a PFR to perform the same conversion. In case of inhibition on the enzyme, the reactor kinetics include the relative inhibition mechanisms and constants (Wang *et al.*, 1979).

1.2.2.4 BIOREACTOR SELECTION, DESIGN AND OPERATION

The rational selection of reactor design and operation is based on the characterisation of the elements of the process and the identification of their constraints. This approach has been successfully employed in the field of animal cell reactors (Bliem *et al.* 1991).

Lilly (1992) and Woodley and Lilly (1994) reviewed the criteria for selection of biocatalyst and reactor choices. The basis of reaction characteristics, the properties of reactants and products, the biocatalyst, the reactor features and their reciprocal interactions has been analysed against the three major types of reactor kinetics (PFR, BSTR, CSTR, Table 1.2).

BSTR and PFR are more beneficial when the enzyme has product inhibition (exposing the catalyst to high product concentration only at the end of the reaction) (Wang *et al*, 1979), where CSTR is more indicated in case of substrate inhibition, since the concentration of substrate in the reactor is equal to that of the outlet stream. These limitations have provided the basis for decision in process synthesis. Woodley and Lilly (1994) have brought forward the concept of logical steps in bioreactor design defining a hierarchy between the characteristics of the system followed by the definition of constraints (on reaction medium, on kinetics and on catalyst performance) that would then be followed by the matching with the mode of operation and the reactor liquid flow pattern. This procedure would enable the rational selection of reactor options. As a result of this approach, the concept of operating window is introduced. The boundaries of the key factors for the whole biotransformation are defined and related to each other in a form of diagram (Woodley and Lilly, 1994, Woodley and Titchener-Hooker, 1996). Windows of operation are a graphical representation of the system and engineering constraints which may define the feasible region of a specific step, part of a process or a complete process (Woodley and Titchener-Hooker, 1996). Their use can be found in early stages to assess initial feasibility. In late design stages, they can be used for process control and sensitivity evaluation of process parameters. Economic

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Table 1.2   **General suitability of reactor types** (after Wang *et al.*, 1979, Lilly, 1992, Woodley and Lilly, 1994).

Characteristics		BSTR	PFR	CSTR
Operational:	good mixing	+	-	+
	feeding	+	-	+
	continuous	-	+	+
Reaction:	pH control	+	-	+
	multi-phase medium	+	-	+
Biocatalyst:	high concentration	-	+	-
Interactions:	substrate inhibition	-	-	+
	product inhibition	+	+	-

+ : suitable , -: not suitable

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optimisation (Ingelby *et al.*, 1986) can be achieved through the selection of an appropriate operating point. These limits might shift in increasing scale, but the biotransformation at process proportion should be maintained within its optimal performance region.

Catalyst preparation is fundamental at the very early stages of bioprocesses. Cloning, over expression, production of the enzyme and possible immobilisation are all steps required even before approaching the reaction conditions at large scale and downstream processing (Mahmoudian *et al.*, 1993).

It is possible to divide bioprocesses into conversion limiting and recovery limiting systems (Cooney, 1983). Minimisation of process costs (Cooney, 1983; Ilanes *et al.*, 1992, Malcata, 1995) is perceived to be the objective of reactor design within the physical and biological rate limitations. It is required to operate at the border of the limits imposed by the system, maximising the desired variables and making the process more economically feasible. The physical and biological constraints incurred in reactor design are usually mass balance, enzyme kinetics, biocatalyst stability and mass transfer limitations (Ilanes *et al.*, 1992, Bódalo *et al.*, 1995, Malcata, 1995). These constraints are expressed in the form of rate equations that are iteratively solved for all the variables. The following parameters have economic consequences in design:

- Maximal volumetric productivity (or space-time yield) would be the target for conversion limiting bioprocesses. Since reactor size is proportional to this value, the importance of productivity is reflected in minimisation of capital investment (Cooney, 1983; Malcata, 1995). This can be improved by increasing specific activity of the biocatalyst and / or high catalyst load.

- Yield is important to minimise raw materials costs. This parameter is dependent on the effective operational stability of the biocatalyst. $R_{P/X}$ (Cooney, 1983), the ratio of product per unit biocatalyst should be then maximised (Equation 1.4):

$$R_{P/X} = X \int_0^{t_c} S_a(t)dt \qquad (1.4)$$

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where:

$R_{P/X}$	-	Ratio of product per unit biocatalyst.
X	-	Fractional conversion of reactant to product or product / substrate yield.
$S_a(t)$	g / g / d	Specific activity as a function of time.
t_c	d	Operational life of the catalyst.

- Product concentration leaving the reactor is an important parameter for downstream separation constraints (Malcatta, 1995), since the separation costs are proportional to the amount of processed water (Cooney, 1983).

These parameters are often interrelated and none of them gives a complete picture of the most appropriate bioreactor design choice and operation. The decision of the most important criterion goes back to economic analysis (Cooney and Raju, 1996) and the characteristics of the system. Usually the process objectives (for instance quantity, purity, yield or enantiomeric excess) are given in the design specifications and to date, biotransformation process design has been reported mainly for cases of enzyme limited processes (Lilly and Woodley, 1996). However, a hierarchical difference between various process limitations is still lacking. Beyond economic constraints, there may be catalyst availability constraints, technology and time limitations that are still not fully assessed.

1.2.3 FROM UNIT OPERATIONS TO BIOPROCESSES

1.2.3.1 RATIONALE

The design of a whole process is the rational choice of production methods and downstream processing in order to obtain the required productivity targets. In industrial biotransformation processes, process design has been accomplished mainly through a case-by-case basis, lacking a systematic approach to the process as a whole. The study of bioprocesses is therefore related to the design theory and philosophies that are required for the implementation of a full-scale biotransformation. Biocatalytic methods hold great industrial potential. Policies on drug design require the development of chiral resolution as an industrial process (Hodgson, 1992; Faber and Frassen, 1993; Margolin, 1993). The integration of chemical and biochemical steps has then become an important achievement in

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pure optical isomer production (Margolin, 1993). In this section, an overview of design methods and strategies relevant to biotransformation processes is presented.

1.2.3.2 DESIGN METHODS AND STRATEGIES

Scale-up has been historically perceived as the increase in size of batches (Valentas *et al.*, 1991), lacking the study for large scale designs. Conceptual planning starts with clear specifications of purity, cost and time. The required scale may already put some constraints on the equipment type, ruling out other possible options. Background data on physico-chemical properties are essential (Wheelright, 1989). Several design methods are available for bioprocesses, the simplest of which is just to adapt a known process to a different product of the same class. Very little development is required in this case.

Heuristics (rules of thumb) are guidelines for approximate design and are very useful in provisional and feasibility studies, to narrow the possible options for process choices. Different kinds of heuristics can be found: method heuristics for specifying the choice of unit operations, design heuristics to specify the order of operations and species heuristics which are based on the properties of reaction species (Nadgir *et al.*, 1983). By way of illustration, it is found in a rational approach to protein separation processes (Wheelright, 1989) that design heuristics for downstream processing (DSP) are all based on volumes and cost of operations. Low resolution is linked with lower operating costs, larger volumes can be processed and therefore these methods are found at early stages of DSP. High resolution steps are expensive, need less volume to process and are found at the end of the process.

Rudd *et al.* (1973) described a first attempt in a systematic step approach in the chemical process synthesis. At the very early stage of the process selection, the reaction to be performed and its possible paths should be already known. It is required, at this stage, to produce data applicable to process conditions (physico-chemical data). The early screening of various paths and recycles is then performed on the basis of economic evaluation.

Expert systems are a design method that uses a series of postulates coming from qualitative analysis and models. This is usually developed for a distinct class of processes with similar characteristics. Artificial intelligence was found particularly suitable in the applications of expert systems in a number of fields of biochemical engineering where multi-variable problems should be resolved. Experimental software packages have been reported in application of metabolic pathway, downstream processing, mammalian cell culture development (Stephanopoulos and Stephanopoulos, 1986), food processing (Bruin, 1992), recombinant protein separation (Gandikota *et al.*, 1992, Asenjo *et al.*, 1996) and

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biotransformation (Dervakos *et al.*, 1989, 1995). In biotransformation process design, Dervakos *et al.* (1995) described an attempt to gather all process informations for the selection of operational parameters in biotransformations through a computer modelled knowledge-based system or KBS (Figure 1.1). The process algorithm proceeded from the data provided for substrate/product properties, the biocatalyst, reaction and their reciprocal interactions to the division of the task by four heuristic knowledge bases (biocatalyst, solvent, reactor, downstream) co-ordinated by a manager unit in order to provide solutions for process selection and design. This tool could be proved effective for speeding up design for a complete biotransformation.

Biotransformation process design has been achieved by modelling (Rohner and Meyer, 1995). The benefits of this approach are a direct estimation of the process variables and a high degree of accuracy. Moreover, the model provides the basis for process control. Flux modelling, Enzyme kinetics, statistics and heuristics are required to develop the model, that would be refined by a number of experiments. This approach has been implemented to full production scale in the oxidation of nicotinic acid and the production of nicotinamide. The algorithm-model design allows investigation of the effects of operating conditions on the process, but multi variable complex models may be required (Wheelwright, 1989).

According to Kragl *et al.* (1992), the elements of biotransformation process design are the mutual interactions between the process conditions (for instance pH, temperature and the concentration of the species), the reaction engineering, such as issues concerning the reactor design and product isolation, the thermodynamics and the kinetics of the reaction. However, these interactions select a suitable process through heuristic rules.

Integration of the reaction with downstream processing operations has been considered as one possible strategy of process synthesis. Process integration has been used in simulations of whole bioprocesses in order to design and operate the process at the lowest capital (Middleberg *et al.*, 1992) or operating costs (Cooney, 1988,1996, Ingelby *et al.*, 1986). *In situ* product removal (ISPR) is a process design in which the bioreactor is integrated with a continuous product separation in order to avoid product accumulation (Daugulis, 1988, Freeman *et al.*, 1993).

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Figure 1.1   Knowledge-based system architecture for biotransformation process design (after Dervakos *et al.*, 1995).

DATA

PROCESS SELECTION AND DESIGN

Figure 1.1 Knowledge-based system architecture for biotransformation process design (after Dervakos *et al.*, 1995).

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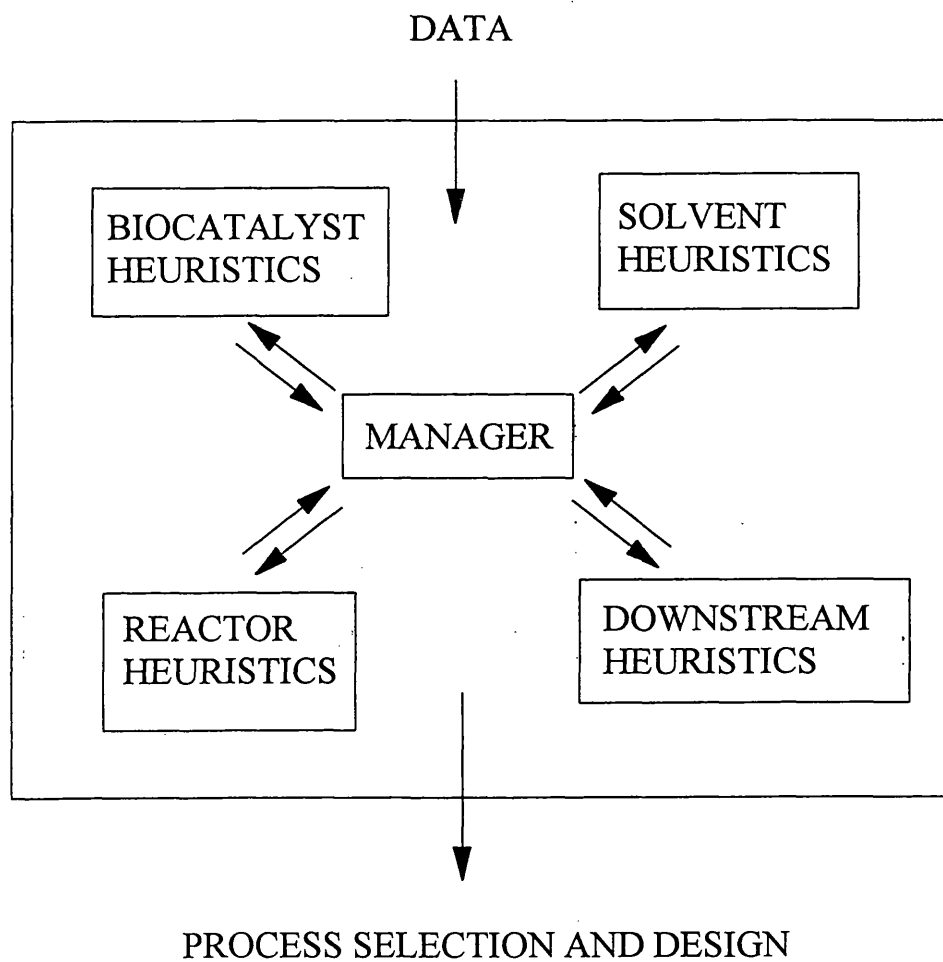
ISPR has been shown beneficial for both yield and productivity improvements since it minimises product interferences with the biocatalyst, product losses due to production environmental conditions and reduces the number of downstream processing steps (Freeman *et al.*, 1993). Moreover, ISPR could shift equilibrium reactions towards product formation (Woodley and Lilly, 1994) and may prove essential in case of intensive product inhibition or toxicity to the biocatalyst (Freeman *et al.*, 1993). A rational approach to ISPR process selection (Daugulis, 1988, Freeman *et al.*, 1993) proceeded from the chemical and biochemical characteristics of the product to the identification of appropriate separation techniques based on the specific product characteristics. Evaluation of different process options and economic feasibility was then required to enable a rational selection. Woodley and Lilly (1994, 1996) approached the process design of biotransformations with a defined sequence of reaction characterisation, constraints identification, and process option evaluation and selection. The design of the whole process includes the reciprocal interactions between the reactor option, product separation and biocatalyst production (Lilly and Woodley, 1996, Figure 1.2). The tools available to support this approach are literature data and specifically designed experiments, heuristics, modelling and simulation to obtain a final design based on the biotransformation characteristics (Woodley and Lilly, 1996).

The criteria for the different steps and stages of the design process define a strategy. All the above methods can be combined and applied at different stages of the project, according to different design strategies. The majority of design cases have been developed for the chemical industry. The flowchart in Figure 1.3 covers the major points of industrial process synthesis, from the conceptual design to transfer to manufacture (Saeder, 1985; Wheelright, 1987; Kaufmann, 1988). In this thesis, a general approach to process design of biotransformations is considered throughout the model system of the chemo-enzymatic synthesis of Neu5Ac.
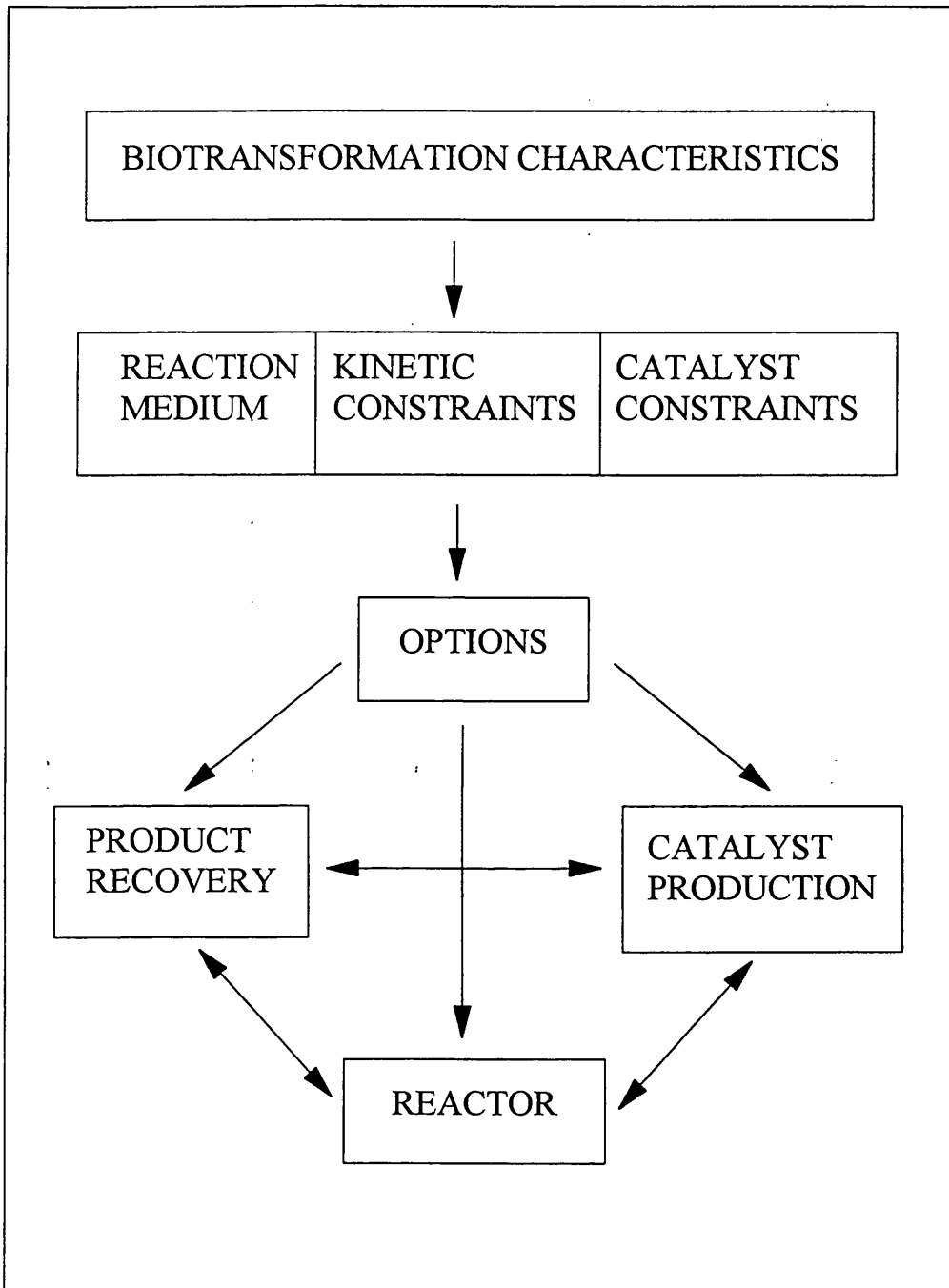
Figure 1.2   Stages in biotransformation process design (after Lilly and Woodley, 1996).

PROCESS SYNTHESIS

↓

DESIGN BASIS

↓

DESIGN STRATEGY

↓

LABORATORY TRIALS

↓

FINANCIAL ANALYSIS

↓

COMPARISON
OPTIMISATION

↓

SCALE-UP

↓

TRANSFER TO MANUFACTURE
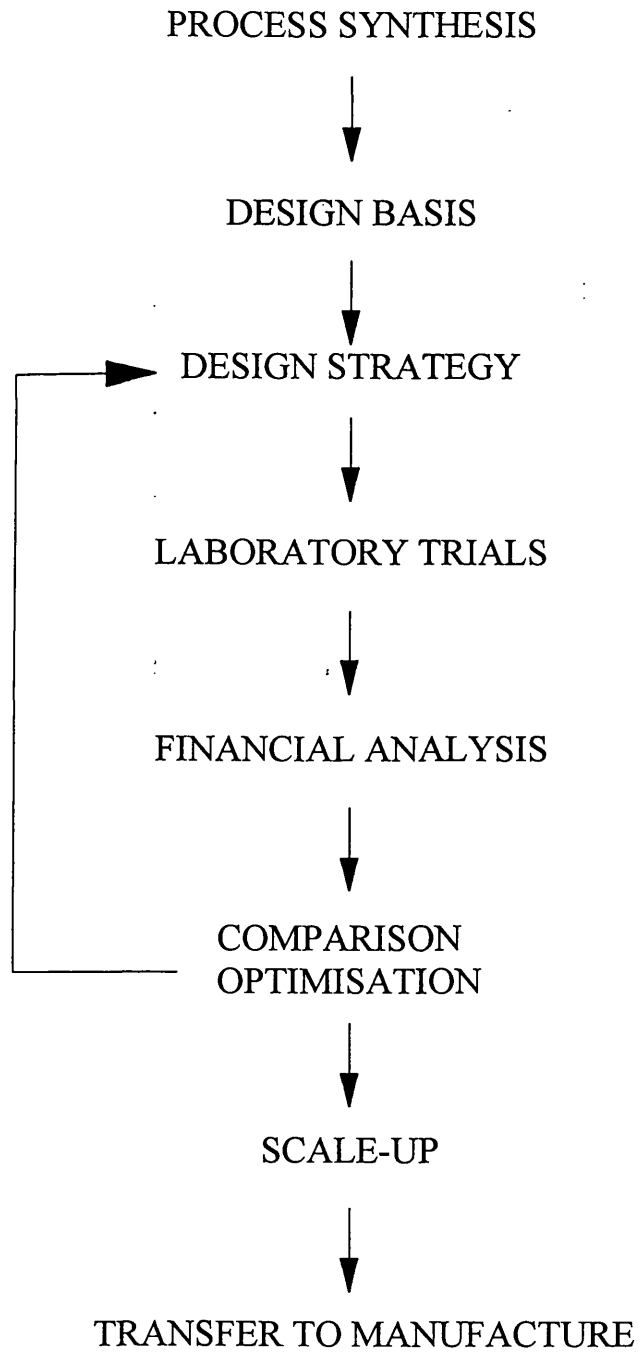
Figure 1.3   Process synthesis flowchart (after Saeder, 1985; Wheelright, 1987; Kaufmann, 1988).

1.3 CHEMO-ENZYMATIC SYNTHESIS OF Neu5Ac

1.3.1 Neu5Ac PRODUCTION

N-Acetyl-D-neuraminic acid (Neu5Ac, NAN, NANA, 5-(acetylamino)-3,5,-dideoxy-D-gycero-D-galacto-2-nonulosonic acid) is the first component of sialic acids, a family of amino sugars containing 9 or more carbons. Neu5Ac is widely present in linked forms, as the glycoside component of complex macromolecules in glycoproteins (blood serum, erythrocyte membrane), oligosaccharides, glycolipids, and natural polymers in various types of linkages, often on a terminal or side chain position (Schauer, 1982). Neu5Ac and its derivatives in linked forms play different biological roles in cell signalling and recognition (Roth, 1992), transport systems and immune response (Schauer, 1978, 1982, 1985). N-Acetyl-D-neuraminic acid can be used as a starting substrate for enzymatic (Simon et al., 1988; Ichikawa et al., 1991; Auge' et al., 1993) or chemical synthesis of derivatives (Schauer, 1970; Schauer and Buscher, 1974; Auge' et al., 1984, 1990; Nagy and Bednarsky, 1991; Sparks et al., 1993) and oligosaccharides, such as Sialyl Lewis X (Le^X), that mediates leukocyte inflammatory response (Ichikawa et al., 1991; Wong and Whitesides, 1994). Due to their importance in biology, sialic acids became valuable compounds in cellular studies and in various areas of therapy, such as potent inhibitors against sialidase of influenza virus (Sparks et al., 1993; Von Itzstein et al., 1993; Wong and Whitesides, 1994).

For large scale preparation, a major source of Neu5Ac is colominic acid from *Escherichia coli* (Barry and Goebel, 1957; McGuire and Binkley, 1964; Barker et al., 1967; NGK insulators, 1994), an exogenous homopolymer of Neu5Ac with α (2→8) or α (2→9) ketosidic linkages (Rodríguez-Aparicio et al., 1989; Gonzáles-Clemente et al., 1990). N-acetyl-D-neuraminic acid is also extracted from submandillar gland glycoproteins (Buscher et al., 1974; Blix and Lindberg, 1960), edible bird nest (Schauer and Buscher, 1974), egg yolk membrane and chalaza (Juneja et al., 1991) and cow milk (Schauer, 1982; Toote et al., 1989). Neu5Ac is mostly prepared by acid hydrolysis. Diluted hydrochloric (Blix and Lindberg, 1960; Juneja et al., 1991) and sulphuric acid (Buscher et al., 1974; Schauer, 1978; Comb and Roseman, 1960) are employed at high temperatures. Yield of free Neu5Ac varies at around 53-66%, mostly due to degradation under these conditions (Barker et al., 1967). Other problems linked to these production methods are the limited amount of raw materials, separation problems occurring after hydrolysis and the high costs of acid preparation.

Chemical synthesis of Neu5Ac and its derivatives, mostly achieved only on experimental scale, is used to prove chemical routes (Kuhn and Baschang 1962; Danishefsky and DeNinno, 1986; for a review, see Schauer, 1982). Chemical methods need protection and deprotection steps (Kim *et al.*, 1988). The β-methyl glycoside is the building block of Neu5Ac derivatives (Schauer *et al.*, 1970) and radioactive sialic acids (Schauer and Buscher, 1974). N-acetyl-D-neuraminic acid can be also synthesised enzymatically by Neu5Ac synthase from N-acetyl-D-mannosamine (ManNAc) and phosphenolpyruvate (PEP), as shown in Equation 1.5:

$$ManNAc + PEP + H_2O \rightarrow Neu5Ac + Pi \qquad (1.5)$$
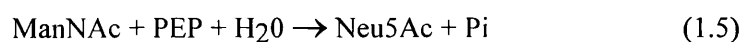
The enzyme has been identified and characterised from *Neisseria meningitidis* (Blacklow and Warren, 1962). However, metal ions and sulfidryl compounds are required in the more purified form and the enzyme has shown a very narrow specificity for both of its substrates (Warren and Blaclow, 1962). These features made Neu5Ac synthase unsuitable as a flexible large scale biocatalyst. These limitations in Neu5Ac production have imposed the need of clear biochemical synthetic methods for the large scale production of Neu5Ac.

1.3.2 RATIONALE FOR USE OF CHEMO-ENZYMATIC SYNTHESIS AS A MODEL

N-Acetyl-D-neuraminic acid (Neu5Ac) is produced from N-acetyl-D-glucosamine (GlcNAc) and pyruvate (Pyr) in two steps (Figure 1.4a). The first step is a base-catalysed epimerisation of GlcNAc to N-acetyl-D-mannosamine (ManNAc) (Roseman and Comb, 1958) and the second is a biotransformation, carried out by the addition of pyruvate to ManNAc, to synthesise a new carbon-carbon bond yielding Neu5Ac (Comb and Roseman, 1960). The latter reaction is catalysed by Neu5Ac aldolase from *E. coli*. This enzyme has been well studied and reported previously (Uchida *et al.*, 1984; Kim *et al.*, 1988; Aisaka *et al.*, 1991; Kragl *et al.*, 1992). The two step synthesis of Neu5Ac may be conceptually divided into an upstream process (USP) to produce the reactant for the biotransformation, a biotransformation step converting reactant to product and a downstream process (DSP) to recover the desired product (Figure 1.4b).
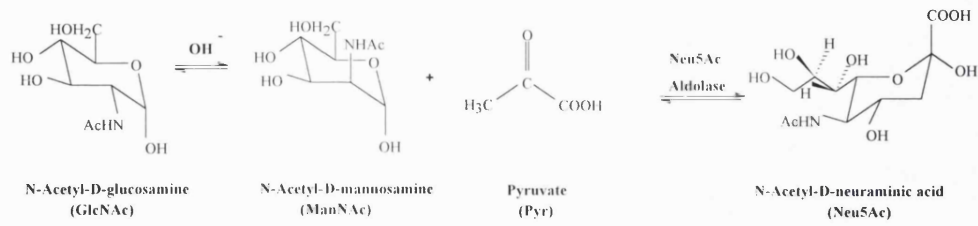
(a)

(b)

Figure 1.4. Chemo-enzymatic synthesis of Neu5Ac (after Roseman and Comb, 1958; Comb and Roseman, 1960); (a) reaction scheme; (b) process scheme.

The following features of the process are typical of many equilibrium controlled biotransformations and integrated chemical and biological reactions:

- The pKa values for Neu5Ac (Kragl, 1992) and pyruvate (Dawson *et al.*, 1993) are similar, which may lead to a potential difficulty in downstream separation unless pyruvate concentration is kept low.

- While neither of the reactions give by-products, both the equilibria for the epimerisation and to a lesser extent the biotransformation are unfavourable for Neu5Ac synthesis (Ghosh and Roseman, 1965; Uchida *et al.*, 1984).

- Since the equilibrium conversion is proportional to the initial reactant concentrations, for both the reactions there is a substantial advantage to operating at high substrate molarities.

- The enzyme is inhibited by both Neu5Ac and GlcNAc and has a very high Km for ManNAc (Kim *et al.*, 1988; Kragl *et al.*, 1992).

Moreover, Neu5Ac aldolase accepts a broad range of aldose sugars as substrates (Fitz *et al.*, 1995), making the chemo-enzymatic synthesis a model system for all the Neu5Ac aldolase catalysed biotransformations. Hence, the synthesis of Neu5Ac serves as a good model for the research in methodology of process design and reaction integration. The components of the chemo-enzymatic synthesis and the alternatives in process selection to date are reviewed in the following sections as the initial step for a rational approach to biotransformation process design (Woodley and Lilly, 1994, Lilly and Woodley, 1996).

1.3.3 REACTION COMPONENTS

1.3.3.1 SUBSTRATES AND PRODUCT

N-Acetyl-D-glucosamine and its epimer, N-acetyl-D-mannosamine are acetylated amino sugars. GlcNAc has been generally produced by chemical synthesis from acid hydrolysis of chitin, which is the structural material in the exoskeleton of insects and crustaceans (Stryer, 1995). ManNAc is prepared by chemical or enzymatic epimerisation (Section 1.3.4.1) from GlcNAc and is a precursor in the biosynthesis of all sialic acids (Schauer, 1978). The degradation of these sugars at alkaline pH has been reported (Schauer, 1973).

Pyruvic acid (2-oxopropanoic acid) is a three carbon acid which is almost fully deprotonated at neutral pH (pKa: 2.39, Dawson *et al.*, 1993). Pyruvate is found in all organisms which utilise carbohydrates for energy production under aerobic and anaerobic conditions. It is a product of glucose degradation, fructose and galactose metabolism (Stryer, 1995). Pyr is likely to be affected at high pH by a mechanism similar to hydroxypyruvate decarboxylation (Hedrick and Sallach, 1961).

Neu5Ac is negatively charged at neutral pH (pKa: 2.2, Kragl, 1992). The pyranose form is more thermodynamically stable. α/β mutorotation occurs through the ketone form, where the β anomer is more present than the α anomer (β/α ratio 92.8/7.2, Dejil and Vliegenthart, 1983; β/α ratio 95-92/5-8, Friebolin *et al.*, 1980). Ca^{2+} and earth alkali ions bind to the β anomer shifting the α/β equilibrium (Kragl, 1992). Decomposition of Neu5Ac occurs in aqueous diluted acids at 80-100 $^{\circ}C$ by a series of possible mechanisms: decarboxylation, enolization, pyrrole ring formation, deacylation, dehydration, and elimination leading to polymerisation reactions (Karkas and Chargaff, 1963; Barker *et al.*, 1967; Schauer, 1978.; Saito *et al.* 1989; Roy and Boratynski, 1990). Likewise, under alkaline conditions, Neu5Ac degradation has been reported, possibly linked with cyclization (Gottschalk 1955; Cornforth *et al.*, 1958; Saito *et al.*, 1989).

1.3.3.2 *E.coli* Neu5Ac ALDOLASE

The family of the lyase enzymes catalyses the formation of a novel C-C bond. Aldolases are responsible for the addition of 3 carbons moiety to an aldehyde; transketolases catalyse the addition of two carbons and oxynitrilases add one carbon to an aldehyde structure (Drueckhammer *et al.*, 1991; Faber, 1992; van der Werf *et al.*, 1994). Lyases hold great potential for industrial use owing to their lack of cofactor requirements, good operational stability and high stereo specificity. The regio- and stereo-selectivity is usually controlled by the enzyme (van der Werf *et al.*, 1994). The aldolases are usually divided in two classes, according to their reaction mechanism. Class I aldolases are usually found in higher animals and plants and do not require a metal cofactor. The reaction involves a Schiff's base formation between pyruvate and the ε-amino group of a lysine residue in the active site (Baumann *et al.*, 1989; Faber, 1992; Wong and Whitesides, 1994). Class II aldolases requires zinc cofactor acting as a Lewis base in the active site and are more abundant in bacteria and fungi (Faber, 1982). Aldolases accept a broad range of unnatural substrates and in many cases the stereo chemistry of products is predicted (Wong and Whitesides, 1994).

Neu5Ac aldolase (E.C. 4.1.3.3) is a Class I aldolase (Aisaka et al., 1991). Uchida and co-workers (1985) detected this intracellular enzyme in various genera of bacteria (among others: *Pseudomonas, Escherichia and Micrococcus*). Neu5Ac aldolase from *Clostridium perfringens* has been purified and characterised (Comb and Roseman, 1960; Baumann *et al.*, 1989). Findings on *Cl. Perfringens* are accepted as indicative for *E. coli*. The biochemical characterisation of *E. coli* aldolase is presented in Table 1.3. The importance of Neu5Ac aldolase reaction lies in the synthesis of sialic acid analogues (Wong and Whitesides, 1994). Faber (1982), Kim *et al.* (1988) and Uchida (1984, 1989) have shown an absolute specificity of the nucleophilic substrate (Pyr), while the electrophilic substrate can vary broadly (Kim *et al.*, 1988; Drueckhammer *et al.* 1991; Sparks *et al.* 1993; Fitz *et al.*, 1995). The hydroxyl group on the C_3 position of the acceptor substrate (the sugar) is critical for the enzyme reactivity. Substitutions at C_4, C_5 and C_6 are flexible (Kragl *et al.*, 1994, Fitz *et al.*, 1995). The enzyme is likely to recognise the α anomers of the sugars as substrates (Dejil and Vliengenthart, 1983), but mutorotation is fast compared to the reaction rates (Kragl, 1992). The research group of Auge' (Auge' *et al.*, 1990; Gautheron-Le Narvor *et al.*, 1991) reported the first changes in stereo selectivity and loss of enantiospecificity due to the substrate D-arabinose, yielding to epimers in similar amounts. It is the first occurrence where the ultimate chemistry was dependent on the substrate acceptor and not on the enzyme. Aldopentoses and aldotetroses have also been accepted as substrates (Fitz *et al.*, 1995).

1.3.4 THE ALDOLASE PROCESS

1.3.4.1 UPSTREAM PROCESSING (USP)

2-acetamido-2-deoxyaldoses epimerise in dilute aqueous alkali. Examples of hexoses and pentoses are reported. The equilibrium is dictated by the relative stabilities of the pyranose chair conformations (Horton and Wander, 1980). GlcNAc is converted to ManNAc by epimerisation through the formation of enolamine and tautomerization (Salo *et al.*, 1976). It is a first order equilibrium reaction. The ManNAc / GlcNAc ratio at equilibrium lies in favour of GlcNAc (1/3.9 - 1/5.0) (Roseman and Comb, 1958; Spivak and Roseman, 1959; Kuhn and Baschang, 1962; Ghosh and Roseman, 1965; Lee, 1990; Kragl, 1992). Ammonia, nickel acetate and pyridine have been employed in the reaction (Kuhn and Baschang, 1962).

Table 1.3   Characteristics of *E. coli* Neu5Ac aldolase.

M.W. and Structure	105-110 KD^a, 98 KD^i; 3 subunits of 35 $KD^{a,f}$ to 33 $KD^{g,i}$, or 4 subunits[c]
Isoelectric Point	4.4-4.6[a]
pH: Activity	Max. pH 6.5-7.0.[a], 7.3[b] or 7.4-7.6[e]. Activity at pH 4: 10%; activity at pH 9.5: 50%[e]
Stability	70 % activity at pH 8.0, 40% activity at pH 5.0 after 20 min[a]. Stable between pH 6.0 and pH 9.0[i]. No activity at pH 10.2 after 24 h, 37 $^oC^i$
Temperature: Activity	Max: 80 $^oC^a$. 90% loss of activity at 10 ^oC (w.r.t. 25^oC)
Stability	Stable for 0.5 h at 70^oC; 52% inactivation at 85^oC (pH 7.0, 15 min.). Enhanced stability with Neu5Ac^a or pyruvate^{a,i} and ManNAc^i
Km_{Neu5Ac}	3.3 $mM^{a,b}$, 9.4 mM^e, 3.6 $mM^{g,i}$
Km_{Pyr}	10 mM^d, 0.136 mM^e
Km_{ManNAc}	700 mM^d, 402.2 mM^e
Vmax synthesis	13.8^e-25^d μmol/min/mg protein
Vmax cleavage	8.51^e-71.4^a μmol/min/mg protein 154.5 μmol/min/mg protein at 37 $^oC^i$
Inhibitors	K_{IPyr}: 2.3-2.5 mM^a, 1.3 mM^e, 1.0 mM^i $K_{IManNAc}$: 23.8 mM^e All inhibitors of Class I Aldolases^a GlcNAc (19% inhibition at 200 mM)^e Ca^{2+} in synthesis direction^e High viscosity solutions^h

a: Aisaka *et al.* (1991) b: Comb and Roseman (1960) c: Izard *et al.* (1994) d: Kim *et al.* (1988) e: Kragl (1992) f: Lilley *et al.* (1987) g: Ohta *et al.* (1989) h: Kragl *et al.* (1991) i: Uchida *et al.* (1984).

Sodium hydroxide has been extensively applied (Roseman and Comb, 1958; Kuhn and Baschang, 1962; Simon *et al.*, 1988; Lee, 1990; Kragl, 1992). Calcium hydroxide has been recently shown a faster catalyst, although calcium has to be removed in the carbonate salt form (Sugai *et al.*, 1995). The chemical kinetic constants are pH and temperature dependent (Lee, 1990: Kragl, 1992). 95% completion is achieved in 33 hours at pH 11 (Kragl, 1992). A sharp increase in rates and equilibrium is reported at pH 12.3 (Lee, 1990). However, above pH 11 browning and non specific degradation takes place (Kragl, 1992). The reaction can be catalysed as well by N-acetyl-D-glucosamine epimerase (E.C. 5.1.3.8). The enzyme is most active at pH 7 and requires Mg^{2+} and adenosine triphosphate (ATP) as cofactors. Pyruvate and Neu5Ac inhibition occurs. A full kinetic model for this enzyme has been already developed (Gosh and Roseman, 1965; Kragl, 1992).

Improvement in ManNAc concentration is required to balance the unfavourable equilibrium. Moreover, GlcNAc has been shown inhibitory to Neu5Ac aldolase (Kragl, 1992; Mahmoudian *et al.*, in press) and maximal conversion of GlcNAc to Neu5Ac is required. ManNAc Enrichment can be achieved exploiting the difference in solubility of the two epimers in lower alcohols. GlcNAc can be removed by methanol addition and subsequent evaporation (Lin *et al.*, 1992). Fractional crystallisation of GlcNAc in ethanol is possible (Roseman and Comb, 1958; Auge' *et al.*, 1988). Hot ethanol extraction (Spivak and Roseman, 1959; Simon *et al.*, 1988) yielded an enriched solution of ManNAc. GlcNAc can also be degraded selectively by *E. coli* fermentation (Spivak and Roseman, 1959). Moreover, propan-2-ol GlcNAc crystallisation and subsequent azeotropic distillation has been used in large scale operations (Dawson *et al.*, 1994; Mahmoudian *et al.*, in press). The enriched mixture can be used directly in the biotransformation reaction.

1.3.4.2 BIOTRANSFORMATION

Lyase-catalysed condensations are equilibrium controlled reactions (Van der Werf *et al.*, 1994). The equilibrium for the synthesis of Neu5Ac by *E. coli* aldolase is 28.7-29.4 M^{-1} (Kragl *et al.*, 1991; Kragl, 1992) and decreases with increasing temperature (Comb and Roseman, 1960; Uchida *et al.*, 1985). Equilibrium shifts towards Neu5Ac formation are achieved either with pyruvate excess (Auge' *et al.*, 1984, 1988; Kim *et al.*, 1988, Lin *et al.*, 1992) due to its lower cost (Sigma, 1996), or by coupling the transformation to another enzyme that utilises Neu5Ac together with the hydrolysis of an activated bond (Ichikawa *et al.*, 1991; Lin *et al.*, 1992). Calcium and other earth alkali bind the β anomer of Neu5Ac

altering the tautomeric equilibrium. This shift, however is inhibitory to the enzyme (Kragl, 1992). The equilibrium conversion is dependent on the initial concentrations of ManNAc and Pyr. Hence, there is a process advantage in operating at high substrate molarities, limiting the excess of pyruvate (Kragl *et al.*, 1992, 1995; Mahmoudian *et al.*, in press). Moreover, pyruvate excess is detrimental to DSP (section 1.3.4.3). The equilibrium is independent from pH, since no pH shifts are involved in the reaction. 25 °C and pH 7.5 have been selected as the optimal reaction conditions (Auge' *et al.*, 1984, 1988; Kim *et al.*, 1988; Lin *et al.*, 1992; Kragl *et al.*, 1992, 1995; Mahmoudian *et al.*, in press). Neu5Ac aldolases from *E. coli* and *Cl. Perfringens* have been immobilised for laboratory synthesis and process aims. Auge' *et al.* (1988, 1990) described the use of cyanogen-bromide activated agarose for immobilisation of *Cl. Perfringens* aldolase, using a protocol developed by Kim and co-workers (1988). Crude *E. coli* enzyme, on a process scale, is immobilised on porous support with epoxide covalent linkages (Mahmoudian *et al.*, in press). Membrane enclosed enzymatic catalysis (MEEC) was presented by Bednarski *et al.* (1987) for laboratory scale synthesis as an alternative to immobilisation. Activated polyacrylamide immobilisation (Pollack *et al.*, 1980; Kim *et al.*, 1988) has been performed on the *E. coli* aldolase. The stability of the immobilised forms appears to be varying upon the reaction conditions at which the enzyme is exposed.

Preparative and large scale biotransformations have been performed in batch reactors with either epimerised or enriched GlcNAc-ManNAc mixtures by Eupergit-C (Röhm & Haas) immobilised aldolase (Dawson *et al.*, 1994; Mahmoudian *et al.*, in press). Kragl and co-workers (1991, 1995) developed a continuous process for Neu5Ac synthesis in an enzyme membrane reactor at preparative scale. Although the reactor had an excellent space-time yield (470 g / L / d) the product stream was diluted (240 mM), requiring large volume separations. The same reactor system has been used for the synthesis of derivatives (Kragl *et al.*, 1993).

1.3.4.3. DOWNSTREAM PROCESSING (DSP)

Various methods for Neu5Ac purification have been reported. The main problem in the separation process is the division of Neu5Ac from pyruvate, since they are both negatively charged and have similar pKa. Excess pyruvate has been either discharged, degraded by yeast or pyruvate decarboxylase (Lin *et al.*, 1992; Sugai *et al.*, 1995) or adsorbed specifically with metabisulphite resins (Mahomoudian *et al.*, in press). Anion exchange chromatography has been widely employed in sugar purifications (Lee, 1990) with elution

reagents volatile under vacuum. Affinity adsorption, such as with lectins, is only now achieving large scale use (NGK, 1994, 1994b). Dowex 2-X8 (Dow Chemical Co.) or Duolite 113 (Röhm & Haas) strong anion exchanger resins in the acetate (Mahmoudian *et al.*, in press) or formate form (Comb and Roseman, 1960; Montreuil *et al.*, 1968; Buscher *et al.*, 1974; Schauer, 1978; Simon *et al.*, 1988; Auge' et al., 1988, 1990; Lin *et al.*, 1992; Kragl, 1992, 1995) have been employed with both gradient and isocratic elution.

Possible candidates for selective separation of Neu5Ac might be found in lectins (Broekaert *et al.*, 1984; Barondes, 1988; Adam *et al.*, 1993). A commercial patent (NGK insulators, 1994, 1994b) described the use of a wheat germ lectin based on silica particles. The silica column was used to purify both colominic acid and free Neu5Ac in yields of 80%.

Neu5Ac solidified in methanol : ether (1:3) solution, when light petroleum was used for seeding (Montreuil *et al.*, 1968). N-Acetylneuraminic acid could be crystallised by aqueous acetic acid (Kuhn and Baschang, 1962). Previous lyophilisation (Comb and Roseman, 1960) or 50% methanol addition (Lin *et al.*, 1992) has been reported. Direct crystallisation from the biotransformation step could be achieved with Neu5Ac concentrations in excess of 125 g / L (Mahmoudian *et al.*, in press).

1.3.4.4. USP-BIOTRANSFORMATION INTEGRATION

Neu5Ac can be produced directly from GlcNAc integrating the upstream processing with the biotransformation step. Pyruvate excess is still required to favour the synthesis reaction. Two different strategies have been reported. Kragl *et al.* (1991) developed a continuous process using N-acetyl-D-glucosamine 2-epimerase (E.C. 5.1.3.8) together with Neu5Ac aldolase in the enzyme membrane reactor. However, in this approach both enzymes are under inhibition, the product stream is diluted and Mg^{++} and ATP required by the epimerase interfere with anion exchange chromatography (Kragl, 1992). Tsukada and Ohta (1994) reacted GlcNAc with pyruvate in alkaline conditions in the presence of Neu5Ac aldolase. This approach led to significant compromises for both the enzyme activity and reaction rates. The epimerisation is not feasible below pH 8 and at pH 10.5 the enzyme stability decreased vigorously. Moreover, losses of Neu5Ac and pyruvate are possible.

1.4 AIM OF THIS WORK

The synthesis of Neu5Ac from GlcNAc and pyruvate composed of chemical epimerization followed by a biotransformation catalysed by an *E. coli* aldolase is taken as a model for the process design of biotransformations. This thesis aims to define a rational approach to the design of enzymatic equilibrium controlled biotransformation processes starting from the current methodology of reaction characterisation, constraints identifications, process options evaluation and process selection (Woodley and Lilly, 1994, 1996, Lilly and Woodley, 1996). The specific objectives of this thesis for the chemo-enzymatic synthesis of Neu5Ac are:

- To characterise the two step synthesis of Neu5Ac and its constraints for process design (Woodley and Lilly, 1992, 1994).
- To evaluate the integration of the biotransformation step with either upstream operations (USP) and downstream processing (DSP).
- To determine the appropriate reactor design and operation for Neu5Ac synthesis according to different processing constraints.

In view of process design speed-up, the work would contribute to the understanding of the major key issues affecting the design of equilibrium biotransformation to full process scale.

2

CHARACTERISATION

2.1 INTRODUCTION

The need for a rational approach to design of bioprocesses was identified in the 80's by Cooney (1983) and Wheelwright (1989) amongst others. More recently, Woodley and Lilly, (1994, 1996) have developed a philosophy for the design of biotransformation processes based on a four part structured approach involving (1) reaction characterisation, (2) constraint identification, (3) process option evaluation and (4) process selection. In this chapter, the rationale for obtaining data to characterise the chemo-enzymatic synthesis of Neu5Ac is presented. The aims of this chapter are:

- To identify process constraints of Neu5Ac synthesis.
- To develop a methodology for data collection in biotransformation process design.

The structured approach to process design is based initially on reaction characterisation (Woodley and Lilly, 1996). Such characterisation requires obtaining data by reference to past information where available and is based on specifically designed experiments. Early decisions on the conditions at which these experiments are carried out are required in order to obtain data relevant to process selection and design. Two sets of data are reported in this chapter. The first set of experiments is designed to obtain data on the physico-chemical characteristics of the reactant(s) and product(s), on the reaction chemistry and on the biocatalyst at the selected conditions. The second set of data has investigated the interactions of the reactant(s) and product(s) with the biocatalyst (e.g. stability, kinetic parameters, reactant(s) and product(s) toxicity). The two set of data have identified constraints both on process synthesis and on reactor design and operation. The identified constraints will be analysed in Chapter 3 and will provide the basis for further investigation (Chapters 4 and 5).

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2.2 RATIONALE

The two step synthesis of Neu5Ac may be conceptually divided into an upstream process to produce reactant for the biotransformation, a biotransformation step converting reactant to product and a downstream process to recover the desired product. Table 2.1 indicates the conditions which need to be investigated in order to examine the potential for process options. Hence experiments need to be carried out at alkaline pH (the two step synthesis could be integrated in a single unit operation, as in Tsukada and Ohta, 1994) and high GlcNAc concentration (the reaction is first order, Lee, 1990), at neutral pH (the enzyme optimum, Uchida *et al.*, 1984) and high substrate concentrations (for thermodynamic equilibrium, see section 2.4.1.2) and acidic pH (crystallisation of Neu5Ac in acetic acid is a potential method of product recovery, Section 2.4.1.2).

2.3 MATERIALS AND METHODS

2.3.1 MATERIALS

All reagents used were analytical grade. Chemicals were obtained from Sigma except for Neu5Ac dihydrate, which was kindly supplied by Glaxo Group Research. *E. coli* Neu5Ac aldolase (24.1 U / mg) was obtained from Toyobo (Toyobo, 1994). All chemicals and suppliers are listed in Appendix 1.

2.3.2 METHODS

2.3.2.1 CHEMICAL STABILITY OF REACTANTS AND PRODUCT

Mixtures of Neu5Ac, GlcNAc, ManNAc and pyruvate were incubated (37 $^{\circ}$C) separately at pH 11.5 at concentrations of 290, 500, 400, and 320 mM, at pH 7.5 at 310, 505, 400 and 370 mM and at pH 2.5 at 310, 530, 416 and 350 mM respectively. Samples were taken after 72 hours and assayed by HPLC (2.3.2.2) for residual Neu5Ac, GlcNAc, ManNAc and pyruvate.

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Table 2.1. Experimental conditions for characterisation of Neu5Ac chemo-enzymatic synthesis.

Process	Operating Conditions	
	pH	**Component Concentrations**
upstream	> 8.5	high GlcNAc
biotransformation	neutral	high Pyr and ManNAc
downstream	< 3.5[1]	high Neu5Ac

1- based on crystallisation with acetic acid (after Lin *et al.*, 1992).

2.3.2.2 HPLC ASSAY

Samples (200 µL) were diluted 100-fold in 6 mM H_2SO_4 to give component concentrations of 2-10 mM and then analysed by isocratic High Performance Liquid Chromatography (HPLC) through an Aminex™ HPX-87H column (300 x 7.8 mm, Bio-Rad). Assay conditions: 65 °C; mobile phase: 6 mM H_2SO_4; flowrate: 0.6 mL / min; refractive index detection (Kragl *et al.*, 1992). Two Aminex columns were used in series with a mobile phase flowrate of 0.45 mL / min for detection of GlcNAc (please refer to Appendix 2 for chromatograph).

2.3.2.3 SOLUBILITY

The aqueous saturation concentrations of reactants and product were determined by incubation of aqueous solutions in duplicates at increasing molarity for 24 hours at 25 °C and pH 7.5, on a bench shaker at 70 r.p.m. Accuracy measured: ± 0.1 M.

2.3.2.4 ENZYME STABILITY

Enzyme solutions (124 µg / mL) were incubated for 8 hours in a pH Stat Radiometer ETS-200 at 25 °C, using 0.5 M NaOH as titrant. Samples (200 µL) were diluted 1:3 in 100 mM Tris-HCl buffer (pH 7.9) and residual activity monitored by spectrophotometric assay (2.3.2.5).

2.3.2.5 SPECTROPHOTOMETRIC ASSAY

Incubation samples (100 µL) were added to 1 mL of assay mixture (945 µL of 100 mM Tris-HCl buffer, pH 7.9; 10 µL NADH , 40 g / L; 40 µL BSA, 9.6 g / L and 5 µL LDH , 10000 U / mL, from beef heart) and incubated at 37 °C (variation on Toyobo, 1994 procedure). The assay was started by addition of 100 µL of Neu5Ac (60 g / L in 100 mM Tris-HCl buffer pH 7.9), and the decrease in absorption at 343 nm was followed with time in a Uvikon 922 spectrophotometer (Kontron Instruments). The activity was calculated according to the formula (Toyobo, 1994):

$$\text{Activity} \left(U / mL \right) = \frac{\Delta A}{t_i} * \frac{1}{\varepsilon} * \frac{V_{tot}}{V_{sampl}} * \frac{1}{L}$$

where:

ΔA	-	decrease in adsorbance between start and end of reaction
t_i	min	incubation time
ε	$cm^2 mol^{-1}$	extinction coefficient for NADH (6.3 $cm^2 mol^{-1}$)
V_{tot}	mL	total cuvette volume
V_{sampl}	mL	sample volume
L	cm	cuvette length (1 cm)

The measurement standard deviation was 2.02 %.

2.3.2.6 BIO-RAD PROTEIN ASSAY

The protein assay followed the protocols described in Bio-Rad, 1994 for protein concentrations up to 25 µg / mL. 800 µL of protein sample were incubated with 200 µL of Bio-Rad protein dye and incubated for 10 minutes. The optical density (OD) at 595 nm was compared to the control (100 mM Tris-HCl buffer pH 7.9) and calibrated against a standard of BSA (Appendix 2).

2.3.2.7 ENZYME ACTIVITY

Pyruvate and ManNAc mixtures (pyruvate concentrations: 0.1, 0.5, 1.0, 2.0 and 3.63 M; ManNAc concentrations: 0.10, 0.25, 0.50, 0.75, 1.00, 1.25 and 1.50 M) at pH 7.5, 25 °C, were incubated with 0.5 mg / mL aldolase and initial Neu5Ac production was monitored by HPLC (2.3.2.2) at 0.5, 1, 2, 5 and 10 minutes.

2.3.2.8 BIOTRANSFORMATION AT HIGH SUBSTRATE CONCENTRATIONS

1.055 M Pyruvate and 0.55 M ManNAc were incubated at 25 °C with 2.2 mg / mL aldolase at pH 7.13 and 7.43 for 200 minutes and the reaction was monitored by HPLC (2.3.2.2). Samples (1 mL) were dialysed (MW cut-off: 20 KDa, Sigma) for 20 hours at 5 °C against 1500 volumes of 100 mM Tris-HCl buffer (pH 7.9). Residual activity was monitored by spectrophotometric assay (2.3.2.5).

2.3.2.9 NACL ENZYME ACTIVITY ASSAY

80 mM Pyr and 40 mM ManNAc were incubated at 25 °C, pH 7.5 with 0.5 mg / mL aldolase in the absence or presence of various NaCl concentrations for 10 minutes and the

reaction was monitored by HPLC. Ionic strength of the substrate solutions was monitored by PW 9505 Philips conductivity meter.

2.3.2.10 NaCL ENZYME STABILITY ASSAY

0.5 mg / mL aldolase was incubated (at 25 °C, pH 7.5) in 15 mL 1 M NaCl solution for 8 hours. In order to separate the aldolase from NaCl, incubation was stopped by addition of 0.5 mL 100 mM Tris-HCl pH 7.9 buffer to the sample (1 mL) and subsequent injection in a size exclusion Hi-Trap ™ desalting column (1.6 x 2.5 cm, Pharmacia), equilibrated with Tris buffer. The protein was eluted with 1.5 mL Tris buffer (10 mL / min) and assayed spectrophotometrically (2.3.2.5). The activity reading was then standardised with Bio-Rad protein assay of the eluate, to account for possible differences in protein concentration (2.3.2.6).

2.4 RESULTS AND DISCUSSION

2.4.1 COMPONENT CHARACTERISATION

2.4.1.1 SUBSTRATES AND PRODUCT

2.4.1.1.1 Solubility

The aqueous saturation concentrations at pH 7.5 and 25 °C were determined for each of the reaction components. The solubility limit of ManNAc (1.6 M in H_2O) decreased with increasing pyruvate concentrations (1.2 M in 2.0 M pyruvate). Maximal solubility values were 1.3 M for GlcNAc, 3.6 M for pyruvate and 1.0 M for Neu5Ac.

2.4.1.1.2 Stability

The stability of a mixture simulating "integrated" (alkaline) and "separated" (neutral) pH biotransformation conditions is shown in Table 2.2. Measurements of stability were made over 72 hours since the combined operations were limited not by the enzymic reaction but by the epimerisation (Kragl, 1992, Tsukada and Ohta, 1994). This provides a realistic worst case scenario on which to base an initial evaluation. In addition, reactant and product stabilities were investigated under acidic conditions to examine the potential for integrated product recovery by acid mediated crystallisation (Lin *et al.*, 1992).

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Table 2.2   **Stability of reaction components (expressed as % original value) remaining after 72 hours incubation at alkaline, neutral and acid pH, 37 °C.** Initial concentrations: 290-310 mM Neu5Ac, 500-530 mM GlcNAc, 400-416 mM ManNAc, 320-375 mM Pyr.

pH	ManNAc	GlcNAc	Pyr	Neu5Ac
11.5	26	68	0	69
7.5	92	95	84	97
2.0	96	90	102	99

The reactants and product are stable at pH 2.0 for 72 hours. Published data on Neu5Ac degradation in acid conditions (Barker *et al.*, 1967; Roy and Boratynski, 1990) indicates that at 80-85 °C, a 30-35% degradation takes place when Neu5Ac is incubated with 0.1 N HCl or 50 mM H_2SO_4 for 2-5 hours. In this study, the sialic acid was found stable, presumably due to the lower incubation temperature (37 °C[1]). However, the option of integration of biocatalysis with crystallisation was eliminated since no enzyme activity (Uchida *et al.*, 1984) was found below pH 3.5 and the conditions required for Neu5Ac aggregation are beneath this value. Under alkaline conditions, while degradation of all components took place, GlcNAc and Neu5Ac were relatively stable compared with ManNAc and pyruvate. It is known that high pH is detrimental to sugars (Gottschalk, 1955; Schauer, 1973). Pyruvate degradation may lead to the formation of polymeric products. These data are important defining constraints on possible design solutions for integration and process limits for operation.

2.4.1.2 REACTION

The Neu5Ac equilibrium conversion is dependent on the initial concentrations of ManNAc and Pyr. Hence there is a process advantage in operating at high substrate molarities, limiting the excess of pyruvate (Kragl *et al.*, 1992, 1995; Mahmoudian *et al.*, in press). For a comprehensive review on the reaction characteristics, please refer to Section 1.3.4.2.

2.4.1.3 BIOCATALYST

2.4.1.3.1 Stability

Neu5Ac aldolase from *E. coli* has been studied previously (Kim *et al.*, 1988; Kragl *et al.*, 1992) and its structure resolved down to 2.2 Å (Izard *et al.*, 1994). The pH-activity profile has also been determined giving maximum activity at pH 7.5 (Comb and Roseman, 1960; Uchida *et al.*, 1985). Complementary studies examined the stability of the catalyst under neutral and alkaline conditions for 8 hours (Figure 2.1). The enzyme was stable at pH 7.15-7.5 throughout the incubation period. However, at alkaline pH there was a substantial loss of activity after 8 hours (30% at pH 10.5 and 75% at pH 11.3).

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[1] Although 25 °C was more favourable to Neu5Ac synthesis due to the reaction equilibrium (Kragl, 1992), the initial experiments on stability were conducted at 37 °C due to conditions reported in the literature (Tsukada and Ohta, 1994). Extended stability experiments (Chapter 4) were performed at 25 °C.
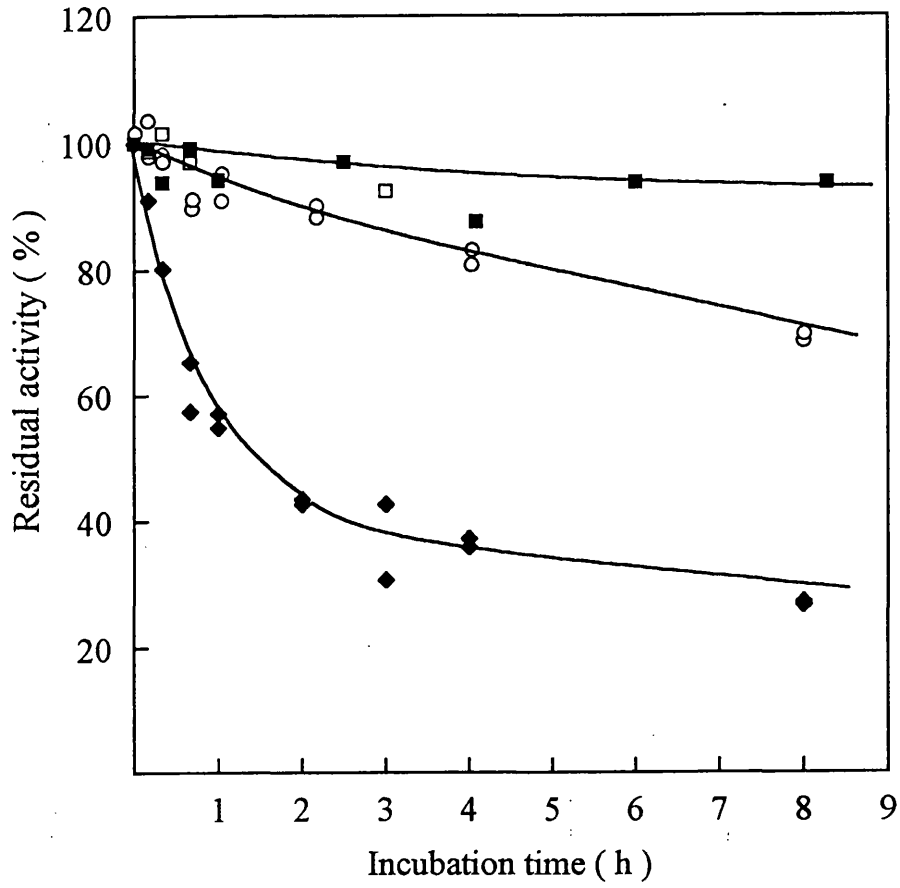
Figure 2.1 Neu5Ac aldolase stability under neutral (■ pH 7.15, □ pH 7.50) and alkaline (O pH 10.5, ◆ pH 11.3) conditions.

2.4.1.3.2 Effects of ionic strength

Enzyme activity and stability are likely to be a function of the ionic strength of the liquid environment (Voet and Voet, 1990). NaCl was used to increase the ionic strength of the substrate solutions. The enzyme was assayed in the synthesis direction (Fig. 2.2) with increasing NaCl concentrations. A decrease in activity was found, with a maximum at 1 M NaCl (77 % of the control reaction). However, the enzyme was found stable at this salt concentration (Figure 2.3). The data suggest that ionic strength might become a determining factor in the kinetics of the aldolase reaction.

2.4.2 INTERACTION CHARACTERISATION

2.4.2.1 KINETIC SIMULATION

A kinetic model of the Neu5Ac aldolase was developed from the steady state theory for double substrates by Kragl et al. (1992), expressing the rate of product formation as a function of substrates and enzyme concentrations (Equation 2.1):

$$\frac{d[P]}{dt} = \frac{[E] * \left(\dfrac{A\,max\,h * [A][B]}{KiA * KiB} - \dfrac{A\,max\,r * [P]}{KmP} \right)}{1 + \dfrac{[A]}{KiA} + \dfrac{[B] * KmA}{KiA * KmB} + \dfrac{[A][B]}{KiA * KmB} + \dfrac{[P]}{KiB * KmP} + \dfrac{[P]}{KmP}} \qquad (2.1)$$

where:

[A]	mol / L	Pyruvate concentration	
[B]	mol / L	ManNAc concentration	
[P]	mol / L	Neu5Ac concentration	
[E]	g / L	Aldolase concentration	
Amaxh	U / mg	Maximal specific activity, synthesis	(13.8 U / mg)
Amaxr	U / mg	Maximal specific activity, cleavage	(8.51 U / mg)
KmA	mol / L	Michaelis-Menten constant, Pyruvate	(0.136 mmol / L)
KmB	mol / L	Michaelis-Menten constant, ManNAc	(402.2 mmol / L)
KmP	mol / L	Michaelis-Menten constant, Neu5Ac	(9.44 mmol / L)
KiA	mol / L	Inhibition constant for Pyruvate	(1.301 mmol / L)
KiB	mol / L	Inhibition constant for ManNAc	(23.76 mmol / L)
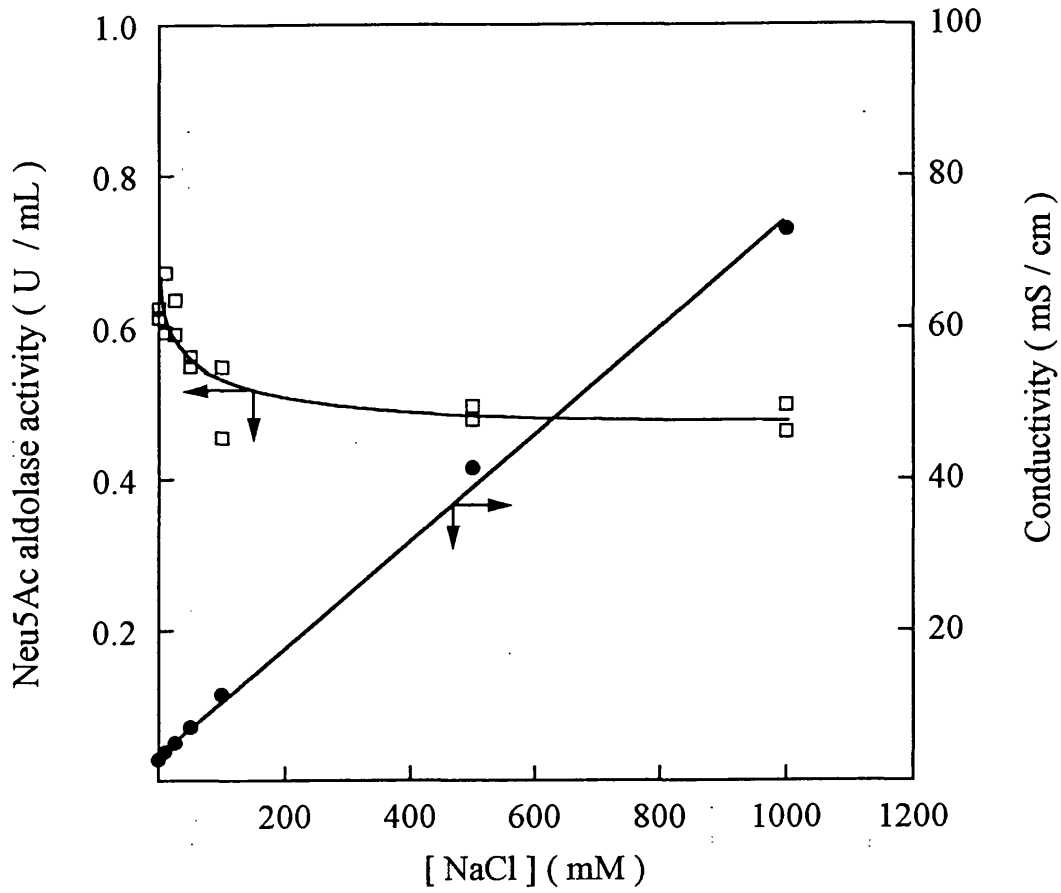
Figure 2.2 Effect of NaCl on Neu5Ac aldolase activity. Neu5Ac Aldolase activity in the synthesis direction (□) was assayed for different NaCl concentrations. Conductivity of substrate solutions (●) is shown as a function of NaCl.
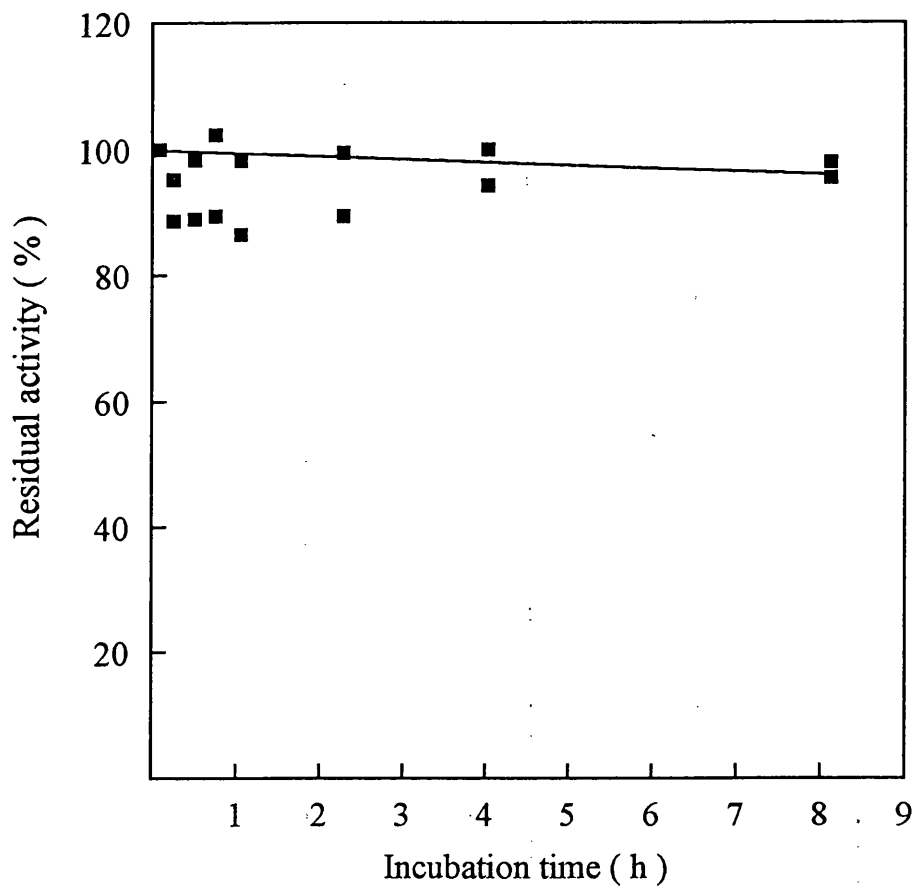
Fig. 2.3   Effect of ionic strength on Neu5Ac aldolase stability. Neu5Ac aldolase residual activity (■) was measured in the presence of 1M NaCl. The incubation was stopped by gel filtration, followed by protein and spectrophotometer assays.

Equation 2.1 described both the synthesis and cleavage of Neu5Ac within the experimental limits of 305 mM ManNAc and 562 mM Pyr (Kragl, 1992). In this thesis, the reaction rate equation (2.1) was integrated with equilibrium and mass conservation assumptions (Equations 2.2, 2.3,2.4):

$$A + B \rightleftharpoons P \qquad (2.2)$$

where:

$[A] = [A]_0 - [P] \qquad (2.3)$

$[B] = [B]_0 - [P] \qquad (2.4)$

where:

$[A]_O$ mol / L   Pyruvate concentration at time = 0

$[B]_O$ mol / L   ManNAc concentration at time = 0

and the integral function was found (2.5):

$$[P] + \frac{\gamma}{2\alpha} * \ln\left|[P]^2 - \delta[P] + [A]_0[B]_0\right| + \frac{\alpha\gamma + 2\beta}{2\alpha\phi} * \ln\frac{\left|[P] - \frac{\delta + \phi}{2}\right|}{\left|[P] - \frac{\delta - \phi}{2}\right|} = \frac{[E] * A\,\max h}{\alpha * KiA * KmB} * t + C \qquad (2.5)$$

where:

$$\alpha = \frac{KiB * KmP - KiA * KmB}{KiA * KmB * KiB * KmP}$$

$$\beta = \frac{A\,\max r}{KmP} * \frac{KiA * KmB}{A\,\max h}$$

$$\gamma = \frac{1}{KmP} - \frac{KmA}{KiA * KmB} - \frac{1}{KiA} - \frac{[A]_O}{KiB * KmP} + \alpha\beta$$

$$\delta = [A]_O + [B]_O + \beta$$

$$\phi = \sqrt{\delta^2 - 4*[A]_O[B]_O}$$

C = integration constant at t = 0, variable with initial conditions.

The above equation was solved numerically with Excel™ spreadsheet computer package. The function was employed to simulate batch reaction profiles. Varying the initial reactants

molarities and the amount of enzyme, the Kragl model was extrapolated to high substrate concentrations. Figure 2.4 shows simulations of a batch reaction starting from different equimolar concentrations of Pyr and ManNAc. The equilibrium point was always below the stoichiometric full conversion. However, a higher conversion was achieved as predicted (Section 2.4.1.2) when operating at high substrate molarities. A clear decrease of reaction rate due to Neu5Ac accumulation was also identified. Hence it was necessary to validate experimentally the model at high substrates concentrations, beyond the limit investigated by Kragl *et al.* (1992).

2.4.2.2 BIOTRANSFORMATION AT HIGH SUBSTRATE CONCENTRATION

In order to investigate the chemical stability of the reaction components and the enzyme activity and stability under potential process conditions, reactions were done at high concentrations of substrates, in order to obtain high product concentrations. Pyruvate was used in excess to obtain a high yield on ManNAc, which is expensive. During a biotransformation with 1.05 M Pyruvate and 0.55 M ManNAc at pH 7.14-7.43 (Figure 2.5) the mass balance was complete, indicating no side reactions or decompositions took place and yielding 94.4 % conversion on ManNAc. The results confirm that the amount of active enzyme during the reaction remained unchanged. Nevertheless, the reaction rate declined as expected on approach to equilibrium. Hence to maintain higher reaction rates removing product while the reaction proceeds (*in situ* product removal, Freeman *et al.*, 1993) may prove beneficial. The reaction profile however was slower than that predicted by the model, suggesting a case of substrate inhibition under these experimental conditions.

2.4.2.3 EFFECT OF ManNAc AND Pyr ON Neu5Ac ALDOLASE ACTIVITY

Aldolase activity as a function of ManNAc and pyruvate concentrations was measured in the direction of Neu5Ac formation (Figure 2.6). Pyruvate has a strong inhibitory effect on initial rates of reaction: the aldolase activity decreased above 0.5 M pyruvate up to 3.6 M (saturation concentration). Likewise, at high ManNAc concentrations the activity fell. A maximum activity was found around 750 mM on this substrate, in accordance with the previously measured Km (402 mM, Kragl *et al.*, 1992). Kragl and co-workers (1992) also described a non-specific inhibition at high molarities of all components of the medium, on account of viscosity increases. Here, a detailed quantitative description of the effect of
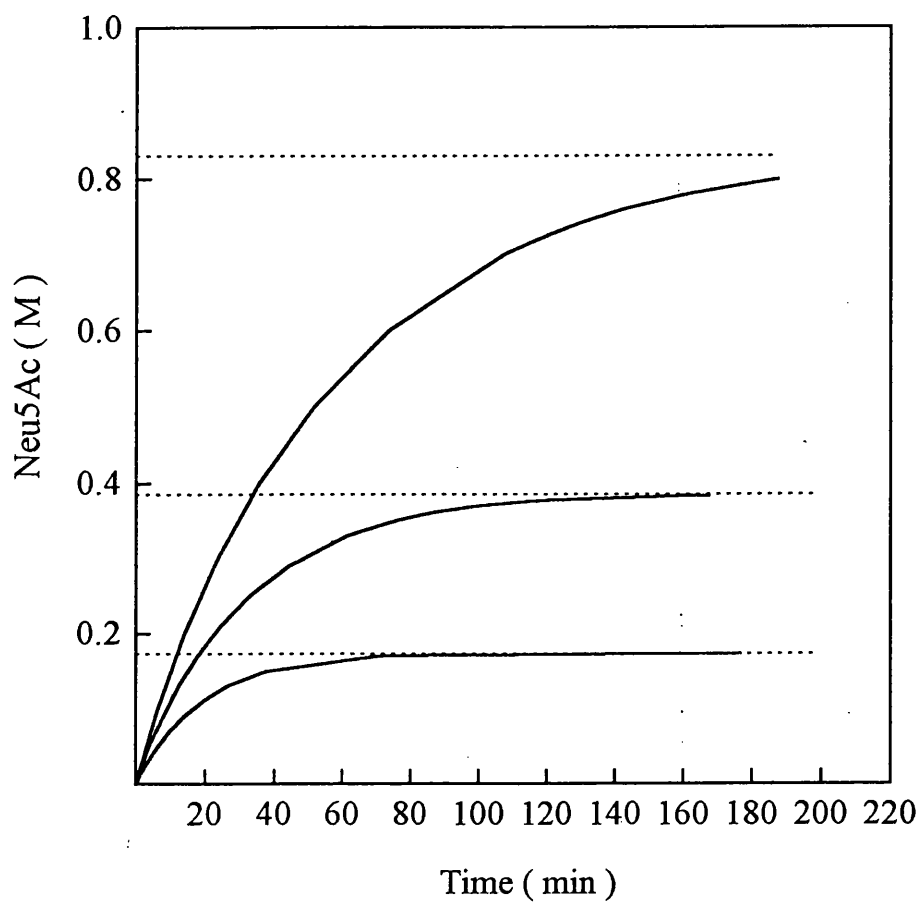
Figure 2.4   Neu5Ac synthesis batch simulation. The reaction profiles were obtained by numerical integration of the model developed by Kragl *et al.* (1992). Simulation conditions: 25 °C, pH 7.5, 1.9 g / L Neu5Ac aldolase, equimolar concentrations of ManNAc and Pyr at 1.0 M, 0.5 M and 0.25 M. Dotted lines: equilibrium concentrations.
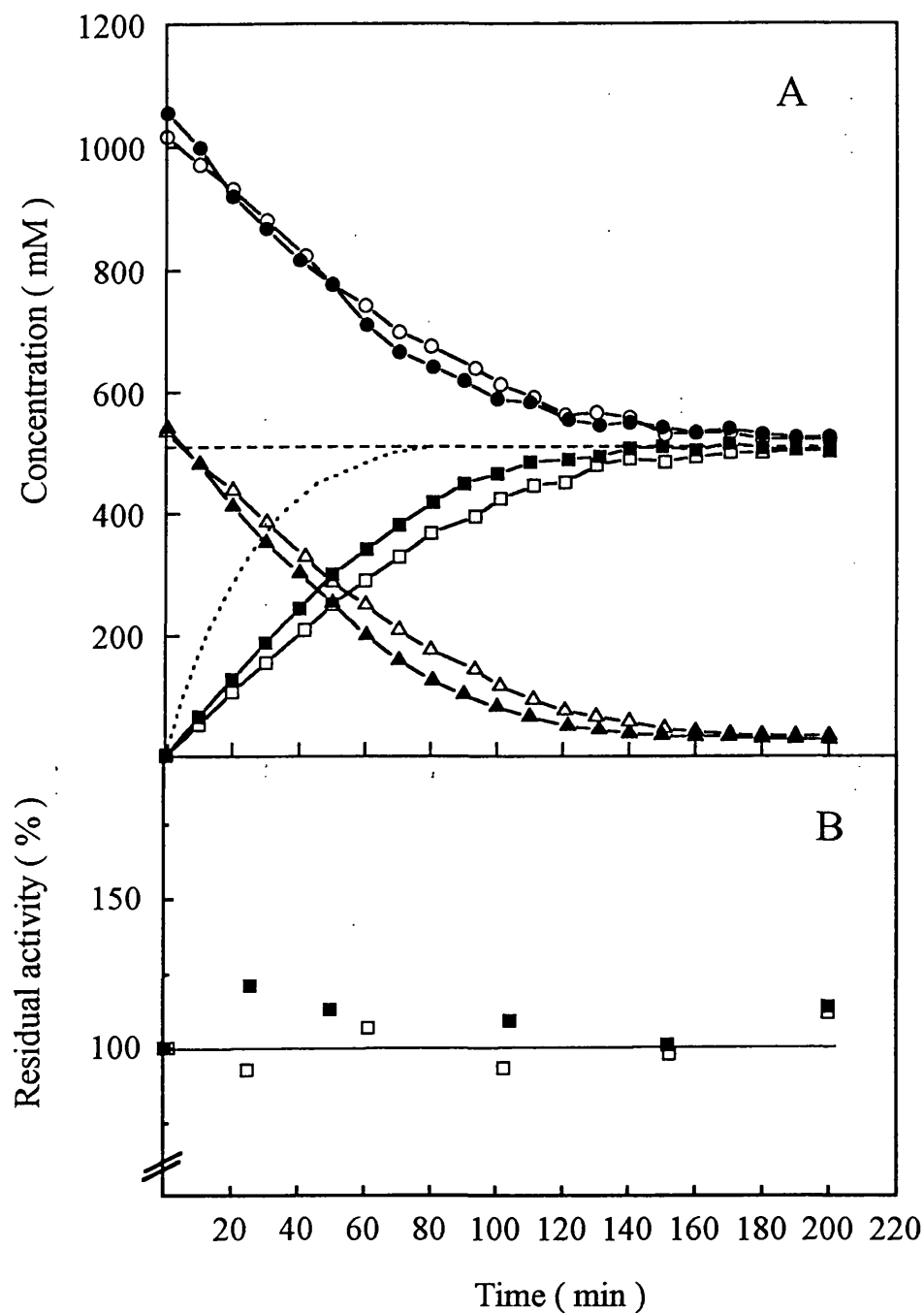
Figure 2.5 Biotransformation kinetic profiles for reactions at pH 7.43 (●, ▲, ■) and pH 7.13 (O, Δ, □) upon approach to equilibrium (-------). Pyruvate (O, ●), ManNAc (Δ, ▲) and Neu5Ac (□, ■) concentrations are given (A) along with Neu5Ac aldolase stability (B: □, ■). Dotted line: Neu5Ac profile simulation based on the model of Kragl and co-workers (1992).
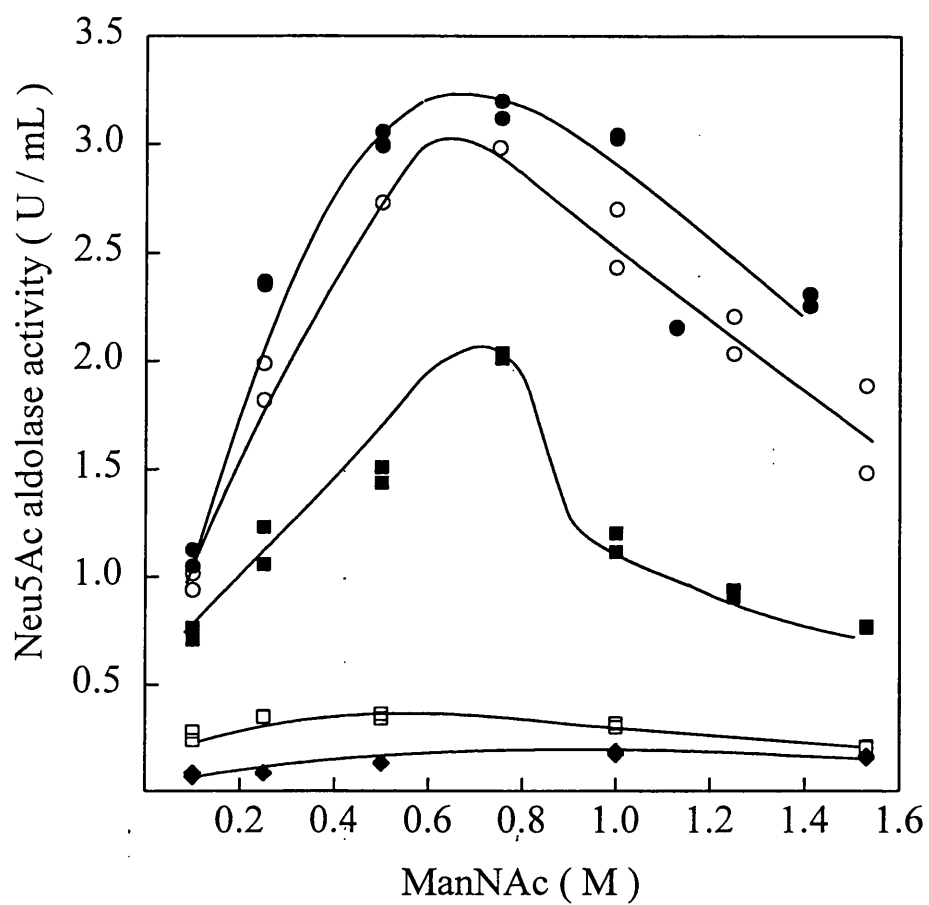
Figure 2.6 Effect of ManNAc and Pyr on Neu5Ac aldolase. Activity of Neu5Ac aldolase (in the synthesis direction) as a function of ManNAc concentration in the presence of 0.1 (●), 0.5 (○), 1 (■), 2 (□), and 3.6 (◆) M pyruvate.

ManNAc and pyruvate concentration on enzyme activity is presented. These data suggest the logic of substrate feeding in order to maintain low concentrations of pyruvate and resultant high reaction rates.

2.4.3 METHODOLOGY

In the design of biotransformation processes, Lilly and Woodley (1996) recognised the importance of collecting data on the properties of substrates and product, the reaction, the biocatalyst and its interactions with substrates and products to identify process constraints. In this thesis, the rationale of data collection for a biotransformation process is proposed (Figure 2.7). Established data on the reaction system would dictate the conditions under which the first set of experiments should be carried out. The component characterisation is designed specifically to collect information on the elements of the process: substrates and products, biocatalyst and reaction. This set of data will indicate advantageous reaction conditions on which to focus subsequent work and eliminate others as unfeasible. In this way the experimental conditions for the second set of experiments are more focused, saving time and accelerating the design procedure. The second set of data concerns the interactions of the reactants and products with the biocatalyst. As a result of this second group of experiments, feasible process options can be then evaluated, selected and further validated. The proposed methodology for data collection would provide the rational basis for the correct process synthesis at very early stages of design.
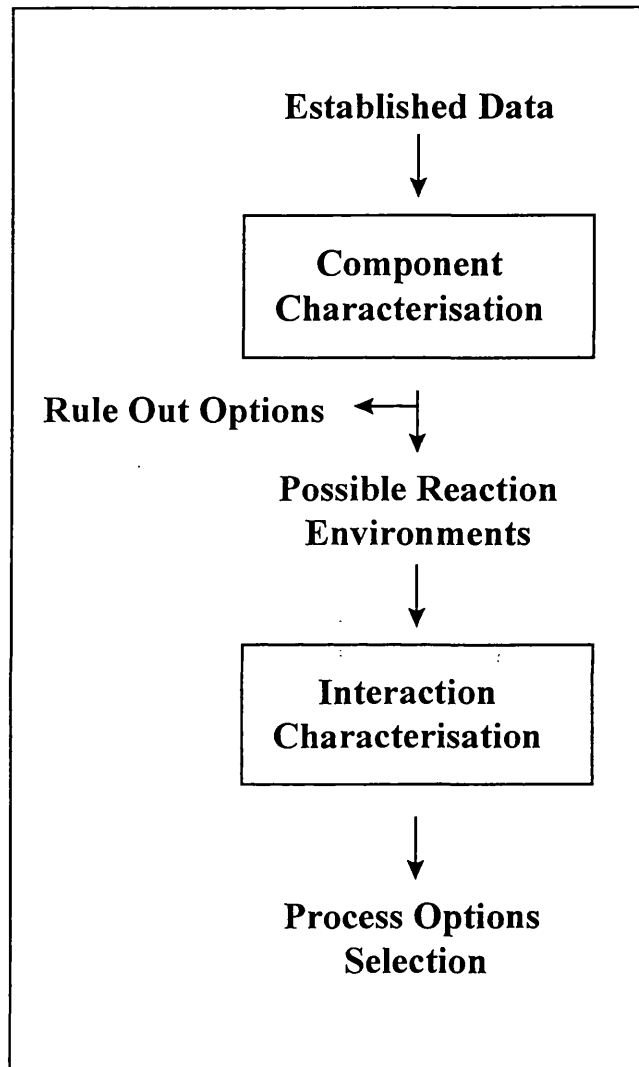
Figure 2.7   Rationale for data collection in process design. The blocks represent experimental work.

2.5 SUMMARY

In the characterisation of the chemo-enzymatic synthesis of Neu5Ac the following results contributed to the definition of process options and constraints:

- The solubility limits of ManNAc (1.6 M) and pyruvate (3.6 M) at pH 7.5 provided a limit for the maximum substrate concentrations in the biotransformation reaction.

- Degradation of all reaction components under alkaline conditions (pH 11.5) and in particular pyruvate imposed a constraint on the feasibility of integrated biotransformation at high pH.

- The substrates and product are stable at acid pH, indicating crystallisation feasibility. However, the integration of crystallisation and biocatalysis was unfeasible since Neu5Ac aldolase is not active below pH 3.5 (Uchida *et al.*, 1984).

- Due to equilibrium, Pyr and ManNAc can not achieve full stoichiometric conversion. There is however a thermodynamic advantage in operating at high substrate molarities.

- The reaction rate decreased on approach to equil ibrium. To maintain higher reaction rates removing product while the reaction proceeds (*in situ* product removal) may prove beneficial.

- Pyruvate had a strong inhibitory effect on initial rates of reaction and ManNAc was found inhibitory above 750 mM. Substrates feeding strategies may overcome kinetic limitations.

A rational approach for data collection in biotransformation process design is presented. The methodology discussed would accelerate process synthesis, ruling out options at the initial phases of design.

3

PROCESS OPTION EVALUATION

3.1 INTRODUCTION

The importance of process evaluation lies in the necessity to identify a process system that could be transferred to manufacturing without significant modifications in later stages of design. Regulatory authority constraints impose little flexibility once the manufacturing process has reached validation and approval (Lee and Robinson, 1995). A framework for the scientific basis is therefore necessary at an early stage of design to rule out unfeasible process options and focus on effective production and separation techniques based on the characteristics of the reaction components, the reaction type and their reciprocal interactions (Lilly and Woodley, 1996). Further quantitative data would be then required to optimise the selection process. In this thesis, the enzymatic conversion of ManNAc and Pyr to Neu5Ac is the subject of further investigations. The aim of this chapter is to provide the framework to the constraints identified in chapter 2 in order to define a region of operation for the biotransformation step (Section 3.2), consider various reactor designs (Section 3.3) and evaluate the rationale for integration between the bioconversion step with both the epimerisation upstream and Neu5Ac recovery (Section 3.4) downstream.

3.2 OPERATING WINDOWS

Knowledge based systems have been developed for the design of protein recovery (Gandikota et al., 1992, Asenjo, 1996) and biotransformation processes (Dervakos et al., 1989). Although initial flow sheeting is achievable with these methods, the interactions of operating variables requires further examination (Woodley and Titchener-Hooker, 1996). It is possible to relate constraints of a particular process operation in a graphical form, defining a "window" of operation. These limits could be dictated by either the system, the process technology or the economics of the process. Operating windows were originally

used as a design tool for qualitative estimation of process boundaries in fermentation (Lilly, 1983). Moreover, operating windows have been developed to select quantitative process conditions in protein separation and two liquid phase biocatalysis (Woodley and Titchener-Hooker, 1996). This approach is here applied to the Neu5Ac aldolase reaction.

For the Neu5Ac biotransformation step, the optimum pH is 7.0-7.5, since the reactants, the product (Table 2.2) and the enzyme (Figures 2.1, 2.5) are all stable in this range and the enzyme is optimally active. The data for enzymatic activity (Figure 2.6) at concentrations of ManNAc and pyruvate up to aqueous saturation values can be presented in the form of an operating window (Woodley and Titchener-Hooker, 1996). The process boundaries shown in Figure 3.1 for a batch reaction are the solubilities of the substrates, the final obtainable product concentration (arbitrarily fixed at 0.2 M Neu5Ac at equilibrium) and the enzymatic activity (arbitrarily fixed at 50 % of maximum). These boundaries have been plotted as a function of the reactor concentrations of ManNAc and pyruvate. Hence a region (operating window) can be defined where the operating conditions meet the necessary productivity requirements. Further limitations can be introduced to examine the sensitivity of the operating window. In order to illustrate this concept three examples are presented in Figure 3.2. As shown in figure 3.2A, a limit may be set by the demands of subsequent downstream processing (here arbitrarily set by a ratio of 10-fold Neu5Ac to pyruvate, important in ion exchange chromatography, Auge' et al., 1984). In this case, pyruvate feeding could prove beneficial, which would also have the effect of reducing enzyme inhibition and further enhancing productivity. Limitations may also be set by economic constraints. For example, the most expensive substrate would need higher conversion (arbitrarily set to 90 % conversion on ManNAc, Figure 3.2B, Sigma, 1996). Another example may require a minimal yield on both the substrates (Figure 3.2C, arbitrarily set at 80 %). Further optimisation of the biotransformation step would operate the process at a single point in the operating window. Moreover, the use of the operating windows in scale-up and transfer to manufacturing would estimate the flexibility of the operation within the set process targets. The boundaries of this operating window are partially fixed (reactants solubility) and partially defined by the engineer.
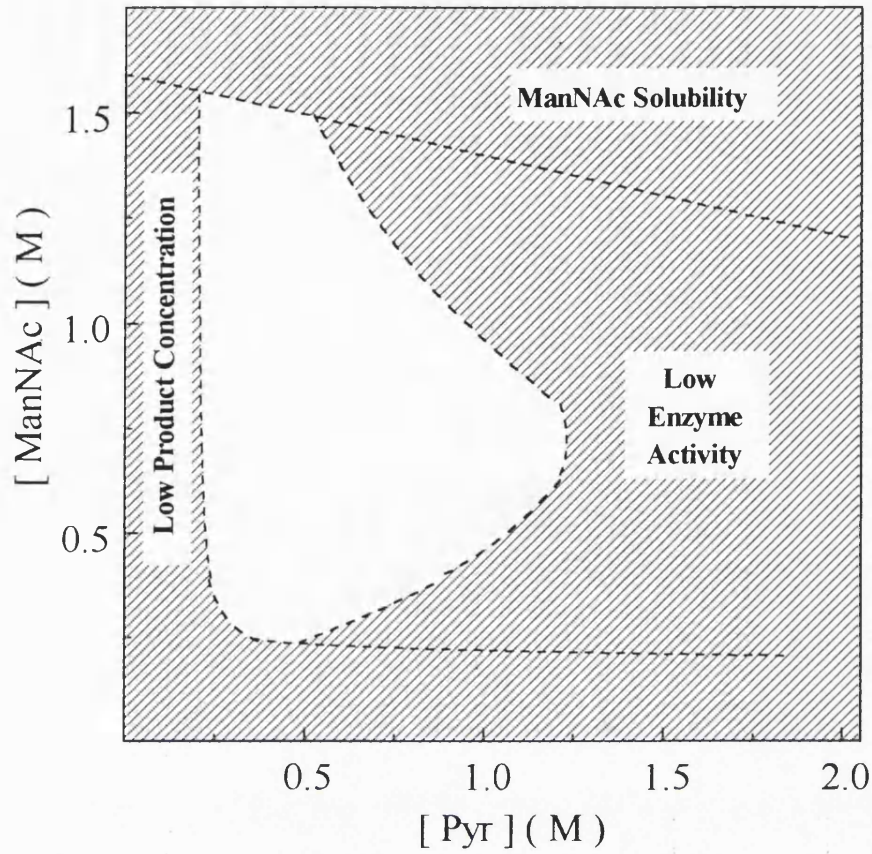
Figure 3.1   Neu5Ac synthesis process limits as a function of ManNAc and pyruvate reactor concentration. Boundaries: substrates solubility, 0.2 M final Neu5Ac concentration (arbitrarily fixed) and 50 % maximum enzymatic activity (arbitrarily fixed). The shaded area indicates inefficient operation.
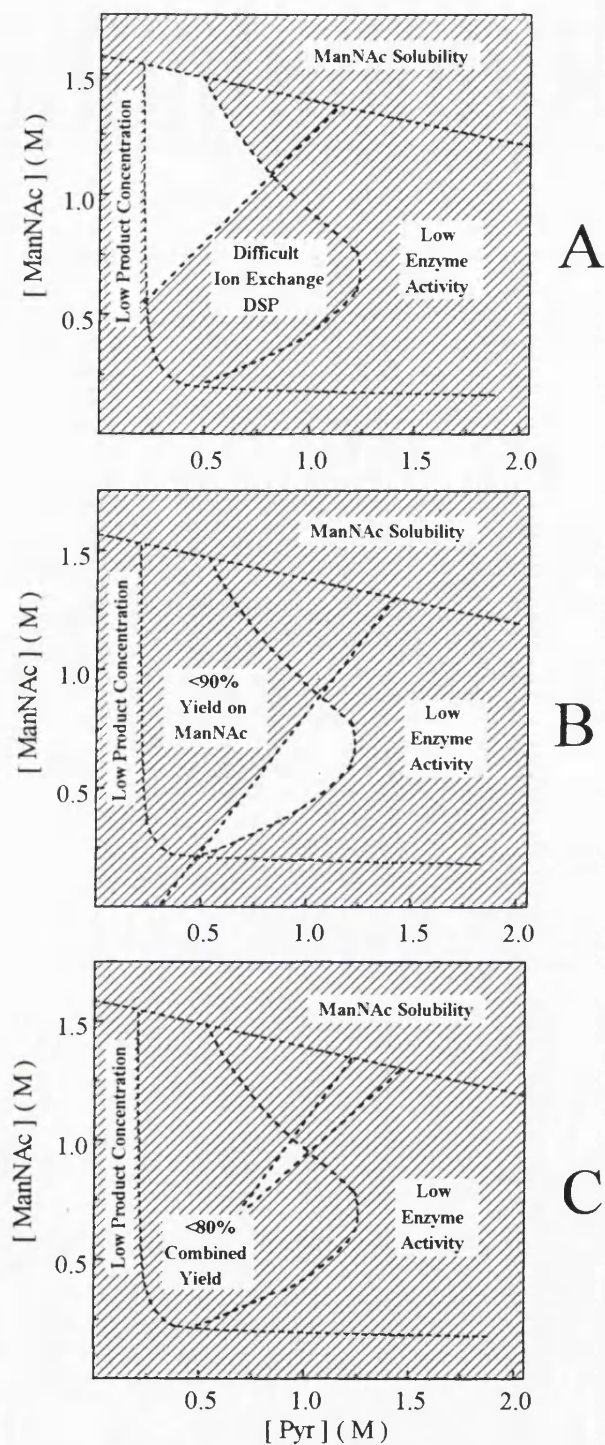
Figure 3.2 Additional constraints on Neu5Ac synthesis operating window (Figure 3.1). Process limits of downstream processing (high Neu5Ac/pyruvate ratio, A), high yield on ManNAc (B) and high yield on both substrates (C).

3.3 REACTOR SELECTION AND OPERATION

The most common reactors employed in biotransformation processes are the batch stirred tank (BSTR), with or without feeding and the plug flow reactor (PFR) (Lilly, 1992). Moreover, the stirred tank reactor could be operated continuously (CSTR), while the plug flow reactor is normally employed as a packed bed (Lilly, 1992). Lilly (1992) and Woodley and Lilly (1994) reviewed the suitability of these reactors for biotransformation according to the reactor operational characteristics, the properties of biotransformation components, the reaction type and their interaction. While the stirred tank is more suitable when homogeneous conditions should be attained (feeding, pH control, two liquid or gas liquid biocatalysis), the PFR has a larger catalyst load and less mechanical damage to the catalyst is achieved. For the Neu5Ac biotransformation step, the constraints for reactor selection and operation are listed in Table 3.1. Reaction components stability indicated pH 7.5 and 25 $^{\circ}$C as a suitable operating condition (Kragl, 1992, Section 2.4.1). The equilibrium requires high substrate concentrations, substrate excess or removal of Neu5Ac to drive the reaction towards Neu5Ac formation. Therefore one substrate fed-batch biotransformation is a feasible option, since the other would be present in excess. The kinetic constraints of the system include Pyr inhibition at high molarities and ManNAc inhibition above enzyme saturation (Figure 2.6), together with the decrease in reaction rate due to Neu5Ac accumulation. Under these constraints, several reactor designs and operations are available, each of them with advantages and drawbacks.

In this thesis, different processing scenarios are considered, based on costs or availability limitations (Table 3.2). In order to emphasize enzyme use, BSTR and PFR are more beneficial considering Neu5Ac inhibition (exposing the catalyst to high product concentration only at the end of the reaction, Wang *et al.*, 1979), where CSTR (as developed by Kragl *et al.*, 1992, 1993) is more advantageous in case of substrate inhibition, since the concentration of substrate in the reactor is equal to that of the outlet stream. Specific productivity per unit catalyst and operational stability would be important parameters in assessing the reactor performance. In case of downstream processing limitations, the presence of contaminants (Section 3.2) and final achiavable product concentration would be relevant paramenters for the choice and subsequent sizing of separation units, while elevated throughput and conversion would be significant for high substrates (Section 3.2) and

Table 3.1   Characteristics and constraints for Neu5Ac biotransformation reactor selection and operation.

Characteristics		Constraints				
		Reactor selection				Reactor operation
		FBSTR	BSTR	CSTR	PFR	
Components	pH lability Enzyme optimum					pH = 7.5 T = 25 °C
Reaction	Equilibrium	○	○		○	High substrate concentration One substrate excess
Interactions	Neu5Ac inhibition	○	○	●	○	Neu5Ac < 8-20 mM[1]
	Pyr inhibition	○	●	○	●	Pyr < 200 mM
	ManNAc inhibition	○	●	○	●	ManNAc < 750 mM

○: suitable ●: not suitable above reactor operation conditions.

1- based on enzyme saturation for Neu5Ac resolution (Uchida *et al.,* 1984, Ohta *et al.,* 1988, Aisaka *et al.,* 1991, Kragl *et al.,* 1992).

Table 3.2 **Processing bases for reactor selection and operation.**

Limit	Criterion for Reactor Evaluation
enzyme	productivity[1] / unit enzyme
	operational stability
DSP	product concentration
	product / contaminants
substrate and product	conversion
	productivity[1] / unit volume
time	productivity[1]
	operation time

1- product / unit time

product costs. Eventually, minimisation of timing would reduce operating expenditures of production units.

The following designs have been selected for further evaluation under possible reaction conditions (Chapter 5):

- Biotransformation with pyruvate feed
- Biotransformation with feed of ManNAc and Pyr to maintain Pyr below 200 mM and ManNAc below 750 mM.
- Biotransformation in a plug flow reactor.
- Biotransformation with *in situ* Neu5Ac removal.

Batch reaction with excess Pyr has been already assessed (Chapter 2). Feeding strategies would be beneficial for enzyme kinetics and possible reactor-downstream integration (Section 3.4). A plug flow reactor can be operated in continuous mode with the benefits of a batch kinetics in relation to Neu5Ac inhibition. In addition, *In situ* product removal would overcome Neu5Ac detrimental effects together with equilibrium constraints and achieve full substrates stoichiometric conversion. The validation of these reactor configurations and their operating characteristics will be investigated in Chapter 5 for different processing frameworks.

3.4 INTEGRATED PROCESSES

In the design of bioprocesses, the potential of a step change in production techniques could overcome the constraints imposed on a particular biotransformation and permit processes otherwise unfeasible (Woodley and Lilly, 1996). Moreover, the integration of production with other steps of the process is required in order to select optimal flow sheet (Cooney *et al.*, 1988; Bruin, 1992) and operating strategies (Ingleby, 1986; Middleberg *et al.*, 1992). There are several process advantages when the production step is integrated with downstream processing operations. Minimisation of interferences due to product accumulation, reduction of product losses due to the reaction conditions and limitation of number of downstream processing are among the most important factors in fermentation and

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biotransformation (Freeman *et al.* 1993). Moreover, an unfavourable thermodynamic equilibrium may be shifted by *in situ* product removal (Woodley and Lilly, 1994).

The characteristics of Neu5Ac biotransformation have been investigated to determine the process options and in particular the feasibility of integration of a biotransformation with either the upstream chemical epimerisation or the downstream product recovery. One of the simplest techniques for recovery of the product, Neu5Ac, is crystallisation in acetic acid at low pH (Lin *et al.*, 1992). Although all the reactants and the product were found to be relatively stable at pH 2 (Table 2.2) the enzyme loses activity below pH 3.5 (Uchida *et al.*, 1984) thus there is no justification for integration of the biotransformation and product recovery, unless an alternative to crystallisation is used. However, other methods of *in situ* product removal (Freeman *et al.*, 1993), such as ion exchange chromatography (Schauer, 1973), may prove possible and would improve the conversion yield and reaction rate. The evaluation of an *in situ* product removal system for the synthesis of Neu5Ac will be developed in Chapter 5.

The advantage of integration between the chemical epimerisation and the biotransformation step is that it overcomes the unfavourable equilibrium for conversion of GlcNAc to ManNAc by removal of the latter compound by the biotransformation. The rate of epimerisation of GlcNAc to ManNAc is highly pH-dependent (Lee, 1990) and operation at pH 10.5-11 is necessary for a reasonable conversion rate. Preliminary experiments to determine the characteristics of the biotransformation showed that at high pH pyruvate was particularly unstable itself (Table 2.2), although it is reported to have a protecting role on Neu5Ac aldolase deactivation (Uchida *et al.*, 1984). Batch biotransformations at pH 9-11 have been reported (Tsukada and Ohta, 1994) but the results reported in Chapter 2 also indicate an inevitable loss of pyruvate through alkaline degradation. Thus selection of the appropriate operating conditions for the combined epimerisation and biotransformation requires further investigation (Chapter 4).

3.5 SUMMARY

In this chapter, the characterisation of the chemo-enzymatic synthesis of Neu5Ac has provided constraints as a basis for a rational approach to process selection, identifying operating limits at an early stage and ruling out potential integrated process options. Pyruvate was found to be inhibitory at high concentrations, suggesting the need for feeding. As a result of this approach, an operating window was defined, relating the process boundaries in the form of a diagram. Feeding strategies for both pyruvate and ManNAc together with plug flow reactor configuration have been selected for further evaluation to the imposed process constraints

(Chapter 5). The rationale of integration between the biotransformation step together with either the epimerisation (Chapter 4) and ion exchange chromatography (Chapter 5) is discussed.

4

EPIMERISATION-BIOTRANSFORMATION INTEGRATION

4.1 INTRODUCTION

In this chapter, the feasibility of integration between the biotransformation step and the chemical epimerisation is investigated in order to assess the process constraints and advantages of an integrated chemo-enzymatic reaction. The biotransformation step under alkaline conditions has been reported in a commercial patent by Tsukada and Ohta (1994). GlcNAc and Pyr were converted to Neu5Ac by Neu5Ac aldolase at alkaline pH over 5 days and % conversion based on limiting substrate is shown in Figure 4.1. pH 10.5 appears to be the most beneficial condition for the combined operation. However, none of the reaction described achieved more than 52 % yield (63 % of calculated equilibrium conversion, based on $Keq = 4.69$ M^{-1}, Kragl, 1992 and Roseman and Comb, 1958[1]). The patent also described the improved stability of the aldolase between pH 10 and 11 due to the presence of Pyr and GlcNAc (Figure 4.2). No detail was given for the source of the enzyme and its form. These data have been considered in the characterisation of the system. Another example of integrated reaction was developed by Kragl and Co-workers (1991) using N-acylglucosamine 2-epimerase (E.C. 5.1.3.8) and Neu5Ac aldolase from *E. coli* in a membrane reactor operated continuously at pH 7.5. However, this design did not prove efficient on large scale since the epimerase is not currently available in large quantities and it requires ATP (Kragl, 1992). Likewise, both the enzymes are inhibited by Neu5Ac, which is at its highest concentration, due to CSTR kinetics. In this chapter, in order to evaluate the constraints of a combined chemo-enzymatic synthesis, the epimerisation reaction is investigated to define its operating limits (Section 4.3.1) and further characterisation of the biotransformation components is undertaken under alkaline conditions (Section 4.3.2). An example of an integrated reaction is then presented in Section 4.3.3. Moreover,

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[1] Reactions performed at 30 °C. Data extracted by linear regression of van't Hoff equation for Neu5Ac synthesis (Kragl, 1992).
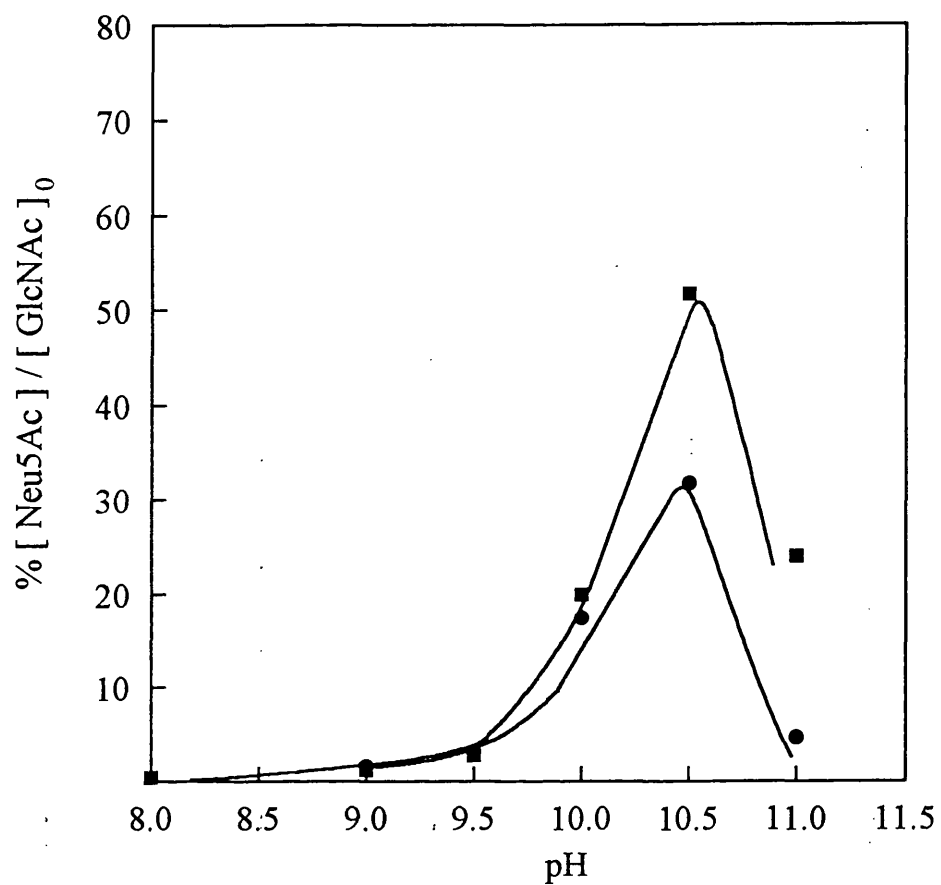
Figure 4.1   Yield of integrated chemo-enzymatic synthesis of Neu5Ac according to Tsukada and Ohta, 1994. (■) 1636 mM Pyr, 813 mM GlcNAc. (●) 409 mM Pyr, 203 mM GlcNAc.
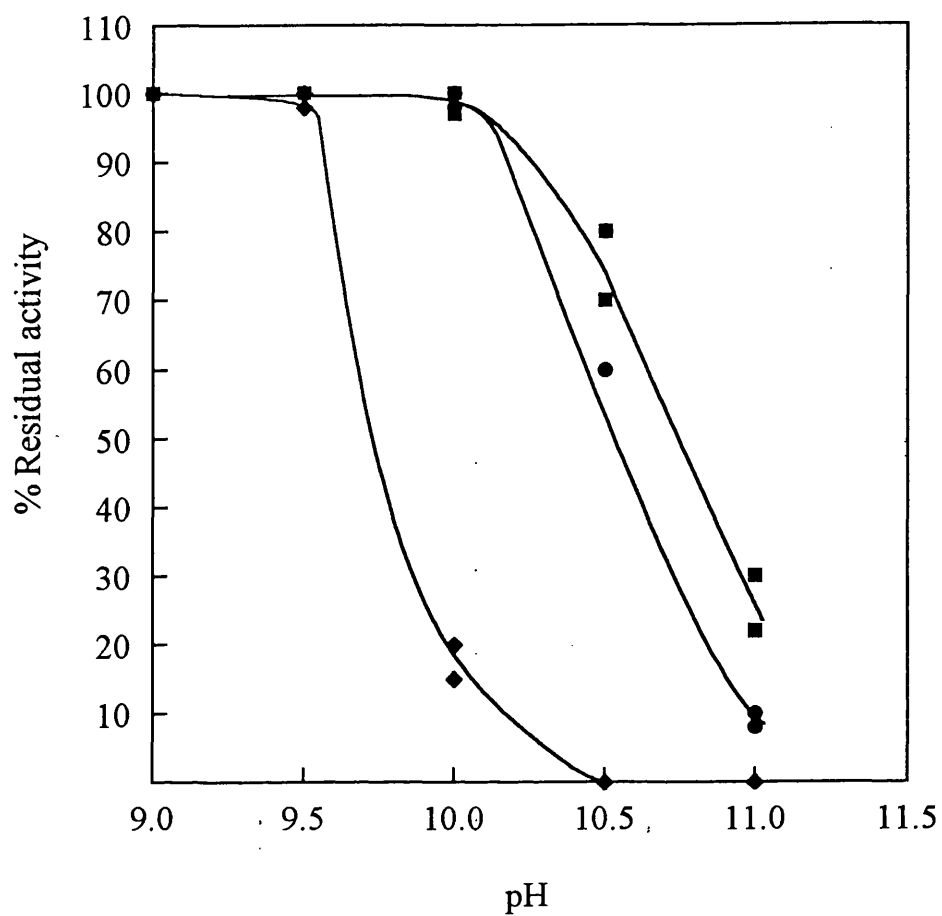
Figure 4.2   Stability profile of Neu5Ac aldolase according to Tsukada and Ohta (1994).

Conditions: 16 hours incubation with 1636 mM Pyr and 813 mM GlcNAc (■), 409 mM Pyr and 203 mM GlcNAc (●), control (◆).

the economic scenarios for further reaction optimisation are discussed.

4.2 MATERIALS AND METHODS

4.2.1 MATERIALS

As described in Section 2.3.1, chemicals were supplied by Sigma except for Neu5Ac dihydrate (from Glaxo Group Research) and *E.coli* Neu5Ac Aldolase (from Toyobo) (Chemical suppliers are given in Appendix 1).

4.2.2 METHODS

4.2.2.1 GlcNAc-ManNAc EPIMERISATION RATE DETERMINATION

20 mL of 260 , 258, 245, 272, 260 and 265 mM GlcNAc were incubated for 8 hours at pH 8.5, 9.5, 9.9, 10.0, 11.0 and 12.0 respectively in a Radiometer ETS 200 pH-Stat, titrant 1 M NaOH, at 25 °C. Samples (200 μL) were taken at time 0, 2, 4 and 8 hours and diluted 1:50 in 6 mM H_2SO_4 followed by HPLC analysis (Section 2.3.2.2). The initial rate of ManNAc formation was calculated from the linear slope of adjusted concentration-time plot. The approximate reaction constant was calculated according to Section 4.3.1.

4.2.2.2 GlcNAc-ManNAc EPIMERISATION REACTIONS

80 mL of 260 mM GlcNAc solution were prepared with pH adjusted to 10.7, 12.0 and 13.2 respectively with 4 M NaOH. The solution was filter sterilised and 5 mL aliquots were transferred aseptically to sterile vials (7 mL). Nitrogen was sparged on the top of each sealed vial to maintain constant pH and the vials were incubated at 25 °C. Each vial was withdrawn from incubation as a sample. The reaction was monitored for 72 hours by HPLC (samples at 0, 2, 4, 8, 24, 48 and 72 hours). 200μL from each sample was diluted 1:50 in 6 mM H_2SO_4, and analysed by HPLC (Section 2.3.2.2). pH was measured on each sample with a Mettler Toledo Delta 340 pH meter. The equilibrium constant was calculated from the average of a quadruple sample at the end of reaction.

4.2.2.3 STABILITY OF REACTION COMPONENTS SIMULATING A BIOTRANSFORMATION

20 mL of mixtures containing 760 mM Pyr and 101 mM ManNAc; 700 mM Pyr, 52.1 mM ManNAc and 47.7 mM Neu5Ac; 700 mM Pyr and 100 mM Neu5Ac respectively were incubated at pH 11.5, 25 °C for 8 hours in a radiometer ETS-200 pH-Stat, titrant 0.1 M NaOH. Samples (200 µL) were diluted 1:100 in 6 mM H_2SO_4 and analysed by HPLC (Section 2.3.2.2). In control experiments, 500 mM Pyr and 100 mM GlcNAc were incubated separately following the above protocol.

4.2.2.4 PYRUVATE STABILITY IN ALKALINE CONDITIONS

20 mL of 105 mM Pyr, 50 mM ManNAc and 202 mM GlcNAc were incubated together with 1.4 g / L Neu5Ac at pH 11.4 for 4 hours under the conditions described in the previous section. In another experiment, 200 mL of 500 mM Pyr were incubated with 1.4 g / L Neu5Ac aldolase at pH 10.5 as above.

4.2.2.5 EFFECT OF NITROGEN ON PYRUVATE DEGRADATION

15 mL of 102 mM Pyr were incubated together with 266 mM GlcNAc and 1.33 g / L Neu5Ac aldolase at 25 °C, pH 11.4 in a ETS-200 pH Stat, Titrant 100 mM NaOH. The experiment followed then the protocol described in Section 4.2.2.3 for 50 hours. In a separate experiment, 70 mL of 100 mM Pyr, 260 mM GlcNAc and 1.3 g /L Neu5Ac aldolase were titrated to pH 11.4 with 4 M NaOH and then the experiment followed the procedure in section 4.2.2.2 for 50 hours.

4.2.2.6 Neu5Ac ALDOLASE STABILITY AT ALKALINE pH

The enzyme stability experiments were performed with 0.5 M ManNAc (pH 10.5 and 11.3) and 0.5 M GlcNAc (pH 8.5, 9.5, 10.0, 10.5, 11.0, and 11.35) according to the protocol described in Section 2.3.2.4. In a separate experiment 124 µg / mL Neu5Ac aldolase was incubated with 0.5 M pyruvate at 25 °C, pH 10.5. Samples were diluted in 100 mM Tris-HCl buffer (pH 7.9) and subsequently injected onto a size exclusion Hi-Trap™ desalting gel filtration column (1.6 x 2.5 cm, Pharmacia), pre-equilibrated with Tris buffer. The experiment then followed the protocol described in Section 2.3.2.10. The gel filtration elution profile and protein assay calibration curve are shown in Appendix 2.

4.2.2.7 BIOTRANSFORMATION AT ALKALINE pH

80 mL of 120 mM Pyr and 250 mM GlcNAc were incubated together with 1.34 g / L Neu5Ac aldolase (24.1 U / mg) at pH 10.53. The experiment then followed the protocol described in Section 4.2.2.2 for 99 hours. Additional 1 mL was taken from each sample and dialysed (MW cut-off: 20 KDa, Sigma) for 24 hours against 1500 volumes of 100 Tris-HCl buffer, pH 7.9. Remaining enzyme activity and protein concentration were measured according to Section 2.3.2.5 and Section 2.3.2.6 respectively. The equilibrium constant was calculated as described in Section 4.2.2.2.

4.3 RESULTS

4.3.1 EPIMERISATION

The conversion of GlcNAc to ManNAc can be achieved chemically (Roseman and Comb, 1958) or enzymatically (Kragl *et al.*, 1991) (Section 1.3.4.1) The base catalysed epimerisation of GlcNAc to ManNAc follows a first order reversible tautomerisation described by Salo *et al.* (1976). The rate of ManNAc formation can be described by the following equations (4.1, 4.2):

$$GlcNAc \underset{k_b}{\overset{k_a}{\rightleftharpoons}} ManNAc \tag{4.1}$$
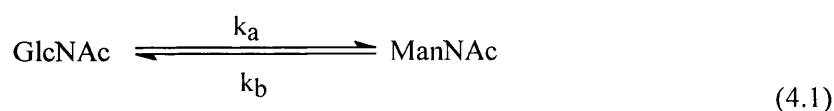
$$\frac{d[ManNAc]}{dt} = k_a[GlcNAc] - k_b[ManNAc] \tag{4.2}$$

where:

k_a h^{-1} Kinetic constant of GlcNAc epimerisation

k_b h^{-1} Kinetic constant of ManNAc epimerisation

The kinetic parameters have been investigated by Lee (1990) at pH 11 and pH 12.3. The higher pH was shown to increase the forward reaction rate 12 times, while the backward reaction constant increased only by 8 fold. In this work, it was necessary to investigate the boundaries of the chemical epimerisation. The rate of ManNAc formation was measured for

8 hours at 25 °C between pH 8.5 and 12 and the approximate kinetic constant of GlcNAc epimerisation (4.3) was obtained for each pH (Figure 4.3). The epimerisation did not occur below pH 9 and the kinetic constant increased exponentially to $39.88 * 10^{-3}$ hours at pH 12.

$$k_{a_{app}} = \frac{\frac{d[ManNAc]}{dt}}{[GlcNAc]} \qquad (4.3)$$

where:

$$[ManNAc] \approx 0, \quad [GlcNAc] \approx [GlcNAc]_o$$

The epimerisation was then followed for 72 hours at pH 10.7, 12.0 and 13.2 (Figure 4.4). At pH 10.7 the mass balance indicated that no decomposition took place (Figure 4.5) and the equilibrium constant was measured:

$$Keq = \frac{[ManNAc]}{[GlcNAc]} = 0.249 \pm 0.0032$$

which is in agreement with published data (Roseman and Comb, 1958; Hanchak *et al.*, 1976, Lee, 1990). At pH 12, 18.1 % carbohydrate degradation took place after 72 hours, while at pH 13.2, 71.7 % decomposition was measured. Although non-specific browning above pH 11 was reported (Kragl, 1992), in this work sugar degradation was observed and therefore it was possible to define pH 12 as the higher limit for epimerisation reactions.

4.3.2. COMPONENTS CHARACTERISATION

4.3.2.1 SUBSTRATES AND PRODUCT

It was necessary to investigate further the stability profile of GlcNAc, ManNAc , Pyr and Neu5Ac under alkaline conditions. As reported in the preliminary observation in Section 2.4.1.1.2, Pyr degradation was the most severe phenomenon when a mixture simulating the reaction was incubated at pH 11.5 and 37 °C (Table 2.2).
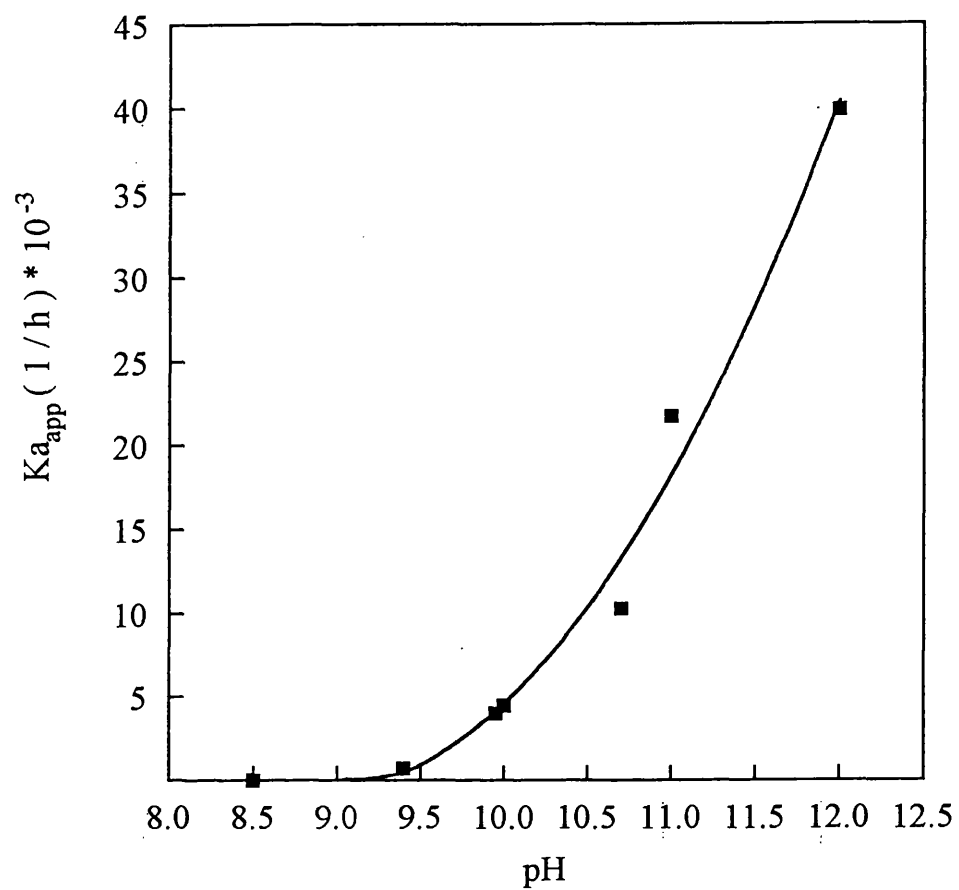
Figure 4.3   Initial GlcNAc-ManNAc epimerisation rate. The approximate kinetic constant for ManNAc formation (■) is shown for pH 8.5-12.
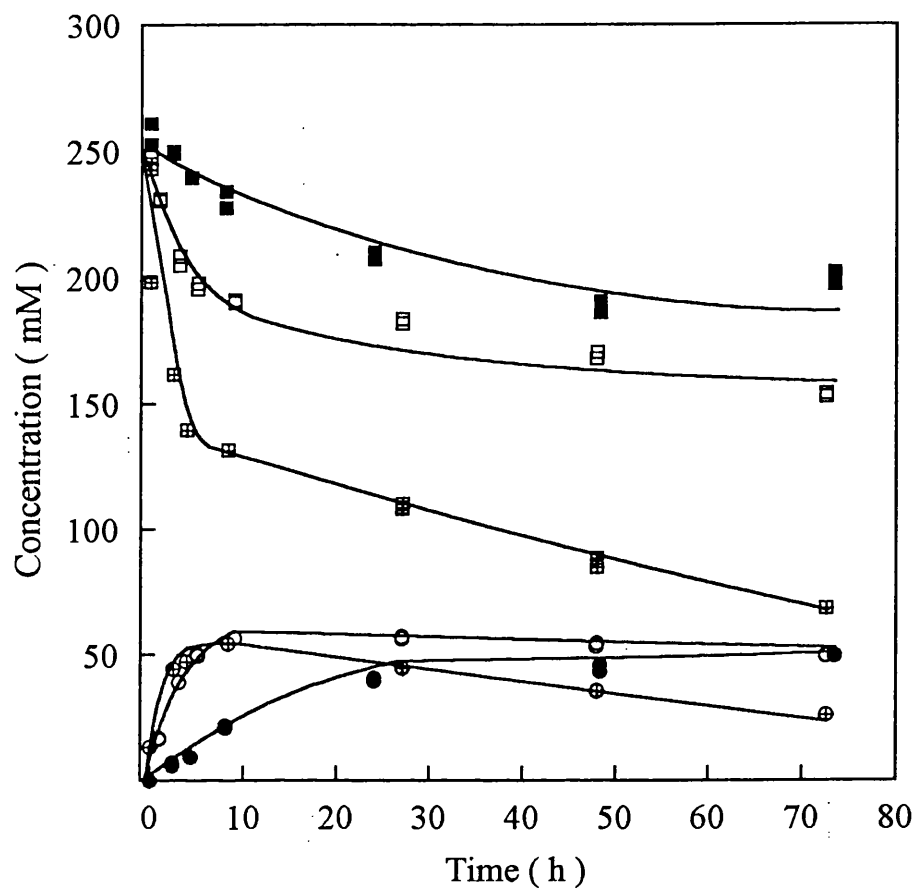
Figure 4.4 GlcNAc - ManNAc epimerisation. GlcNAc (■, □, □) and ManNAc (O, ●,

⊕) are measured at pH 10.7 (■, ●), pH 12 (□, O) and pH 13.2 (▦, ⊕).
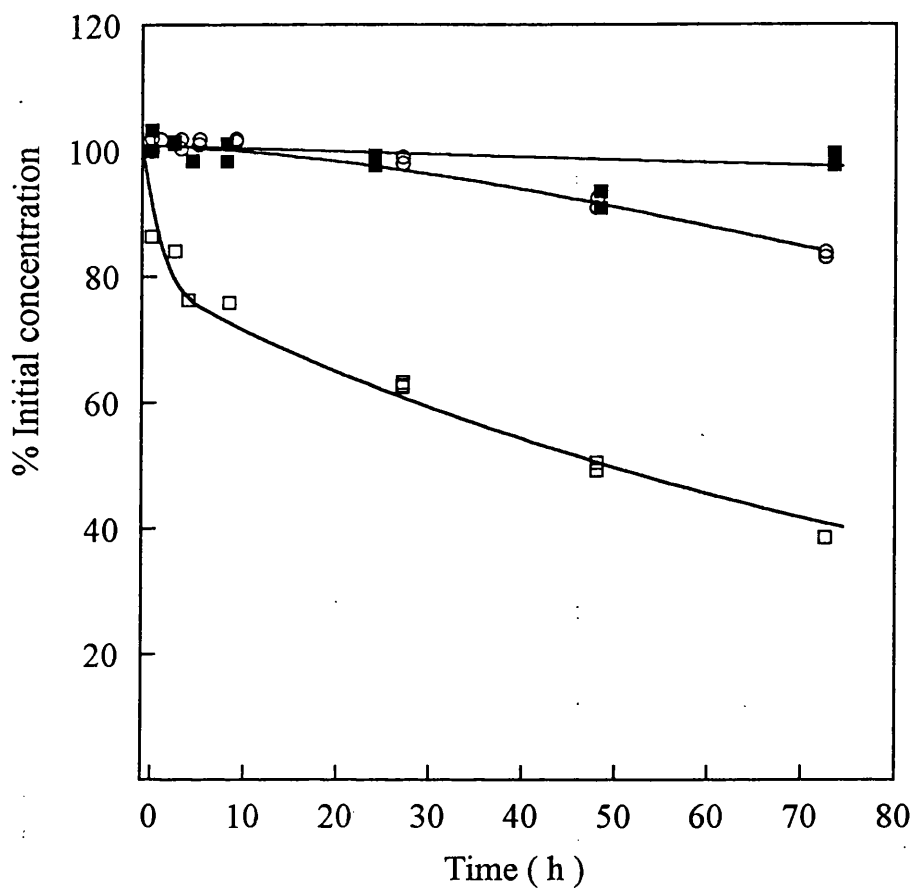
Figure 4.5   Carbohydrate mass balance during GlcNAc - ManNAc epimerisation. % Initial GlcNAc concentration is shown for reactions at pH 10.7 (■), pH 12 (O) and pH 13.2 (□) (Figure 4.4).

Table 4.1 shows the stability of the reaction components at pH 11.5, 25 °C after 8 hours of incubation. Three experiments were designed to investigate possible reciprocal effects on degradation in mixtures simulating a biotransformation reaction at its start, middle and end with excess pyruvate. 760 mM Pyr and 100 mM ManNAc were selected as start conditions for the simulated biotransformation. 700 mM Pyr, 52 mM ManNAc and 48 mM Neu5Ac were mimicking the biotransformation in the middle of the reaction and 700 mM Pyr and 100 mM Neu5Ac simulated a biotransformation at the end of the reaction. While pyruvate degradation was 74.2 - 82.5 % after 8 hours, ManNAc and GlcNAc formed during the experiment were degraded by 42 - 45.4 % and Neu5Ac was stable under these conditions. However, under similar conditions the sugars mass balance was complete. Hence GlcNAc-ManNAc degradation is likely to be affected by the presence of other reaction components. The stability of pyruvate under potential reaction environments was investigated in detail.

The kinetics of Pyr degradation in the array of conditions described in Table 4.1 was compared to that of 104 mM Pyr in the presence of 1.4 g / L Neu5Ac aldolase, 50 mM ManNAc and 202 mM GlcNAc at pH 11.5 and to that of 500 mM pyruvate with 1.4 g / L Neu5Ac aldolase at pH 10.5 (Figure 4.6). Neu5Ac aldolase was beneficial to Pyr. After 4 hours at pH 11.5, the aldolase reduced pyruvate degradation by 3.6 folds, but no Neu5Ac was detected, indicating enzyme denaturation under these conditions. Moreover, Pyr degradation was affected by pH. At pH 10.5, 76 % of initial pyruvate was measured after 4 hours, while at 11.3 only 63 % remained. Pyr degradation may occur according to decarboxilation as suggested by Hedrick and Sallach (1961). Alternatively nucleophilic attacks of ionised molecules could form polymeric products (partially detected by H^1NMR, data not shown). Titration with a base or nitrogen head sparging was required to maintain alkaline pH in the reaction vessel[2]. The effect of air and nitrogen on 100 mM pyruvate degradation was investigated for 50 hours under possible integrated conditions (with 1.3 g / L Neu5Ac aldolase and 265 mM GlcNAc) at pH 11.4 (Figure 4.7). Neu5Ac was not formed, indicating again enzyme deactivation at this pH. Air had no effect on the degradation and nitrogen sparging was an efficient mode to control pH. The overall degradation of pyruvate under alkaline pH suggests either combined epimerisation and biotransformation at pH 9-10 or pyruvate feeding strategies in order to minimise loss during reaction.

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[2] To prevent the lowering of pH due to the carbonate effect, once CO_2 from air enters in solution (Bailey and Ollis, 1986).

Table 4.1   Stability of reaction components (expressed as % original value) after 8 hours incubation at pH 11.5, 25 °C simulating a biotransformation at the start, middle and end of reaction.

Component	Start[1]	Middle[2]	End[3]	Control
Pyr	18.5	25.5	24.7	26.8[4]
GlcNAc + ManNAc	-	56.6	58.0	100[5]
Neu5Ac	-	99.2	98.0	-

1-760 mM Pyr, 100 mM ManNAc, 2- 700 mM Pyr, 52 mM ManNAc, 48 mM Neu5Ac, 3- 700 mM Pyr, 100 mM Neu5Ac, 4- 500 mM Pyr, 5- 100 mM GlcNAc.
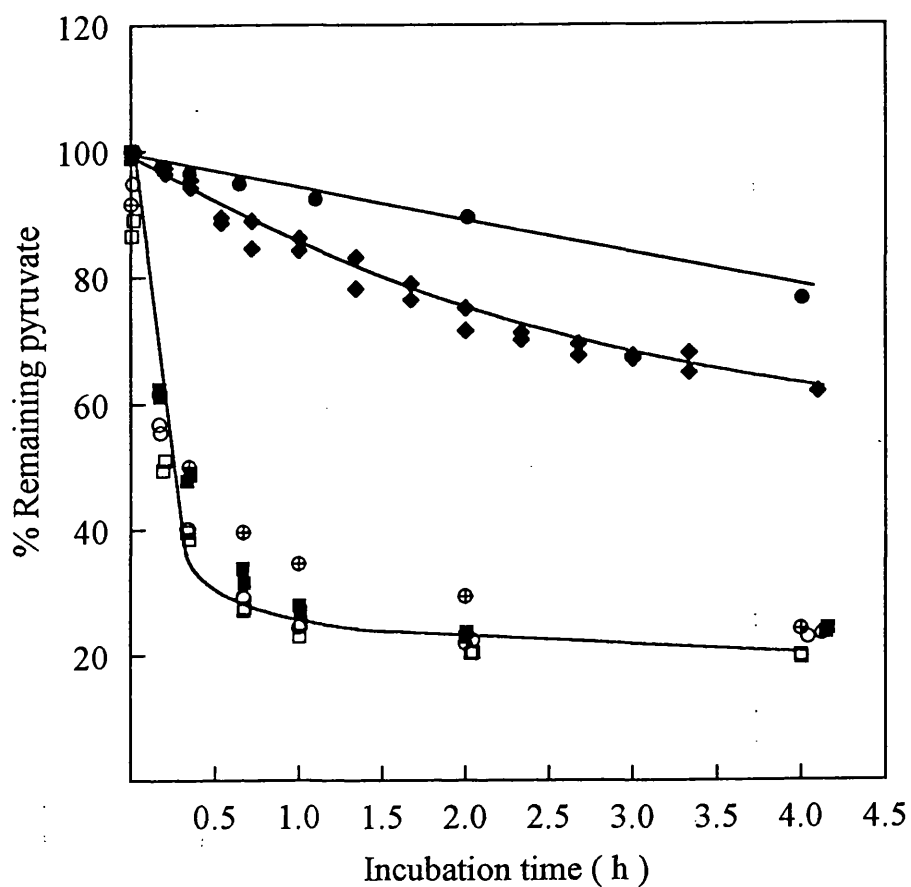
Figure 4.6   Effect of Neu5Ac aldolase on pyruvate stability at alkaline pH. Conditions without aldolase (pH 11.5): 760 mM Pyr, 101 mM ManNAc (□); 700 mM Pyr, 52 mM ManNAc, 48 mM Neu5Ac (O); 700 mM Pyr, 100 mM Neu5Ac (■); 500 mM Pyr (⊕). With Neu5Ac aldolase (1.4 g / L): pH 11.47, 104 mM Pyr, 50 mM ManNAc, 202 mM GlcNAc (◆); pH 10.5, 500 mM Pyr (●)
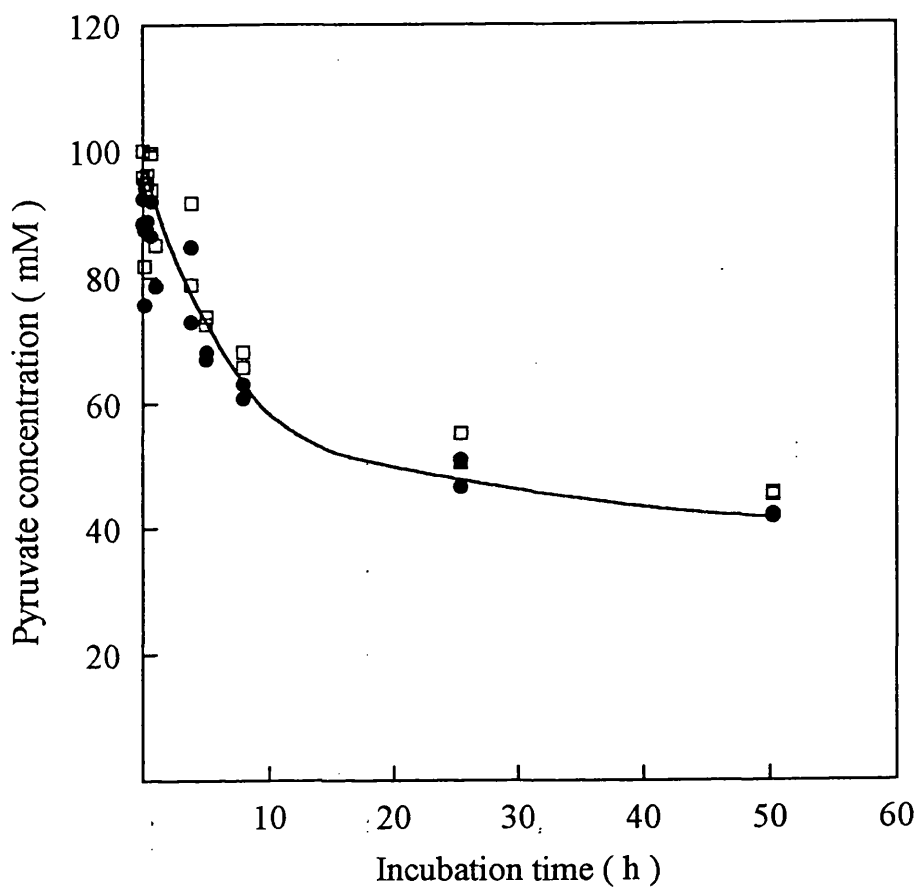
Figure 4.7   Pyruvate stability under possible integrated reaction conditions. Incubation at pH 11.4 under nitrogen (□) and air with pH control (●) in the presence of 1.33 g / L Neu5Ac aldolase and 266 mM GlcNAc.

The results presented by Tsukada and Ohta (1994) for the combined operations could then be explained considering pyruvate decay.

4.3.2.2 Neu5Ac ALDOLASE

In chapter 2, the stability of Neu5Ac Aldolase was investigated at pH 10.5 and 11.3 (70 % activity left after 8 hours at pH 10.5, 25 % at pH 11.3). In order to assess the feasibility of a biotransformation under alkaline conditions, further data on enzyme stability were required under conditions that would simulate an integrated epimerisation and biotransformation as a single step. At pH 11.3, in the presence of either 0.5 M ManNAc or 0.5 M pyruvate, the enzyme was completely inactivated after 2 hours (Figure 4.8). The option of an integrated reaction at pH 11.3 was then ruled out as unfeasible. However, the experimental results were very sensitive to pH values. The effect of ManNAc, pyruvate and GlcNAc on enzyme stability was examined at pH 10.5 (Figure 4.9). Under these conditions, incubation with ManNAc and GlcNAc caused a more severe drop in activity than incubation in water (in the presence of 0.5 M GlcNAc a 70 % decrease in activity was measured after 8 hours, with 0.5 M ManNAc a 52 % decrease was found). At the same pH, the presence of pyruvate, or pyruvate degradation products, apparently increased the enzyme stability. This finding is in agreement with the established protective role of pyruvate on aldolase thermal deactivation (Uchida *et al.*, 1984) and the commercial patent for Neu5Ac production under alkaline conditions (Tsukada and Ohta, 1994). Therefore a reaction with pyruvate supplied as a batch would be beneficial for Neu5Ac aldolase stability. The degradation of pyruvate and its protective role in enzyme stability support conflicting choices in possible fed-batch biotransformations at alkaline pH. The effect of pH on Neu5Ac aldolase was then monitored in the presence of 500 mM GlcNAc for 8 hours to account for the most deleterious environment to the enzyme (Figure 4.10). The enzyme maintained 85 - 75 % of initial activity after incubation at pH 8.5, 9.5 and pH 10. However, a severe drop in activity resulted from pH 10.5 onward (At pH 10.5 - 11, 22 % initial activity remained; the enzyme was fully deactivated at pH 11.3 after 8 hours). The profile of enzyme stability provided a boundary to the operating conditions of the biotransformation integrated with alkaline epimerisation.
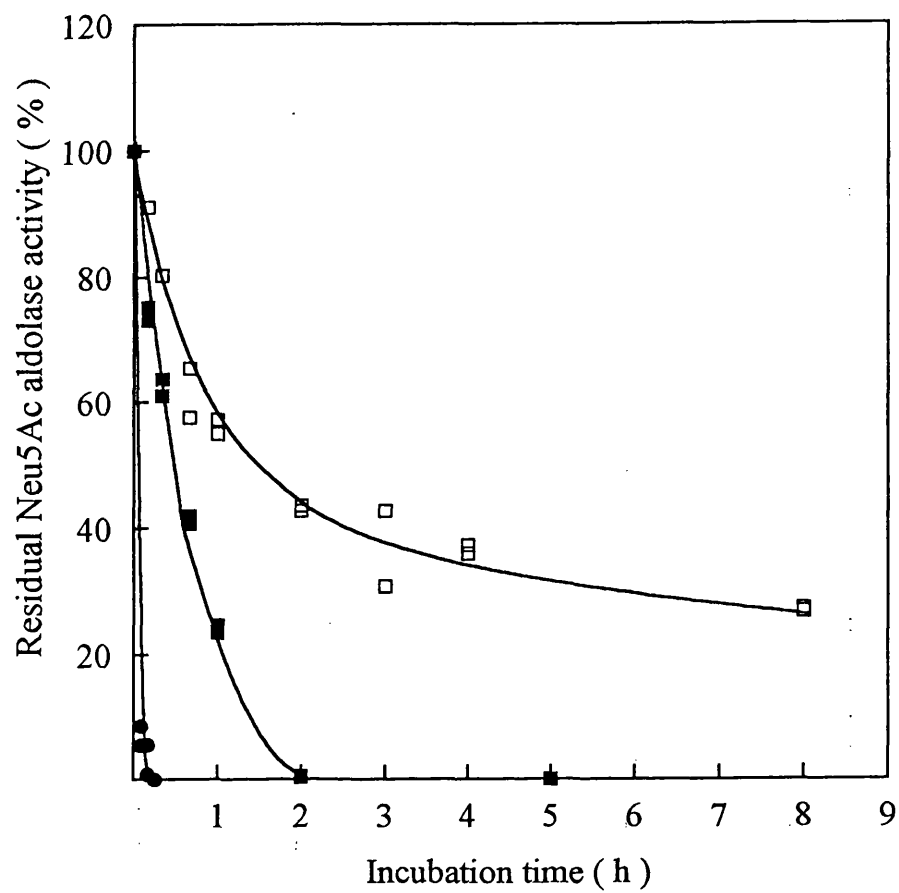
Figure 4.8 Neu5Ac aldolase stability at pH 11.3. Neu5Ac aldolase incubation in water (□), 500 mM ManNAc (■) and 500 mM Pyr (●).
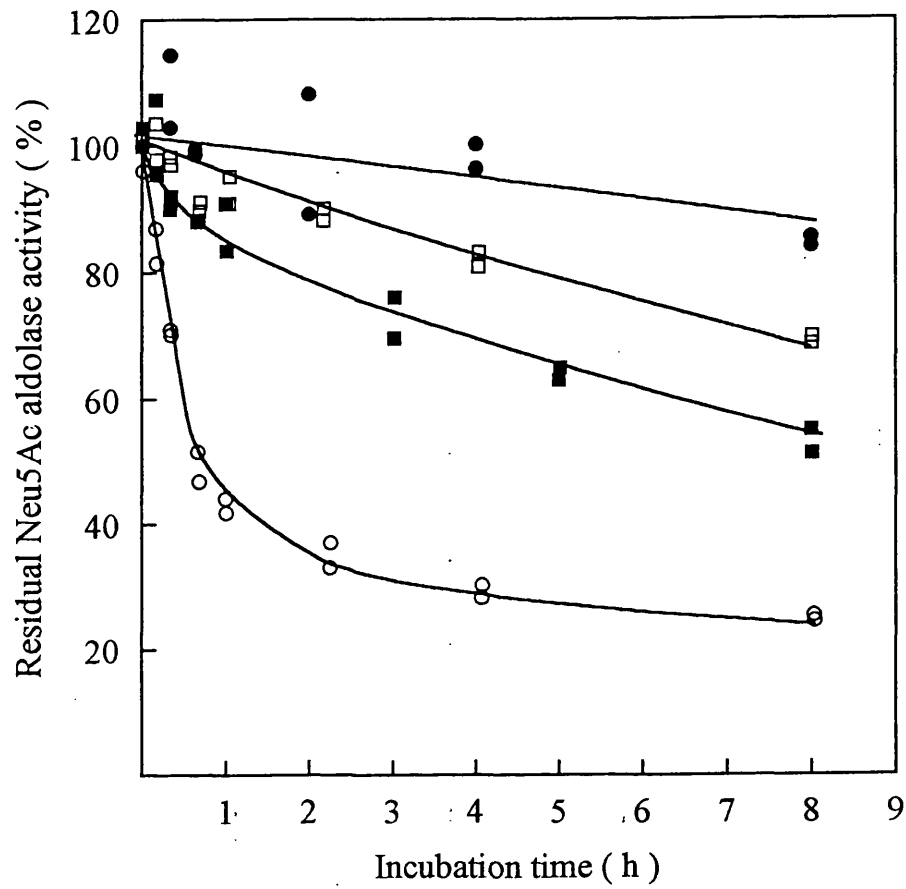
Figure 4.9   Neu5Ac aldolase stability at pH 10.5. Neu5Ac aldolase incubation in water

(□), 500 mM Pyr (●), 500 mM ManNAc (■) and 500 mM GlcNAc (○).
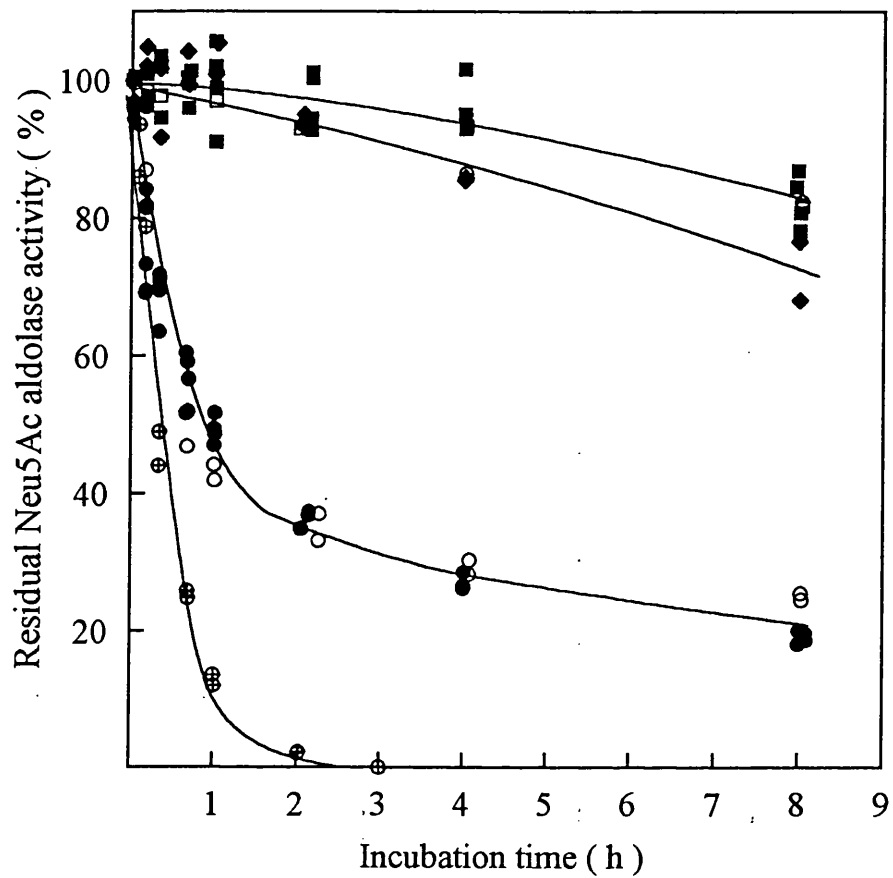
Figure 4.10   Influence of pH on Neu5Ac aldolase stability in the presence of 500 mM GlcNAc. pH 8.5 (■), pH 9.5 (□), pH 10.0 (◆), pH 10.5 (O), pH 11 (●), pH 11.35 (⊕).

4.3.3 INTEGRATED REACTION

The characterisation of the epimerisation and the biotransformation components in the previous section has identified the constraints acting on the combined operation in terms of substrate degradation and enzyme activity loss. It was then possible to relate these limits to define an operating region for the integrated process. Figure 4.11 shows the approximate reaction constant together with the remaining Neu5Ac aldolase activity after 8 hours incubation with GlcNAc as a function of pH. The combined reaction therefore could take place between pH 9 (no epimerisation) and pH 10.5 (arbitrary limit set at 50% enzyme loss). Pyruvate degradation suggests substrate feeding while its beneficial role to Neu5Ac aldolase stability could point towards batch mode of operations. The different approaches would be dictated by economic scenarios, as discussed in the following section.

A biotransformation with excess GlcNAc was then performed at pH 10.53 for 99 hours as a compromise example between Pyr operating strategies (Figure 4.12). The reaction revealed to be kinetically limited by the chemical epimerisation. 31.3 % conversion on Pyr took place after 72 hours, when the reaction appeared to achieve a steady state. The combined equilibrium constant was:

$$K_{eq} = \frac{[\text{Neu5Ac}]}{[\text{GlcNAc}][\text{Pyr}]} = 6.728 \pm 0.003 \text{ M}^{-1}$$

which is the product between the chemical equilibrium constant and the Neu5Ac synthesis equilibrium constant (Kragl, 1992 and Roseman and Comb, 1958). The value was found in accordance with the data obtained in a double enzymatic synthesis (6.76 M^{-1}, Kragl et al., 1991). The mass balance of the sugars was complete (Figure 4.13), showing that neither GlcNAc, ManNAc nor Neu5Ac decomposition took place. However, 30 % pyruvate degradation was measured alongside the conversion to Neu5Ac. The apparent slow kinetics of Pyr decomposition after 20 hours of reaction enabled the reaction to maintain a "pseudo" steady state after 72 hours. The amount of active enzyme measured during the reaction also decreased to 41 % within the first 24 hours and remained unvaried afterwards. Neu5Ac present after the first 24 hours could play a role towards enzyme stabilisation. Even within unfavourable conditions, the measured conversion on the limiting substrate (31.3 %) was comparable with the enzymatic process yield (41 %, Kragl et al., 1991).
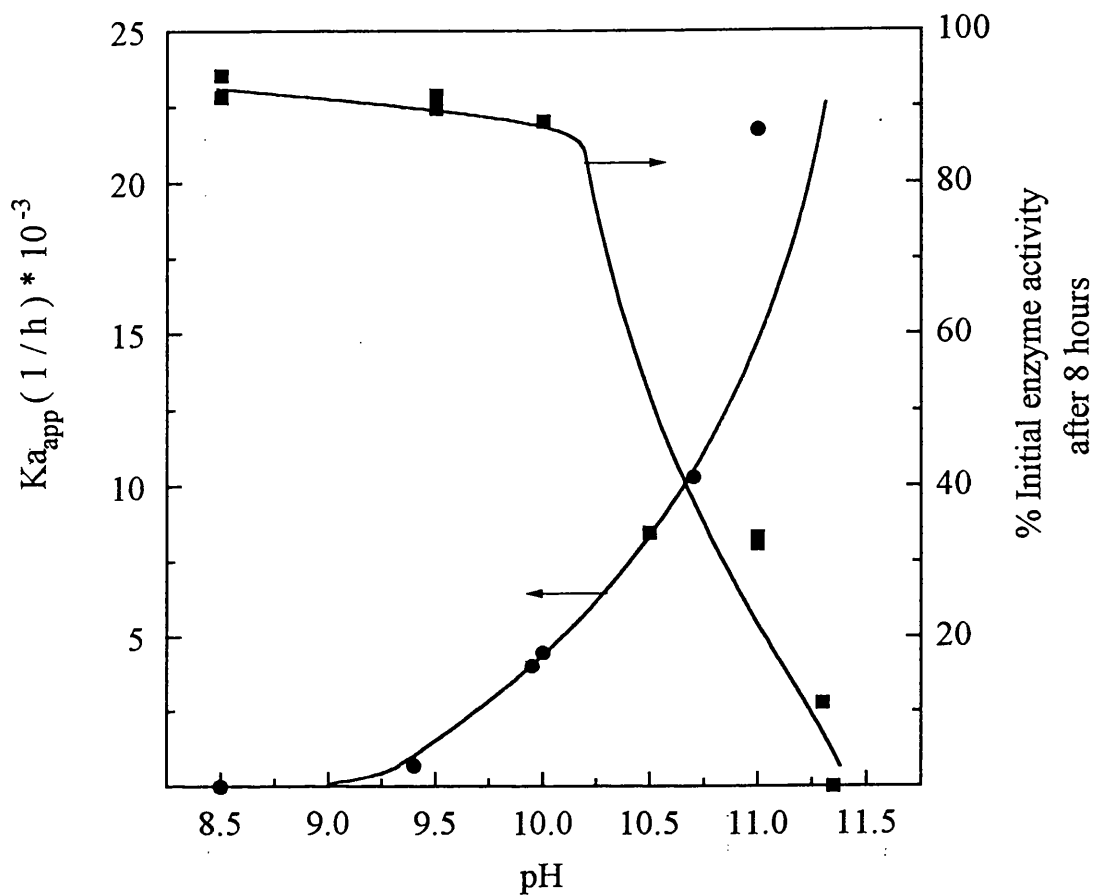
Figure 4.11 Operating regions of the integrated process. Approximate ManNAc formation rate constant and Neu5Ac aldolase stability in the presence of GlcNAc are here plotted as a function of pH.
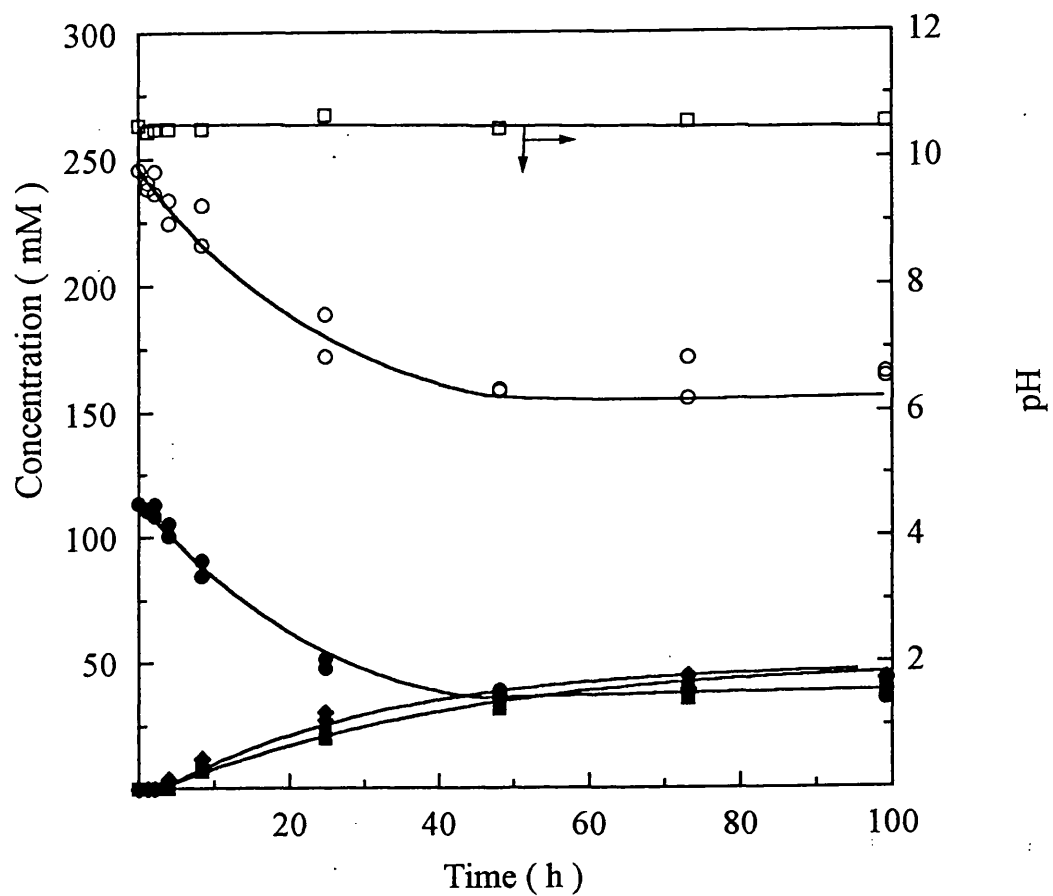
Figure 4.12 Biotransformation at alkaline conditions. GlcNAc (O), pyruvate (●), ManNAc (■) and Neu5Ac (◆) were monitored for 99 hours by HPLC. pH (□) was kept constant with nitrogen head sparging.
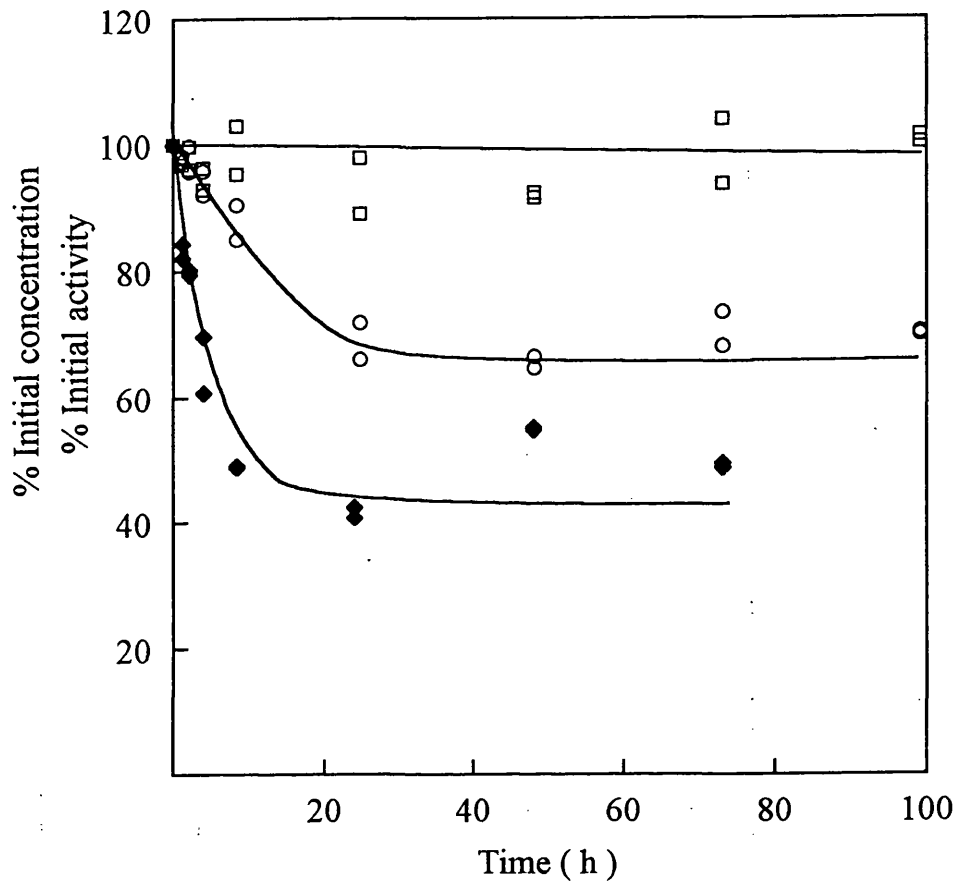
Figure 4.13 Stability and mass balance of components of biotransformation at alkaline conditions (Fig. 4.12). Sugars (□), pyruvate (O), Neu5Ac aldolase (◆).

4.6 DISCUSSION

In this chapter, the feasibility of a combined reaction between the chemical epimerisation step and the biotransformation step was investigated in order to evaluate the process constraints and advantages with respect to separate operations. The overall synthesis of Neu5Ac in alkaline conditions has proved feasible. However, enzyme activity and pyruvate loss took place. Moreover, the concentration of reactants and product at the end of reaction was dictated by the combined equilibrium of the two reactions, providing no real thermodynamic advantage for Neu5Ac synthesis unless *in situ* product removal could be implemented. The epimerisation rate was found exponentially pH-dependent (Lee, 1990, Section 4.3.1) up to pH 12 and operation above pH 9 is necessary. Experiments to determine the characteristics of the biotransformation provided further boundaries for the combined reaction. At alkaline pH pyruvate was particularly unstable although it partly stabilised the enzyme at pH 10.5. The enzyme was much less stable in the presence of GlcNAc or ManNAc. Feeding pyruvate would reduce losses of this substrate but might lower its stabilising effect on the enzyme and would also reduce the equilibrium product concentration achieved. Thus selection of the appropriate operating conditions for the combined epimerisation and biotransformation is complex. The results reported in combined biotransformations at pH 9-11 by Tsukada and Ohta (1994) can be explained with the loss of pyruvate through alkaline degradation. The advantages of the integrated reaction are that it shifts the unfavourable equilibrium from GlcNAc to ManNAc by removal of the latter by the biotransformation step. Moreover, the number of operations between the chemical step and the biotransformation (Lin *et al.*, 1992, Dawson *et al.*, 1994) are reduced. The compromised operating conditions should ultimately be selected on an economic basis. Relative GlcNAc, pyruvate and enzyme costs should be compared for the most appropriate operating strategy. Batch operations would be appropriate for high enzyme process costs, but will inevitably have a severe effect on pyruvate. Fed batch would favour yield on pyruvate at the expense of enzyme stability. The choice of pH within the imposed constraints could be optimised on the basis of productivity, decreasing processing times. Recently, it has been reported (Sugai *et al.*, 1995) that the epimerization of GlcNAc to ManNAc by calcium hydroxide was five to six times faster than the reaction catalysed by sodium hydroxide. This approach may prove attractive, but will suffer from the inhibitory effects of the calcium ion upon the enzyme activity (Kragl, 1992) when operated in an integrated process. Eventually, the characterisation of the constraints in this feasibility study

could be taken as an example in methodology to assess the integration of chemical and biochemical synthesis steps.

4.7 SUMMARY

In this chapter, the integration between chemical epimerisation and Neu5Ac aldolase biotransformation has been assessed. The following results were relevant to process boundaries:

- GlcNAc epimerisation took place above pH 9.0 and the reaction rate increased exponentially with pH. However, above pH 12 sugar degradation occurred.

- Pyruvate was degraded above pH 10.5 and Neu5Ac aldolase has been proved beneficial to pyruvate stability, while degradation was not related to the presence of air.

- Neu5Ac was found stable at alkaline pH.

- Neu5Ac aldolase was denatured at pH 11.3 while Pyr was shown to increase enzyme stability at pH 10.5. Batch pyruvate would be required to stabilise the enzyme. GlcNAc increased enzyme degradation.

- 31.3 % conversion on Pyr was achieved in an integrated biotransformation. The "pseudo" steady state at the end of reaction was comparable to the equilibrium achieved with enzymatic synthesis (Kragl et al., 1991).

The integrated reaction was proved feasible at the expense of pyruvate and Neu5Ac aldolase degradation. The combined reaction overcame the unfavourable GlcNAc-ManNAc epimerisation equilibrium by removing the latter through the formation of Neu5Ac. Final Neu5Ac concentration in the chemo-enzymatic synthesis at alkaline pH was influenced by the equilibrium product of the separate reactions. Hence, no real advantage was found in the production of Neu5Ac unless in situ product removal could be implemented.

5

REACTOR CONFIGURATIONS FOR Neu5Ac SYNTHESIS

5.1 INTRODUCTION

In this chapter, the Neu5Ac biotransformation is carried out in different reactor configurations to accomplish the characterisation results (Chapter 2) and the process option evaluation and selection (Chapter 3). Two sections are found in this chapter. The first part concerns the characterisation for an *in situ* product removal (ISPR) system and leads to a design example of ISPR for Neu5Ac synthesis. The second section is an evaluation of different reactors, under potential process conditions, for the production of Neu5Ac. In this section, a case of ISPR is presented. In the discussion, different reactor parameters are compared for different manufacturing cases: enzyme limitations, downstream processing limitations and product limitations.

5.2 MATERIALS AND METHODS

5.2.1 MATERIALS

All reagents used were analytical grade. Chemicals were obtained from Sigma except for Neu5Ac dihydrate, which was kindly supplied by Glaxo Group Research and Eupergit-C™, supplied by Röhm Pharma. Purified *E. coli* Neu5Ac Aldolase (24.1 U / mg) was obtained from Toyobo. All chemicals and suppliers are listed in Appendix 1.

5.2.2 METHODS

5.2.2.1 BREAKTHROUGH ANALYSIS

5 g of Dowex 1x2 400 (4 % cross-linkage, Sigma) was hydrated to 7.5 mL gel and packed into an Econo-Pack column (Bio-Rad, diameter: 15 mm). The resin was converted overnight with 1 Molar NaOH, HCOONa, CH_3COONa, $NaHCO_3$ or NaCl at 0.5 mL / min, 4 bed volumes / hour (bv / h) (Gilson Minipuls 3 peristaltic pump) to obtain respectively the hydroxide, formate, acetate, bicarbonate or chloride counterion form. The column was then washed with 20 bv of double dionised water (DDW) prior to the loading step. For each experiment, the component solution at pH 7.5 was applied to the top of the column at 4 bv / h and the eluate collected in 1 mL fractions, followed by HPLC analysis (2.3.2.2) until the output concentration was equal to that of the input and the resin was fully loaded. The starting concentration for each experiment follows. Hydroxide form: 72 mM Neu5Ac; acetate form: 158 mM Neu5Ac; formate form: 189 mM Neu5Ac, 98 mM GlcNAc, 2120 mM ManNAc; bicarbonate form: 190 mM Neu5Ac, chloride form: 256 mM Neu5Ac. After 5 bv wash, the loaded resin was eluted with 2 M of the respective salt in 1 mL fractions and analysed by HPLC (2.3.2.2).

5.2.2.2 COUNTER-ION EFFECT ON Neu5Ac ALDOLASE ACTIVITY

10 mL of 80 mM Pyr and 40 mM ManNAc were incubated at 25 °C in 50 mM potassium phosphate buffer, pH 7.5 with 0.5 g / L Neu5Ac Aldolase and increasing concentrations of sodium acetate and sodium formate. The reaction was monitored by HPLC (2.3.2.2) for 10 minutes. The initial reaction rate was obtained by the slope of the reaction profile and standardised against the control (without salts).

5.2.2.3 COUNTER-ION EFFECT ON Neu5Ac ALDOLASE STABILITY

0.5 g / L Neu5Ac Aldolase was incubated in 15 mL at 25 °C, pH 7.5 either in 1 M sodium formate or sodium acetate solution for 8 hours. In order to separate the aldolase from the salt, incubation was stopped by addition of 0.5 mL 100 mM Tris-HCl pH 7.9 to the sample (1 mL) and subsequent injection in a size exclusion Hi-Trap ™ desalting column (Pharmacia) equilibrated with Tris buffer (2.3.2.10). Subsequent analysis was performed as described in Section 2.3.2.10.

5.2.2.4 EFFECT OF DOWEX 1X2 400 ON Neu5Ac ALDOLASE

3 U / mL Neu5Ac Aldolase (25 mL) were incubated at 25 °C, pH 7.5 in a ETS 400 pH-Stat (Radiometer) and circulated at 0.5 mL / min through a 5 mL column of Dowex 1x2 400 in the formate form, prepared as described in Section 5.2.2.1. The aldolase solution was assayed for enzyme activity (2.3.2.5) and protein content (2.3.2.6) for 4 hours.

5.2.2.5 NEU5AC ELUTION COMPETITION

5 g of Dowex 1x2 400 (4 % cross-linkage, Sigma) were packed and converted in the Cl⁻ form according to the procedure described in Section 5.2.2.1. 257 mM Neu5Ac, pH 7.5 were loaded at 4 bv / h onto the column until saturation was achieved. After column wash (10 bv), a gradient of NaCl (starting reservoir: 2 M NaCl, pH 7.5) was applied at 4 bv / h. The eluate was collected in 1 mL fractions and analysed by HPLC (2.3.2.2). The same procedure was then applied for a formate column: 235 mM Neu5Ac, pH 7.5 were loaded on the column, with 4 M HCOONa reservoir concentration for gradient elution. In a third experiment, the above protocol was followed to load a formate column to 10 % capacity with 125 mM Neu5Ac, pH 7.5, with 2 M HCOONa reservoir concentration for gradient elution.

5.2.2.6 NEU5AC BINDING COMPETITION

5 g of Dowex 1x2 400 were prepared in the HCOO⁻ form according to the procedure described in Section 5.2.2.1 and loaded with 100 mM Neu5Ac with or without 50 mM HCOONa, pH 7.5 at 4 bv / h up to column saturation. The eluate was collected in 1 mL fractions and analysed by HPLC (2.3.2.2).

5.2.2.7 DOWEX 1X2 PROCESS CONDITIONS SIMULATION

5 g of Dowex 1x2 resin were converted in the HCO₃⁻ form according to the procedure described in Section 5.2.2.1. The column was loaded with 85 mM Neu5Ac, 5 mM Pyr and 710 mM GlcNAc, pH 7.5. All operations were performed at 4 bv / h. After 4 bv of DDW wash the resin was eluted with 2 M NaHCO₃. The eluate was analysed as in the previous section.

5.2.2.8 BATCH BIOTRANSFORMATION

10.13 g of Eupergit beads (Röhm Pharma) with crude Neu5Ac aldolase immobilised (0.8 U / g, see appendix 3, hereafter: Eupergit) were incubated for 540 minutes together with 23 mL of 456 mM ManNAc and 990 mM Pyr at pH 7.5, 25 °C. Samples (100μL) were spun for 1

minute at 5000 r.p.m. on a bench centrifuge. The supernatant (50 µL) was diluted 1:200 and analysed by HPLC (2.3.2.2).

5.2.2.9 INTERMITTENT FED-BATCH BIOTRANSFORMATIONS

10.08 g of Eupergit were incubated in 24 mL DDW together with 2.990 g ManNAc (520 mM) and 0.55 g Pyr (208 mM) at pH 7.5, 25 °C. After 93 min 550 mg solid Pyr were added to the reaction and at 180 min 1.651 g Pyr were added as well. The reaction was followed for 540 min by HPLC as described in section 5.2.8. In another experiment 10.42 g Eupergit were incubated in 24.2 mL DDW together with 2.98 g (515 mM) ManNAc and 0.54 g (202 mM) Pyr as above. At 150 min, 965 mg ManNAc and 446 mg Pyr were added to the reaction mixture. At 360 min, 967 mg ManNAc and 445 mg Pyr were added. The reaction was followed for 600 minutes as above.

5.2.2.10 CONTINUOUS FED-BATCH BIOTRANSFORMATIONS

10.1 g Eupergit were incubated together with 23.6 mL of 530 mM ManNAc at pH 7.5, 25 °C. 3030 mM Pyr, pH 7.5, was fed at 2.81 µL / min with a Minipuls 3 pump (Gilson) through 0.25 mm diameter tubing. In another experiment, 24.01 mL of 516 mM ManNAc were incubated with 10.55 g Eupergit as above and Pyr solution was fed at 5.62 µL / min. In a third experiment, 23.2 mL of 520 mM ManNAc and 102 mM Pyr were incubated with 10.35 g Eupergit as above and Pyr solution was fed to maintain constant 130 mM Pyr. All the reaction were monitored for 600 min as in section 5.2.2.8.

5.2.2.11 SMALL SCALE PLUG FLOW REACTOR BIOTRANSFORMATIONS

10.67 g Eupergit were packed in an Econo-pac (Bio-Rad) column, 15 mm diameter, with a total column volume of 19.5 mL. The substrates solutions (Table 5.4) were pumped at 0.15-1.0 mL / min with a Minipuls 3 Peristaltic pump (Gilson) at 25 °C, pH 7.5 and 10-15 bed volumes of eluate were collected in fractions of 1 mL with a Frac 100 (Pharmacia) fraction collector. 50 µL from each sample was diluted 1:200 and analysed by HPLC (2.3.2.2) with a Beckman 166 UV detector at 205 nm.

5.2.2.12 LARGE SCALE PLUG FLOW REACTOR BIOTRANSFORMATIONS

56.94 g Eupergit were packed in a 104 mL column, 25 mm diameter. ManNAc was produced from GlcNAc and subsequent enrichment as described in Mahmoudian *et al.* (in press). 465 mM ManNAc, 210 mM Pyr and 170 mM GlcNAc at 25 °C, pH 7.5 were applied from a 1960 mL reservoir onto the column at 0.5 and 1 mL / min for 21 hours with a Minipuls 3 peristaltic pump (Gilson). The eluate was collected in fractions of 10 mL with a Frac 100 (Pharmacia) fraction collector. 100 μL from each sample were diluted 1:100 and analysed by HPLC (2.3.2.2) as described in the previous section.

5.2.2.13 *IN SITU* PRODUCT REMOVAL BIOTRANSFORMATION

Two Econo-Pack columns (Bio-Rad) packed with Dowex 1x2 400 (10.7 g, 15 mL and 10.11 g, 13.5 mL) in the bicarbonate form (5.2.2.1) were linked in parallel to the enzyme column (5.2.2.12). After 8 hours in the experiment described in section 5.2.2.12 at 0.5 mL / min flowrate, the outlet from the enzyme column was switched to the first column for 60 min and then to the second column for 54 minutes. 100 μL samples were taken every 6 minutes from the outlet of the ion exchanger and analysed by HPLC (5.2.2.10). The reaction mixture collected from the ion exchanger columns (56 mL) was titrated back to pH 7.5 with 10 M pyruvic acid. 1.05 g ManNAc and 484 mg Pyr were added to the mixture which was added back to the substrate reservoir. After 114 min, the columns were moved off line. Each column was washed with 3 bv of water and eluted and regenerated with 6 bv of 2 M NaHCO$_3$ at 1 mL / min flowrate. The eluate from the ion exchangers was collected in 2 mL aliquots and analysed by HPLC (5.2.2.10).

5.3 RESULTS

5.3.1 CHARACTERISATION OF *IN SITU* PRODUCT REMOVAL SYSTEM

5.3.1.1 RATIONALE

5.3.1.1.1 Introduction

A reactor design that would remove Neu5Ac while the reaction proceeds would have both kinetic and thermodynamic advantages. *In situ* product removal (ISPR) could maintain the reaction rate at its initial level since the Neu5Ac formation rate decreases on approach to equilibrium. By operating the reaction away from equilibrium and recycling the unused substrates, a full stoichiometric conversion is possible, overcoming the thermodynamic constraint imposed by K_{eq} (Section 1.2.4.2). In this section, a rational approach for the selection and operation of ISPR technique (Chauhan *et al.*, 1996) follows the design methodology developed in Chapter 2 and consists of four parts. The first part aims to identify the components properties and separation techniques which could be used in ISPR. The second part is the separation system characterisation, where operating parameters are investigated. The third part evaluates the reciprocal interferences between the production and separation elements and the fourth part simulates the dynamic interactions of the components under possible production conditions. This rational approach identifies process constraints, rules out unfeasible process options and provides the design criteria for ISPR reactors.

5.3.1.1.2 Component characterisation

The chemical and physical characteristics of the reaction components are listed in Table 5.1. While the molecular weights are all in the range of 100-300 Daltons, making a separation based on size difficult, both Pyr and Neu5Ac are negatively charged at neutral pH. Differences in water and acetic acid solubility are the basis for Neu5Ac crystallisation. The lability of the molecules imposes a constraint on the possible operating conditions for Neu5Ac separation.

Table 5.1   Neu5Ac synthesis biotransformation component characteristics for *in-situ* product removal.

	Pyr	GlcNAc	ManNAc	Neu5Ac
Charge[1]	negative pKa = 2.39	/	/	negative pKa = 2.0
Hydrophobicity[2]	No	No	No	No
Volatility[2]	No	No	No	No
Specific group[2]	1 carboxyl 1 acetyl	4 hydroxyls 1 N-acetyl	4 hydroxyls 1 N-acetyl	1 carboxyl 1 N-acetyl 5 hydroxyls
Solubility	water (3.6 M)[3] EtOH[1]	water (1.3 M)[3], EtOH[1], low solubility in propanol[4]	water (1.6 M)[3], EtOH[1]	water (0.95 M)[3], EtOH[1] low solubility in acetic acid[5]
Others	alkali very labile[3]	pyranose configuration[1] alkali labile[3] α/β mutorotation[6]	pyranose configuration[1] hydrate form[1] alkali labile[3] α/β mutorotation[6]	pyranose configuration[1] α/β mutorotation[7] heat labile[8] alkali[3] and acid[8] labile

1- Dawson *et al.*, 1993, 2- Budavari *et al.*, 1989, 3- Chapter 2, 4- Mahmoudian *et al.*, in press, 5- Lin *et al.*, 1992, 6- Kragl, 1992, 7- Friebolin *et al.*, 1980, 8- Varkas and Chargaff, 1963.

5.3.1.1.3 Product separation choice

A variety of options for Neu5Ac purification are reported in the literature (Chapter 1). The possible operations that can be used for *in situ* product removal (ISPR) are summarised in Table 5.2. Ion exchanger resins and electrodialysis are both based on non-specific charge interactions. Hence a low concentration of Pyruvate in the stream leaving the reactor is required to make an effective use of these techniques. Different vegetal lectins recognise specifically Neu5Ac and the first industrial applications have been reported (NGK, 1994) for their use in affinity chromatography. Neu5Ac crystallisation was proved a selective separation method. However, it was already ruled out (Chapter 2) for ISPR. Increased specificity in the separation technique imposes fewer constraints on the design and operation of the biotransformation reactor. Anion exchange chromatography has been chosen to evaluate process constraints of non specific Neu5Ac ISPR. A strong anion exchanger with quaternary ammonium groups (Dowex 1x2 400, Dow Chemical) has been selected since it is an established method for sialic acid separation (Comb and Roseman, 1960), the material is suitable for scale-up (Dorfner, 1972) and continuous operation is possible.

This model system aims to characterise the constraints due to biotransformation-downstream integration with a non selective separation technique that is already implemented at preparative scale (Kragl *et al.*, 1995).

5.3.1.2 DOWEX 1x2 400 CHARACTERISATION

In a quaternary ammonium anion exchanger, the selectivity is (Dorfner, 1972; Diaion, 1993):

$$Cl^- > HCO_3^- > HCOO^- > CH_3COO^- > OH^-$$

The resin capacity is an essential parameter in ion-exchange separation design and was first measured with the breakthrough point (Pharmacia, 1990; Diaion 1993). Figure 5.1 shows the breakthrough curve for GlcNAc, ManNAc and Neu5Ac components for Dowex 1x2 400 resin in the formate form. ManNAc elution profile was then selected as the control (no

Table 5.2   Possible *in situ* product removal methods.

Separation	Basis	Notes
anion exchange	negative charge	Pyr binding due to charge, feeding strategy required. Counter anions leakage into the system.
electrodialysis	negative charge	Pyr binding and need of enzyme retention owing to high electrical potential.
lectins	Neu5Ac diol	Possible problems with Neu5Ac elution, availability.
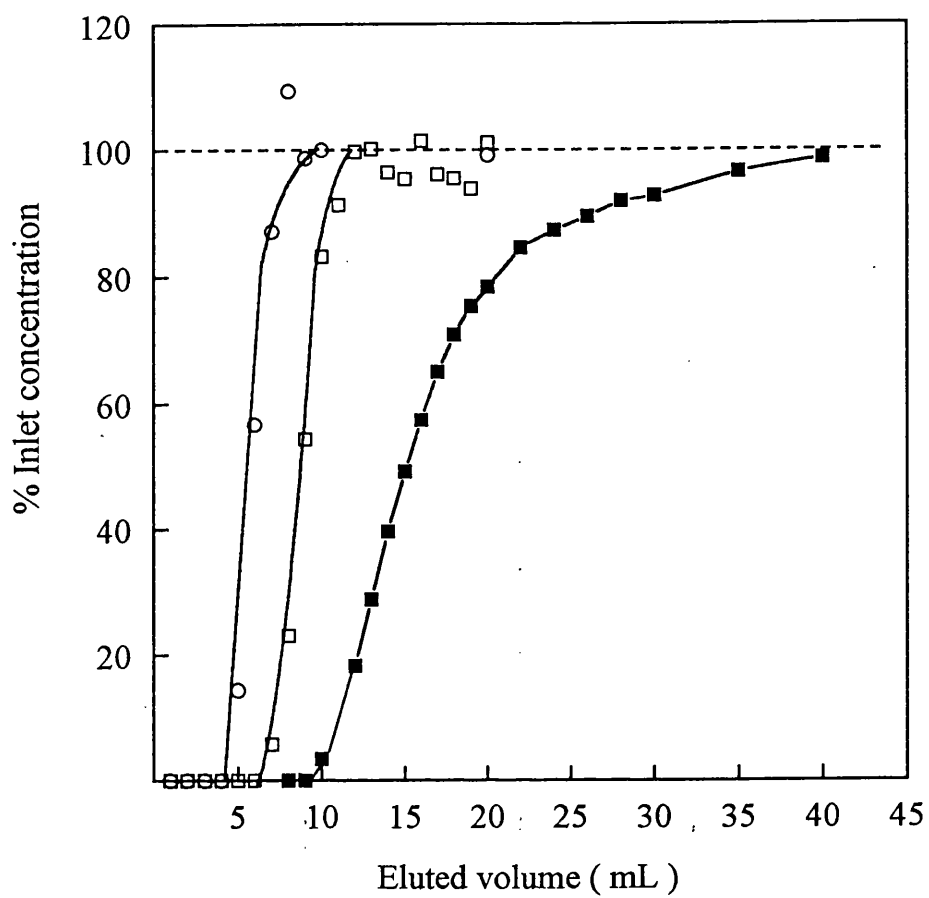
Figure 5.1   Elution profile of ManNAc (O), GlcNAc (□) and Neu5Ac (■) from Dowex

1x2 400 anion exchanger, formate form.

binding) for frontal uptake analysis (FUA) capacity determination. Figure 5.2 shows different Neu5Ac elution curves for hydroxide, acetate, bicarbonate and chloride forms. The frontal uptake analysis was performed according to the formula:

$$Capacity = \frac{V_{F\ ManNAc}[ManNAc] - V_{F\ Neu5Ac}[Neu5Ac]}{V_C} * \frac{1}{\rho_C}$$

Where:

V_F	(mL)	frontal volume (eluted volume at half column saturation)
V_C	(mL)	column volume
[ManNAc]	(mol / L)	inlet ManNAc concentration
[Neu5Ac]	(mol / L)	inlet Neu5Ac concentration
ρ_C	(g / mL)	resin density

to obtain the capacity of the resin at half saturation. The resin was then overloaded, washed and eluted with 2 M counter ion salt (Figure 5.3: full column cycle for a Dowex resin in the formate form). The saturation capacity for Neu5Ac was then calculated from the integration of the elution peak. The summary of the capacity results is shown in Table 5.3. The overall capacity of the resin in different forms was lower than expected from literature data (Dorfner, 1972). This may be due to the different standards used to measure the exchange capacity. The capacity of the resins decreases as expected with increasing counterion affinity. This effect is more dominant in the breakthrough capacity since the resin did not yet achieve equilibrium. The different capacity measurements can be compared to estimate the stability of the components on the resin. Alkaline conditions are detrimental to Neu5Ac (Chapter 2) and the decrease in capacity from breakthrough to saturation in the hydroxide form can be due to Neu5Ac degradation. Hence, the design option of an anion exchanger in the hydroxide form is ruled out from further characterisation.
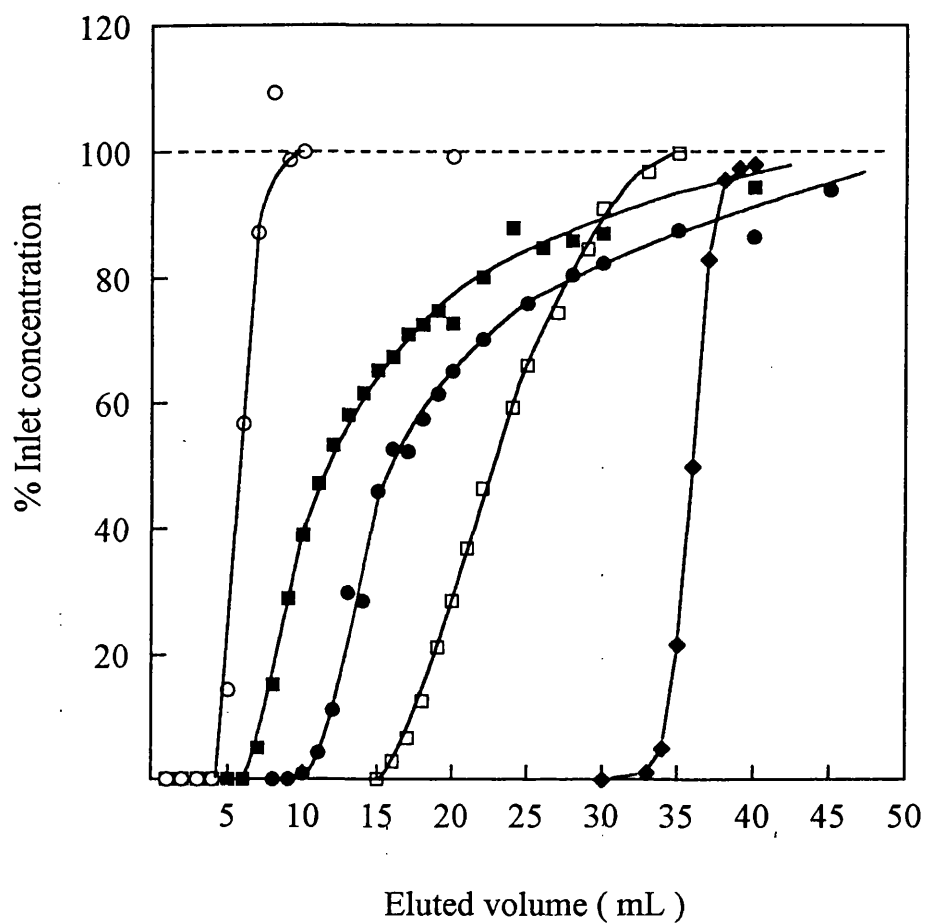
Figure 5.2   Elution profile of Neu5Ac from Dowex 1x2 400 anion exchanger in the bicarbonate (●), acetate (□), chloride (■), and hydroxide (◆) forms. Control: ManNAc (O) on formate resin form.
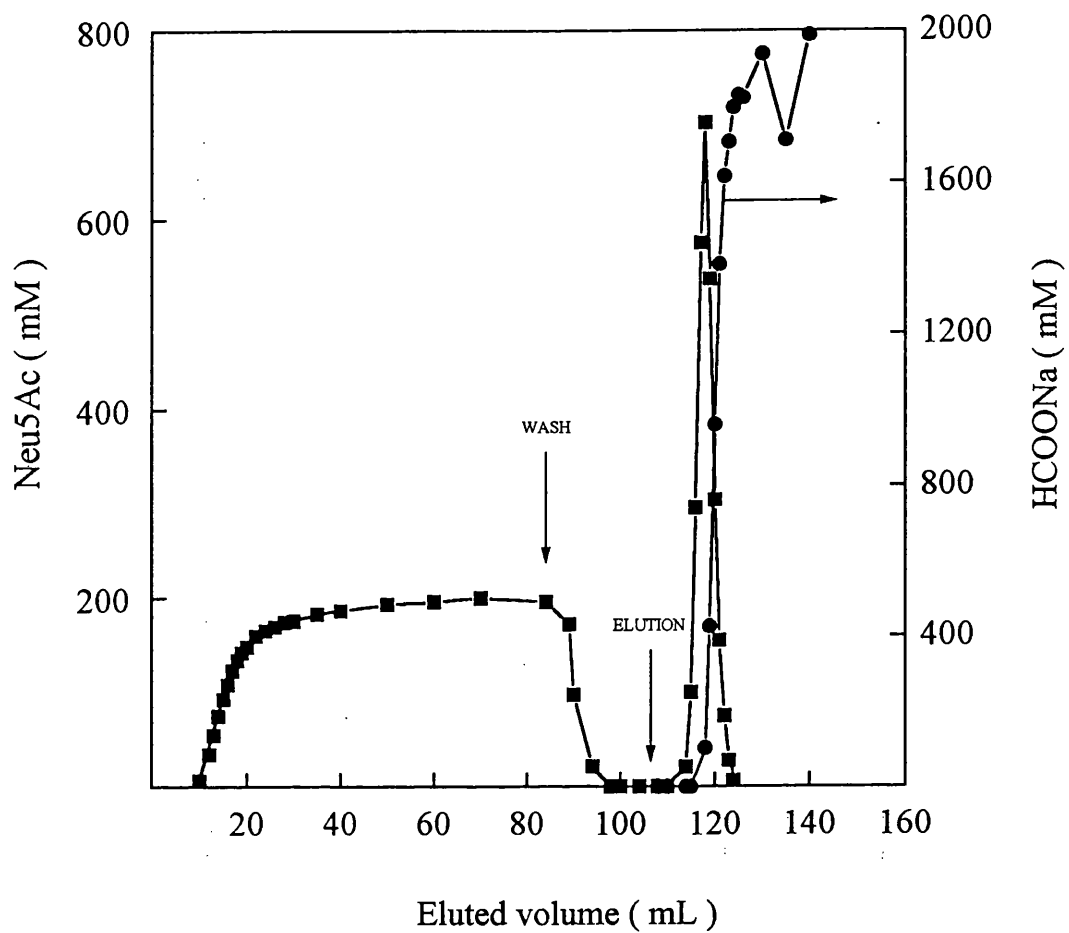
Figure 5.3   Dowex 1x2 400 formate form column cycle. 190 mM Neu5Ac applied at pH 7.5 and Neu5Ac (■) monitored out of the column. Wash with DDW, elution with 2 M formate (●).

Table 5.3   **Capacity determination for Dowex 1x2 400 anion exchange resin.**

Resin Form	Component	Breakthrough Capacity (meq / g)	FUA[1] Capacity (meq / g)	Saturation Capacity (meq / g)
OH⁻	Neu5Ac	0.46	0.27	0.12
CH3COO⁻	Neu5Ac	0.47	0.46	0.72
HCOO⁻	Neu5Ac	0.34	0.32	0.56
	GlcNAc	0.12	0.08	-
	ManNAc	0.17	0.00	-
HCO3⁻	Neu5Ac	0.34	0.37	0.61
Cl⁻	Neu5Ac	0.30	0.33	0.67

1- Frontal uptake analysis

5.3.1.3 EVALUATION OF INTERFERENCES

5.3.1.3.1 Effects of counter-ions on Neu5Ac aldolase

In ISPR the production of counter-ions as a result of the ion exchange process has important implications. The effect of counter-ions on the enzyme was investigated in order to select the resin form which ions recirculating in the system would not interfere with the enzyme activity and stability. Neu5Ac synthesis biotransformations were performed with increasing concentrations of sodium acetate and sodium formate and the resulting initial aldolase activity monitored (Figure 5.4). Both the salts caused a decrease of enzyme activity. However, while the aldolase maintained 80 % of its initial activity in the presence of 1 M formate, the activity with 1 M acetate dropped to 42 %. Figure 5.5 shows the stability profiles of Neu5Ac aldolase in the presence of 1 M sodium acetate and sodium formate over 8 hours. Neither formate nor acetate did cause a relevant decrease in enzyme stability. Consequently, it is likely that acetate could behave as an enzyme inhibitor. This effect might be due to the structural similarity of acetate and pyruvate ions. The design option of anion exchanger in the acetate form is therefore ruled out as unfeasible. Formate ions and chloride ions (Section 2.4.1.3.2) did not interfere significantly with Neu5Ac aldolase up to 1 molar concentration. Although a lower capacity was found for the resins in these forms, both chloride and formate anion exchangers might be implemented in continuous operations with the aldolase and therefore they were selected for further characterisation. Moreover, the stability data provide a worse case scenario for the length of the production time based on enzyme activity in a closed system with counterion build-up. Since the affinity of the chloride ion is higher than that of formate, experimental design limited the data required for the interference of the resin in Cl^- form.

5.3.1.3.2 Resin effect on enzyme

In order to assess the feasibility of free enzyme circulating through the ISPR reactor and the possibility of product removal within the vessel, Neu5Ac aldolase was incubated at pH 7.5 and passed through a Dowex 1x2 400 column in the formate form. Figure 5.6 shows that the aldolase is withdrawn and adsorbed on the column. The isoelectric point of the enzyme is 4.5 (Aisaka et al., 1991). Therefore at pH 7.5 the protein is negatively charged, allowing the removal on the ion exchange column. Enzyme separation from the ion exchanger through retention or immobilisation is therefore a necessary constraint for this ISPR option.
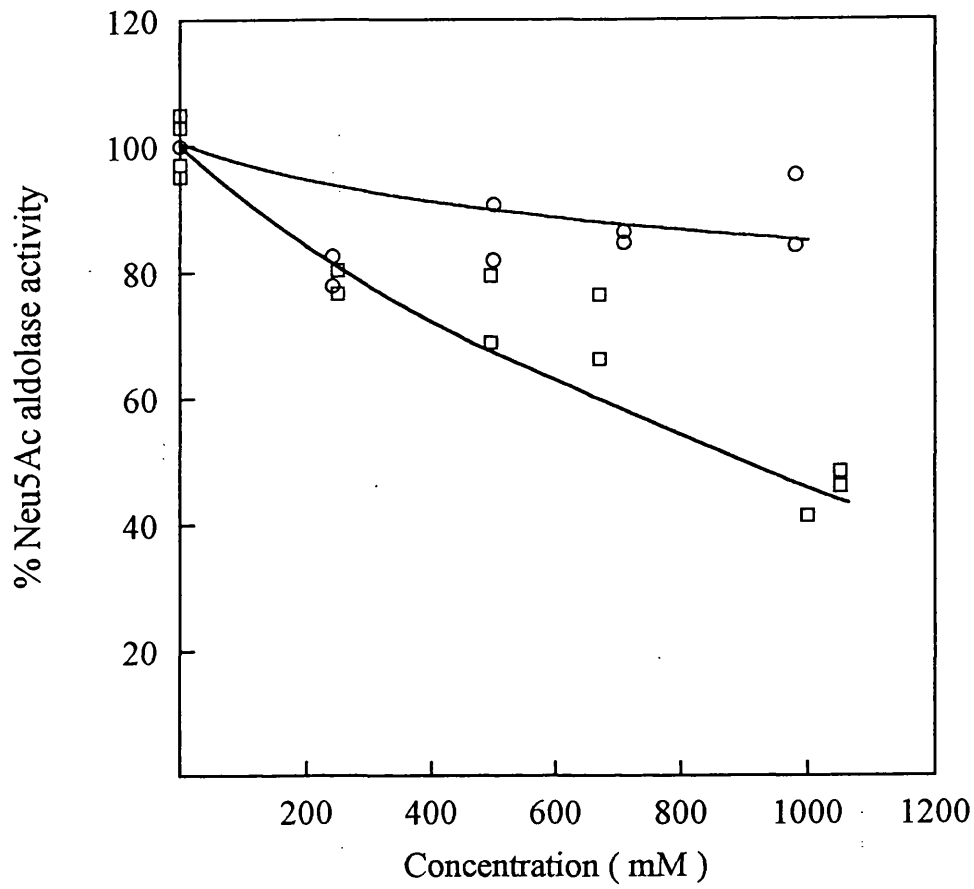
Figure 5.4 Effect of acetate and formate ions on Neu5Ac aldolase activity. % Initial rate of Neu5Ac formation is shown for reactions with increasing acetate (□) and formate (O) concentrations
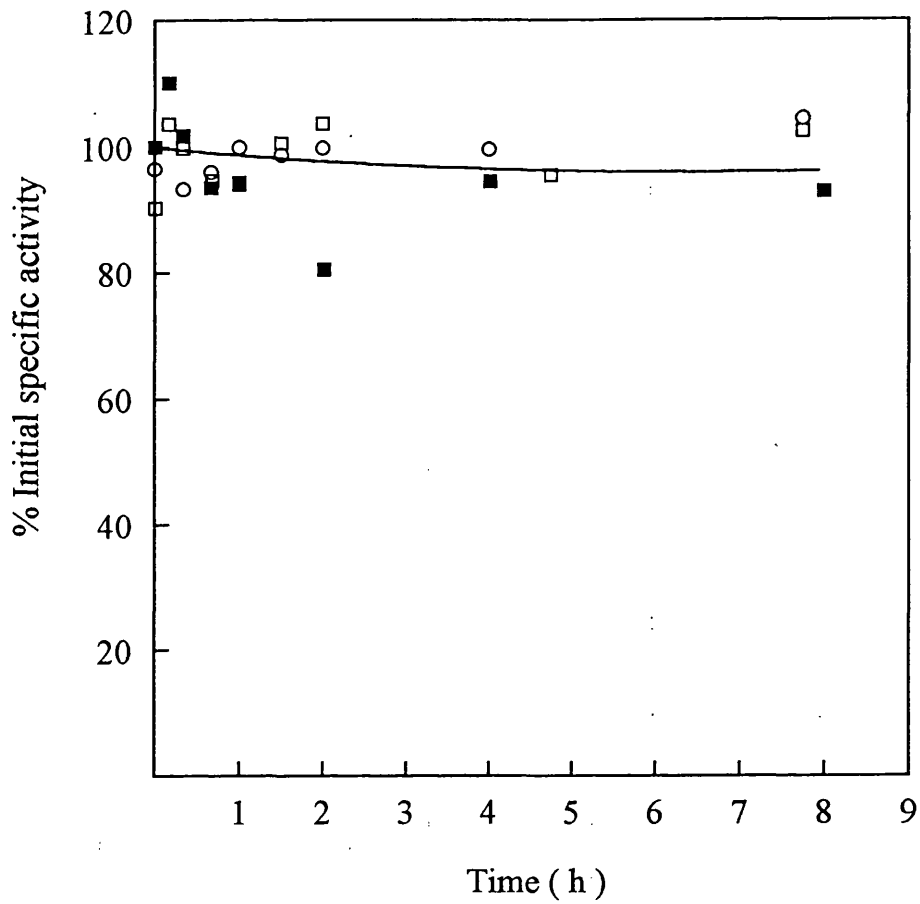
Figure 5.5 Effect of acetate and formate ions on Neu5Ac aldolase stability. Neu5Ac aldolase was incubated with 1M formate (■) and 1 M acetate (□). Samples were desalted and then assayed for remaining activity and protein concentration. Control: DDW incubation (O).
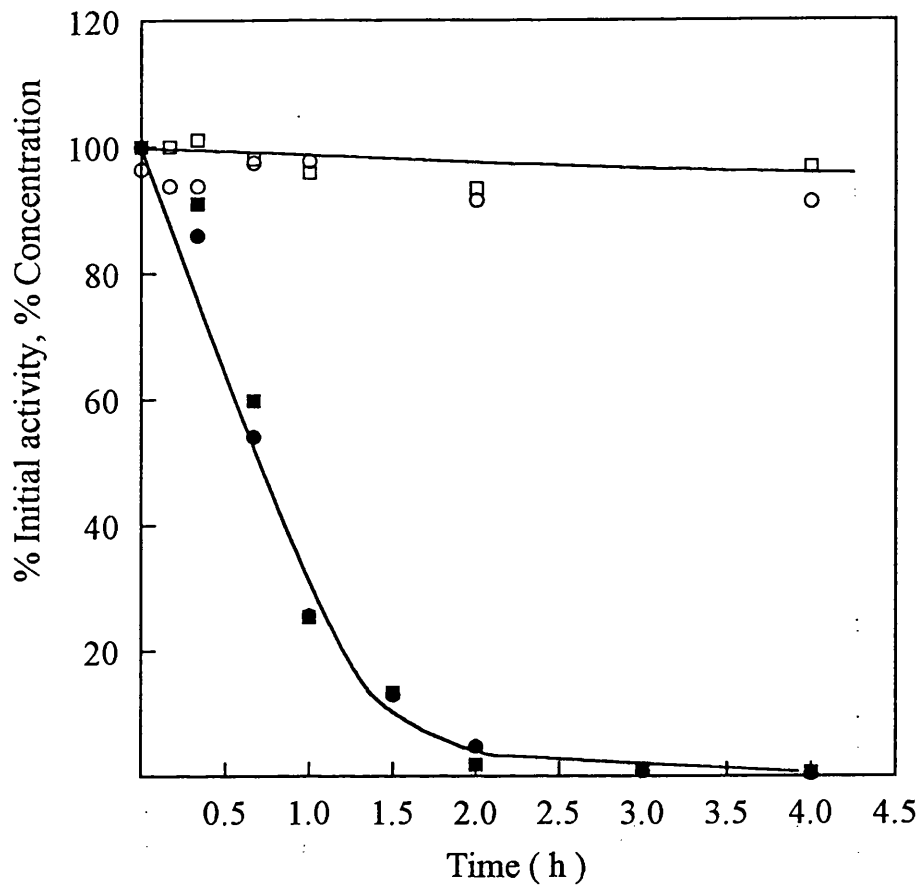
Figure 5.6   Neu5Ac aldolase stability during *in-situ* product removal by a Dowex 1x2 400 column. % initial enzyme activity (●) and protein concentration (■) of Neu5Ac aldolase recirculating through a Dowex 1x2 400 ion exchanger column. Open symbols (O, □): control without recirculation.

5.3.1.4 DYNAMIC INTERACTIONS

5.3.1.4.1 Elution

Counter-ions recirculating in the system could cause competition in the ion exchange process, displacing Neu5Ac that is already bound to the column. The effect of chloride and formate ion gradients was investigated on Dowex 1x2 400 columns in the chloride and formate form fully loaded with Neu5Ac. Both chloride and formate ions depleted Neu5Ac, causing product elution at the beginning of the gradient. Moreover, the same effect was not dependent on the column load since the same result was observed for a column at 10 % saturation in the formate form (Figure 5.7). The constraint of ion competition and subsequent Neu5Ac elution suggested the need for counterion removal from the production system. Otherwise, the selection of a counterion less inhibitory to Neu5Ac binding could provide a suitable solution.

5.3.1.4.2 Binding

The effect of counter-ion inhibition on Neu5Ac binding was investigated on the Dowex 1x2 400 anion exchange resin. The breakthrough profile of 100 mM Neu5Ac was followed with and without 50 mM formate from the anion exchanger in the formate form (Figure 5.8). A frontal uptake analysis was performed (Section 5.3.1.2) on the column profiles. The resin capacity dropped from 350 μmoles Neu5Ac / g resin to 153 μmoles Neu5Ac / g resin, indicating 43 % binding inhibition. The process option of Neu5Ac anion exchanger followed by counter ion removal was then selected.

5.3.1.4.3 Process conditions simulations

The necessity of counter-ion removal imposed further constraints on the choice and operations of anion exchanger ISPR. The use of another unit operation to remove counter ions downstream of the anion exchanger (such as electrodialysis) was avoided by selection of the appropriate resin form. Bicarbonate counter-ion was chosen on account of the carbon dioxide-carbonate equilibrium:

$$CO_2 \text{ (g)} \rightleftharpoons CO_2 \text{ (l)} + H_2O \rightleftharpoons HCO_3^- \text{ (l)} + H^+ \rightleftharpoons CO_3^{2-} \text{ (l)} + 2H^+$$
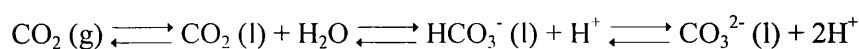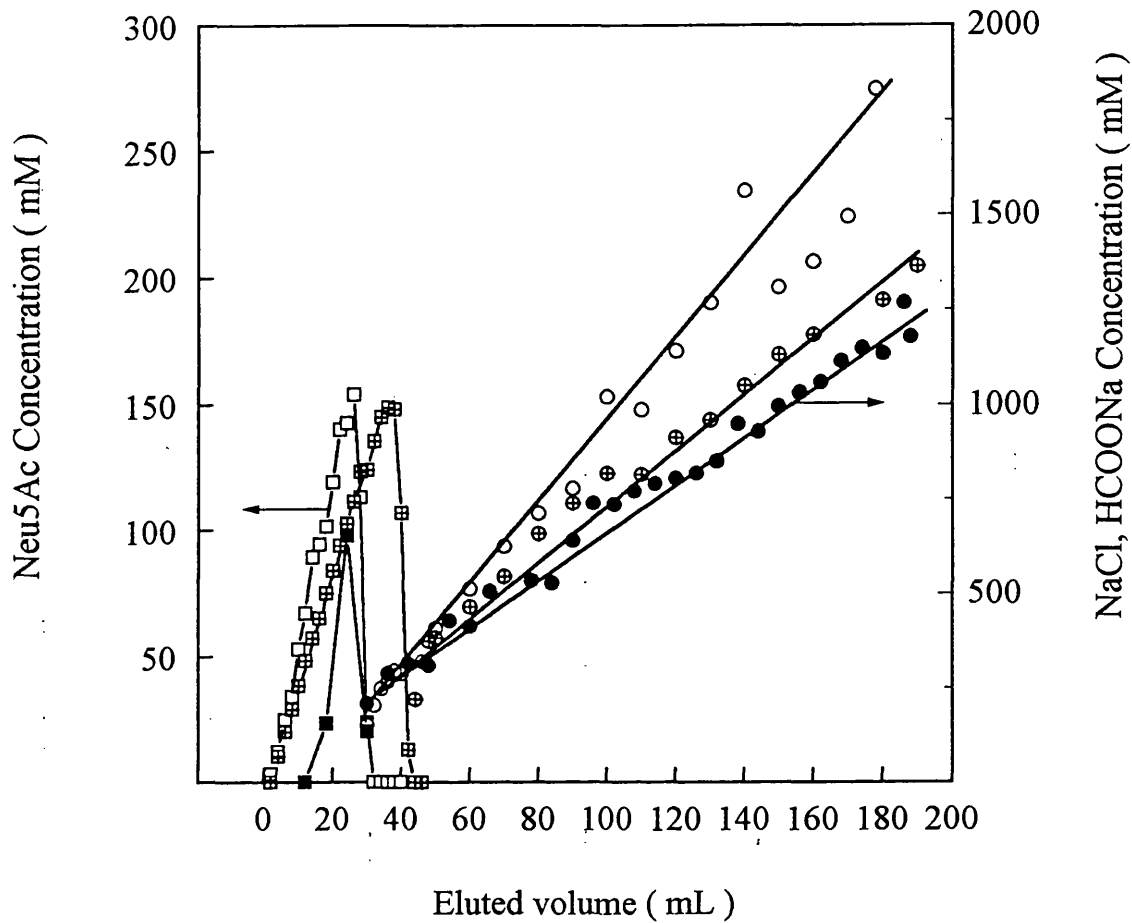
Figure 5.7 Gradient Neu5Ac elution from Dowex 1x2 400 anion exchanger. Neu5Ac was eluted from a formate column at saturation (□) and 10 % saturation (■) and from a saturated column in the chloride form (□). Gradient elutions with HCOONa (●, O) and NaCl (⊕).
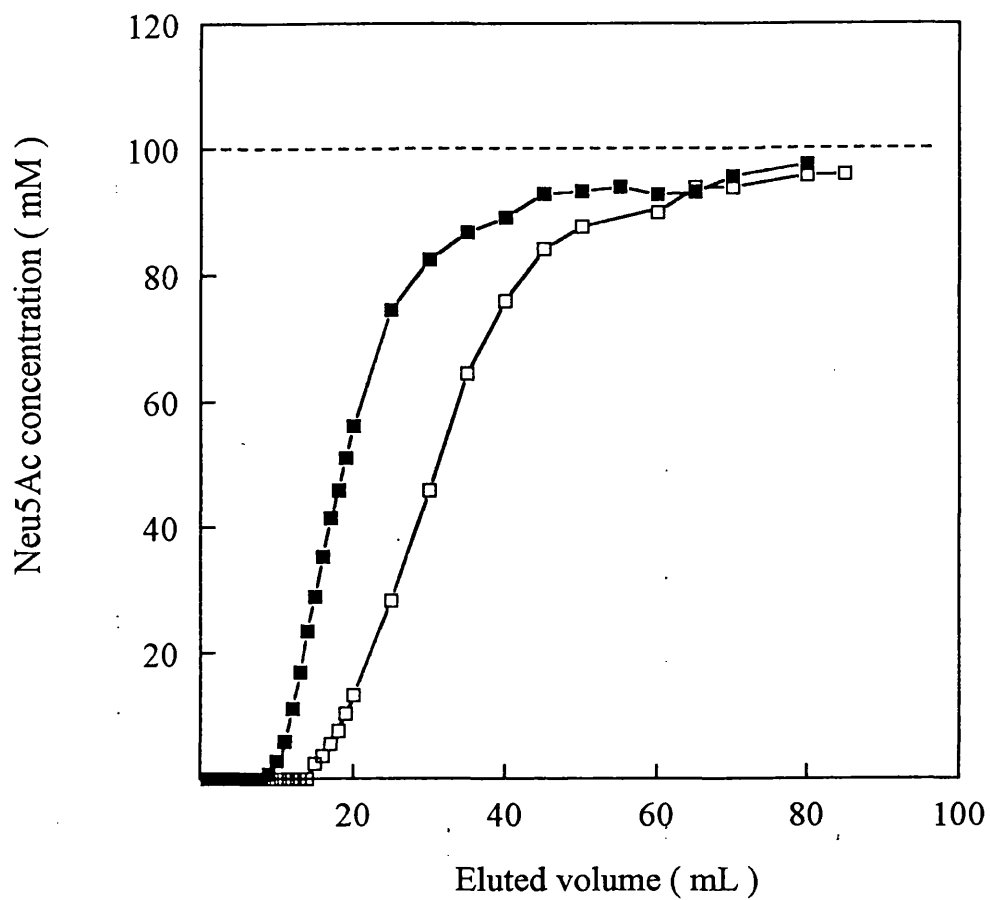
Figure 5.8   Formate competition on Neu5Ac binding. Breakthrough profile of Dowex 1x2 400 column in formate form with 100 mM Neu5Ac (□) and with 100 mM Neu5Ac and 50 mM HCOONa (■).

The equilibrium concentrations of dissolved CO_2, HCO_3^- and CO_3^{2-} in water are a function of pH. At pH 7.5, the bicarbonate concentration is in the range of millimolarity (Bailey and Ollis, 1986). Titration to pH 7.5 after the ion exchanger step would shift the equilibrium towards CO_2 gas formation, displacing the excess bicarbonate ions. Titration with pyruvic acid would avoid the accumulation of other competing anions in the system.

In order to evaluate the performance of Dowex resin in the bicarbonate form under simulated process conditions, a column was loaded with 710 mM GlcNAc, 85 mM Neu5Ac and 5 mM Pyr at pH 7.5 up to saturation and eluted with 1 M $NaHCO_3$ (Figure 5.9). While GlcNAc was not adsorbed on the column, both Pyr and Neu5Ac were retained. A mass balance on the column indicated that the resin capacity was consistent with previous data (0.34 meq / g). Pyr was a strong competitor of Neu5Ac and the elution profiles of the two species overlaid. Hence, a reactor design that minimises pyruvate concentration leaving the reactor is required to make an effective use of anion exchange chromatography. Column geometry and gradient elution procedures could improve the separation of Neu5Ac from Pyr.

5.3.1.5 CONSTRAINTS OF *IN SITU* PRODUCT REMOVAL SYSTEM

The characterisation of a system for Neu5Ac *in situ* product removal was based on previous data and specifically designed experiments in order to identify process constraints and options for reactor development. The constraints are here summarised:

- COMPONENTS PROPERTIES: Separation based on charge. Non-specific separation requires design to minimise Pyr concentration leaving the reactor.
- RESIN CHARACTERISATION: resin capacity decreases with increasing counterion affinity. The hydroxide form of Dowex 1x2 400 resin is ruled out for Neu5Ac degradation.
- EVALUATION OF INTERFERENCES: The acetate form of Dowex 1x2 400 resin is ruled out since acetate ions inhibit enzyme activity. Internal ISPR ruled out and enzyme retention necessary, due to Neu5Ac Aldolase adsorption on the resin.
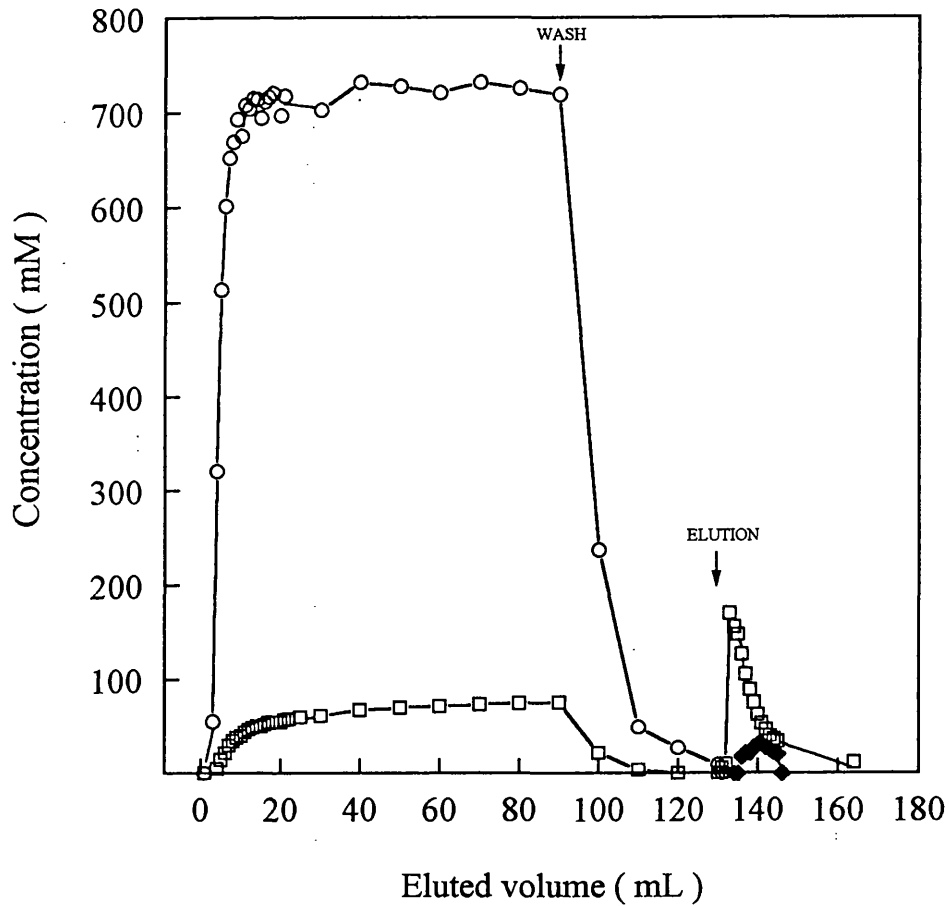
Figure 5.9   Column cycle of Dowex 1x2, bicarbonate form. 5g of Dowex 1x2 in the bicarbonate form were loaded with 85 mM Neu5Ac, 5 mM Pyr and 710 mM GlcNAc.

The concentrations of Neu5Ac (□), GlcNAc (O) and Pyr (◆) are shown in the eluted fraction. Wash step: DDW. Elution step: 1 M NaHCO₃.

- DYNAMIC INTERACTIONS: Neu5Ac elution and binding competition impose the removal of counter-ion after the ion exchanger column. Selection of bicarbonate resin form and pyruvic acid titration. Need for minimal Pyr to maximise column capacity.

Many design solutions can be applied to the above constraints. As a process example, a design for a Neu5Ac ISPR production system (Figure 5.10) will be evaluated in the following section together with different reactor configurations for the synthesis of Neu5Ac.

5.3.2 OPERATING STRATEGIES

5.3.2.1 RATIONALE

The aim of this section is to assess the reactor options for the synthesis of Neu5Ac identified in Chapters 2 and 3. The evaluation of possible designs and operating procedures is achieved under conditions that mimic production scale. Neu5Ac aldolase was over-expressed in *E. coli* NAN 24-F (Dawson *et al.*, 1994) and covalently immobilised as crude extract on porous beads (Eupergit-C™, Röhm Pharma; Mahmoudian *et al.*, 1993, Appendix 3) with a specific activity of 800 U / kg. This catalyst was chosen to assess bioreactor design due to the following considerations:

- Enzyme re-use
- Separation for downstream processing
- Retention in continuous operation
- possible ISPR

Scale-up availability, support robustness and lack of enzyme leaking are additional features of this system. In the synthesis reaction, Pyruvate has been extensively used as the excess substrate to drive the equilibrium towards higher ManNAc conversions, due to its lower costs (Sigma, 1996). Moreover, high substrates molarities are thermodynamically preferred. Pyr however was found strongly inhibitive on initial rates of reactions and ManNAc had an inhibitory effect above enzyme saturation (Section 2.4.2.3). Advantageous experimental conditions have been selected for reaction kinetics and yield. Feeding strategies are likely to overcome kinetic limitations (advantageous for enzyme limiting processes) and provide conditions beneficial to ion exchange separation (favourable for DSP limiting processes).
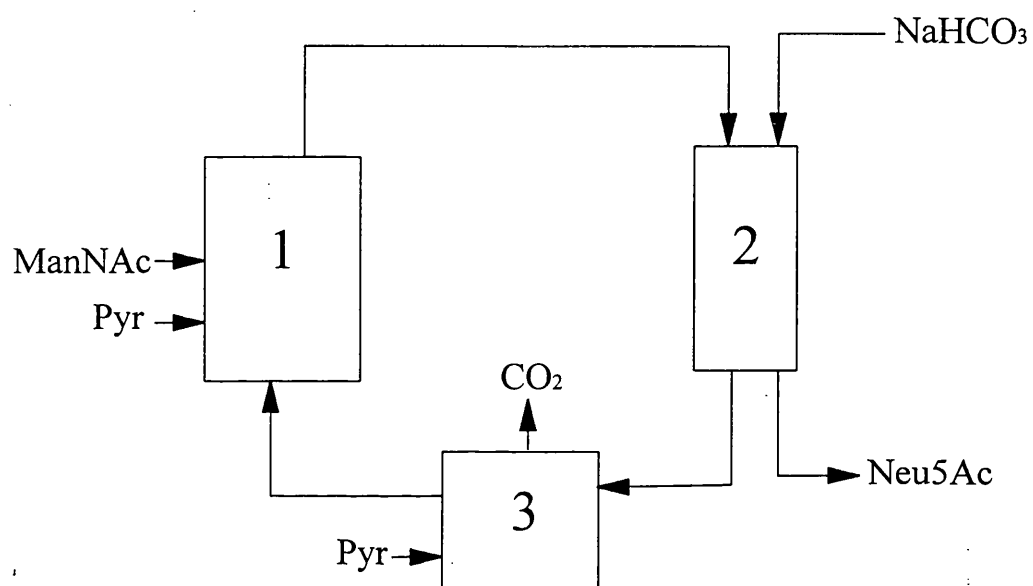
Figure 5.10 ISPR Neu5Ac production design. 1- reactor, 2- Dowex 1x2 400 anion exchanger, bicarbonate form, 3- titration unit.

Batch, fed-batch and continuous operations are here considered in a stirred tank reactor (STR). Plug flow reactor (PFR) was also assessed as an example of continuous operation at different conditions favourable to either yield, enzyme limiting and DSP limiting processes (Section 3.3). The reactor options would be then evaluated for different processing scenarios in Section 5.4.2.

5.3.2.2 STIRRED TANK REACTOR

5.3.2.2.1 Batch

A batch biotransformation was performed with crude Neu5Ac aldolase immobilised on Eupergit beads (Appendix 3) with 990 mM Pyr and 456 mM ManNAc. The 2.17 fold Pyr molar excess was used to drive the equilibrium towards a higher yield on ManNAc, which is more expensive (Sigma, 1996). An immobilised aldolase concentration of 43 % w / v was chosen to test the higher limits of operation. The mass balance (Figure 5.11) indicated that no degradation or adsorption within the beads took place and equilibrium was reached. These conditions have been selected as the control experiment for the following fed-batch biotransformations.

5.3.2.2.2 Fed-batch

Pyruvate feeding would minimise the detrimental effects on enzyme kinetics and evaluate reactor options for the implementation of continuous ion exchange chromatography integrated with the biotransformation step. Hence 200 mM Pyr and 500 mM ManNAc were selected as initial conditions. In order to compare the effects of Pyruvate feeding with batch kinetics, a biotransformation with Pyr pulse was performed with the same amount of substrates (Figure 5.12) as those used in the batch reaction. A 34 % increase in the initial rate of reaction was observed. As expected, the reaction rate decreased when the reaction approached equilibrium. Therefore, both ManNAc and Pyr were pulse fed in the reactor (Figure 5.13) in order to maintain the advantageous initial rate, to minimise both the effect of ManNAc consumption on rate and Pyr concentration at the end of reaction and to observe the effect of Neu5Ac accumulation. A net decrease in reaction rate was observed due to build up of Neu5Ac. This effect was more dominant towards equilibrium. Comparison of batch with double substrate pulsed fed biotransformation (Figures 5.11 and 5.13) indicated the fed-batch could produce the same amount of Neu5Ac with a decrease of 90 % of the
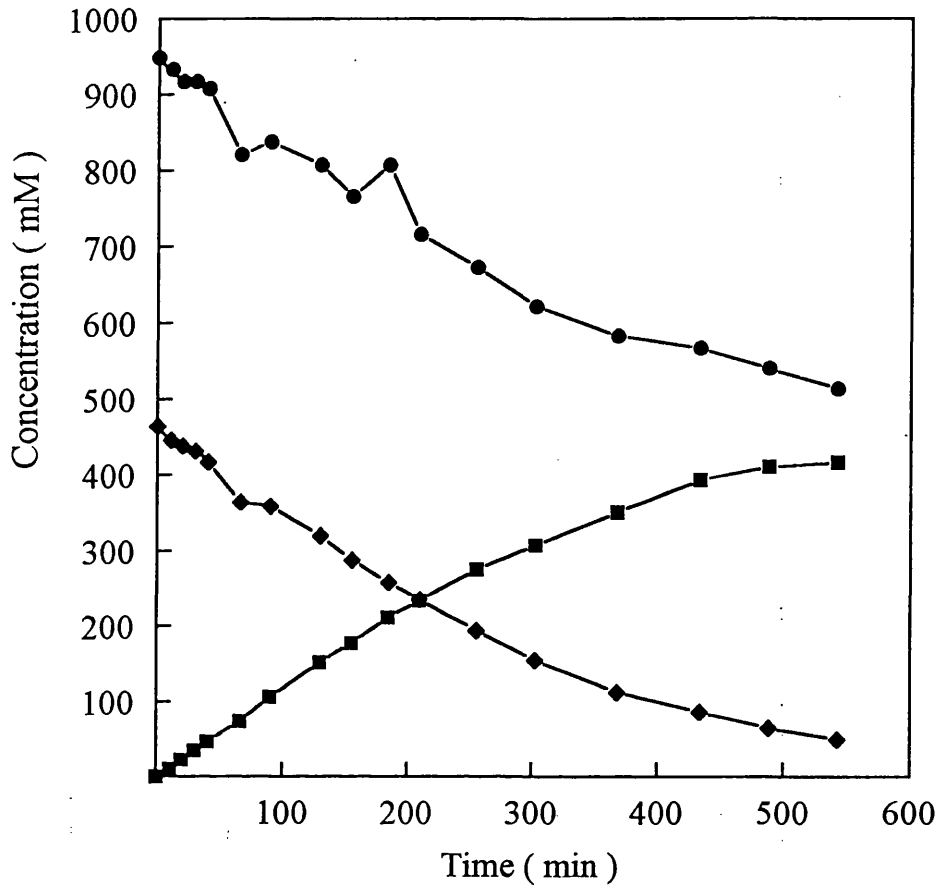
Figure 5.11　Neu5Ac synthesis batch biotransformation. Neu5Ac (■), Pyr (●) and ManNAc (◆) profiles monitored with time.
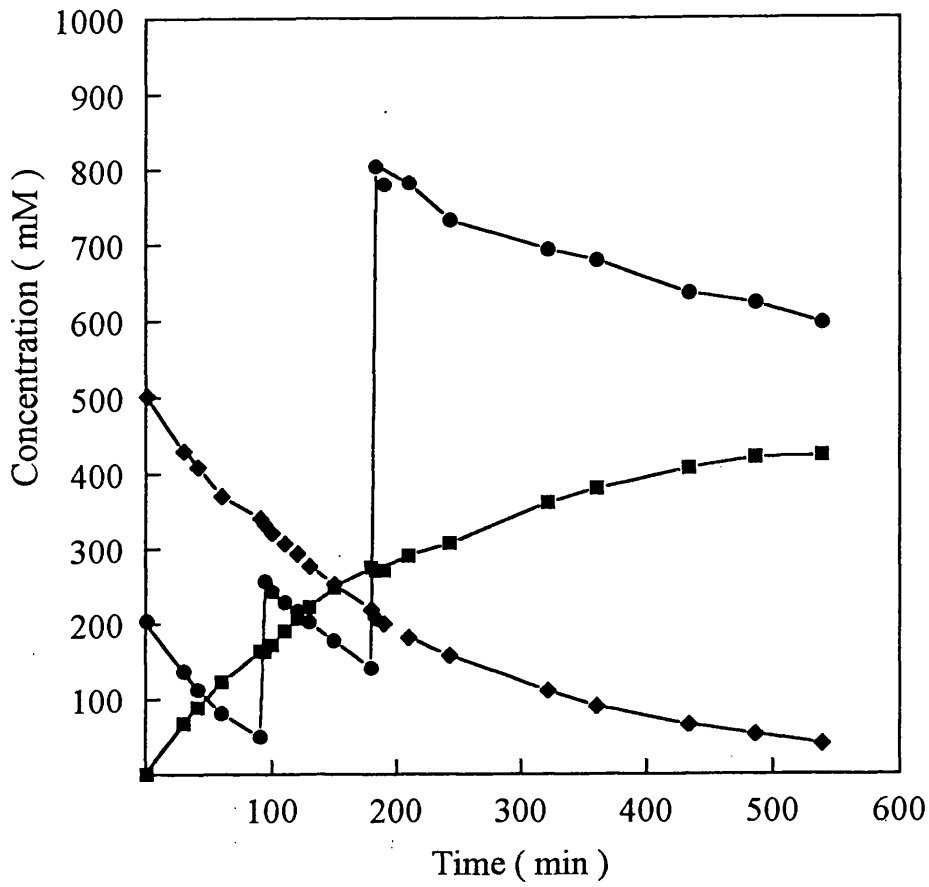
Figure 5.12 Neu5Ac synthesis intermittent fed-batch biotransformation. Neu5Ac (■),
Pyr (●) and ManNAc (◆) profiles monitored with time.
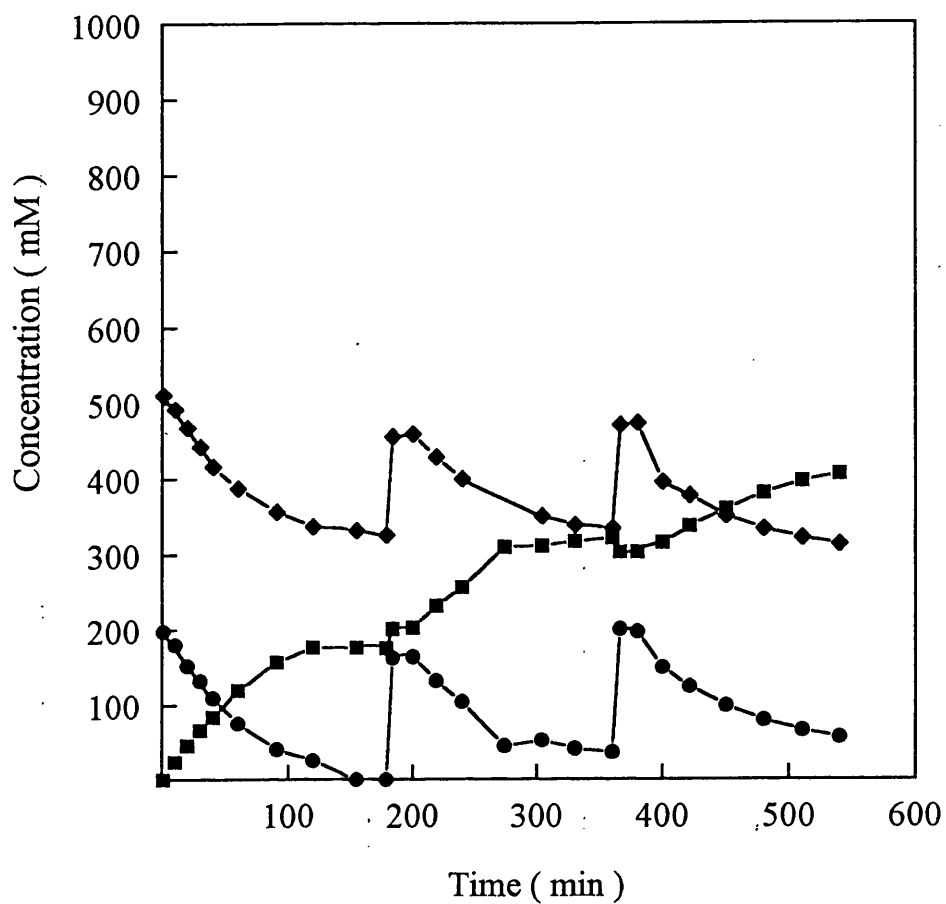
Figure 5.13   Neu5Ac synthesis repetitive fed-batch biotransformation. Neu5Ac (■), Pyr (●) and ManNAc (◆) profiles monitored with time.

residual Pyr at the end of reaction. This decrease in residual pyruvate concentration is clearly beneficial to ion exchange chromatography. Repetitive fed-batch timing could also be optimised further to improve reactor productivity. Moreover, the initial concentrations of ManNAc and Pyr in the pulse fed-batches conferred a kinetic advantage at the beginning of reaction. These conditions were chosen for plug flow reactor kinetic evaluation. Continuous Pyr feeding was also investigated. Since the Km of Neu5Ac aldolase for Pyr is 0.136 mM (Kragl, 1992), the enzyme would be saturated with Pyr concentrations around 14 mM and reaction rates would be limited initially only by ManNAc concentration and pyruvate supply to the reactor. Figure 5.14 shows a fed-batch biotransformation with 550 mM ManNAc where 3 M Pyruvate solution was fed at 2.81 µL / min. The reaction rate was limited by substrate feeding as expected. In order to achieve higher conversion rates, Pyr feeding was increased two fold (Fig. 5.15), however the reaction rate became enzyme limited and Pyr accumulation took place. The cumulative effect of Neu5Ac build up and Pyr limitations imposed constraints on feeding such that beneficial effects on reaction rate were achieved only when Pyr was fed at a constant concentration of 130 mM (Fig. 5.16). In this case, a 59 % increase over the batch initial reaction rate was achieved together with a Neu5Ac / Pyr ratio of 2.7 at the end of reaction. The advantages of feeding strategies on reactor productivity and downstream integration will be discussed in Section 5.4.2.

5.3.2.3 PLUG FLOW REACTOR

5.3.2.3.1 Small scale plug flow reactor

A plug flow reactor (PFR) for the synthesis of Neu5Ac has several benefits. PFR design has the advantages of both continuous operation and batch kinetics (Wang *et al.*, 1979). By operating a substrate feed with excess ManNAc, the PFR can achieve high conversion rates and maintain low pyruvate leaving the reactor, achieving high yields on this limiting substrate. The PFR design was investigated in order to determine its operating conditions and assess the effect of high substrate molarities on enzyme performance. For reactor design, refer to Appendix 3. A 19.5 mL column packed with Neu5Ac aldolase immobilised on Eupergit-C ™ was evaluated under different substrate concentrations and flowrates (Table 5.4). It was possible to observe the effect of excess pyruvate on Neu5Ac aldolase performance (experiments 1, 2 Table 5.4) At a flowrate of 0.46 bv / h the aldolase column achieved 91 % equilibrium conversion when 459 mM ManNAc and 182 mM Pyr were fed
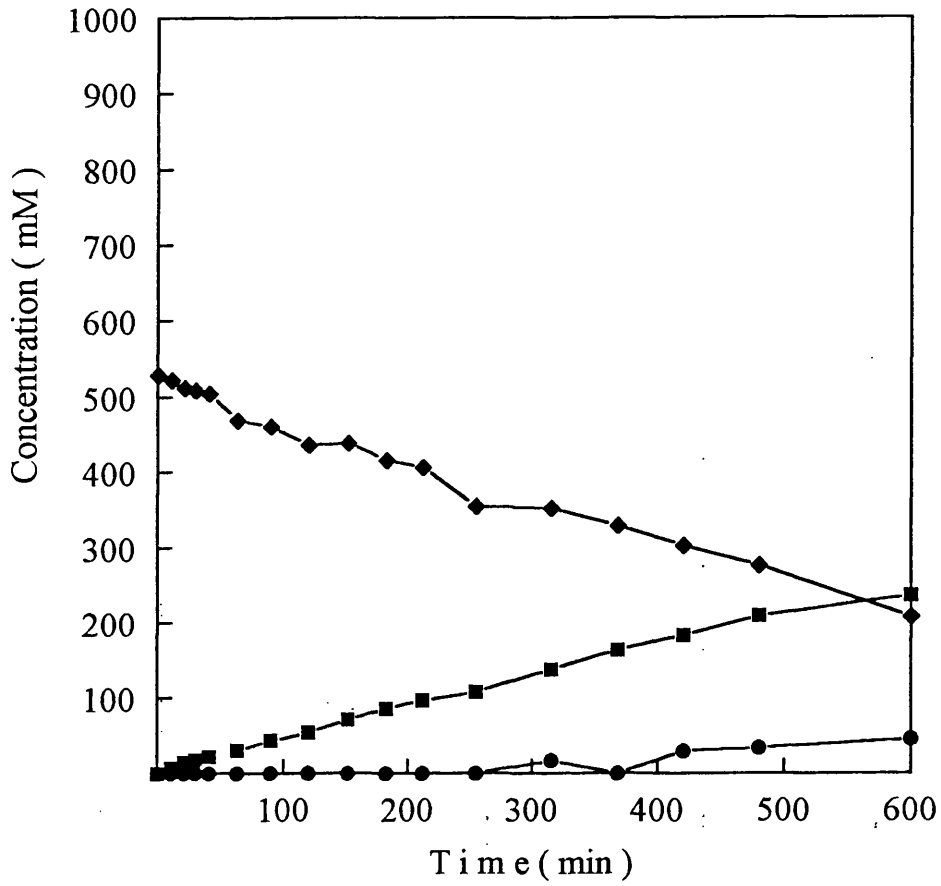
Figure 5.14 Neu5Ac synthesis continuous fed-batch biotransformation, feeding limiting regime. Neu5Ac (■), Pyr (●) and ManNAc (◆) profiles monitored with time. Pyruvate feed: 3 M, 2.81 µL / min.
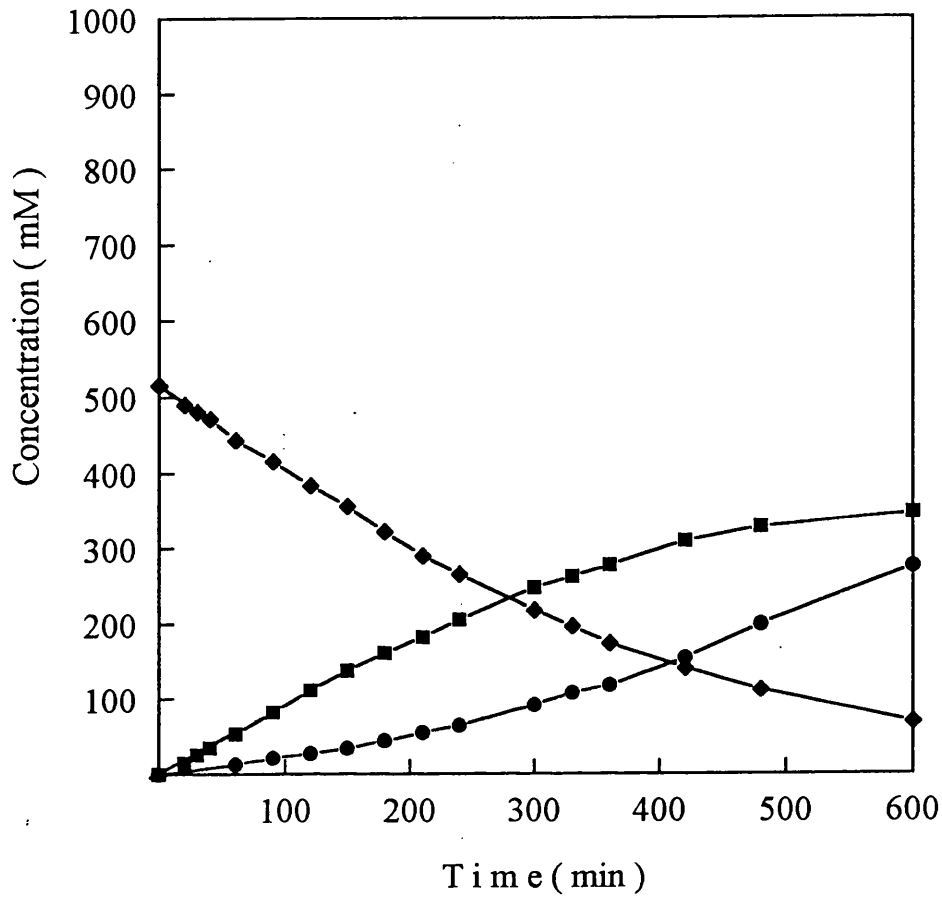
Figure 5.15 Neu5Ac synthesis continuous fed-batch biotransformation, reaction limiting regime. Neu5Ac (■), Pyr (●) and ManNAc (◆) profiles monitored with time. Pyruvate feed: 3 M, 5.62 μL / min.
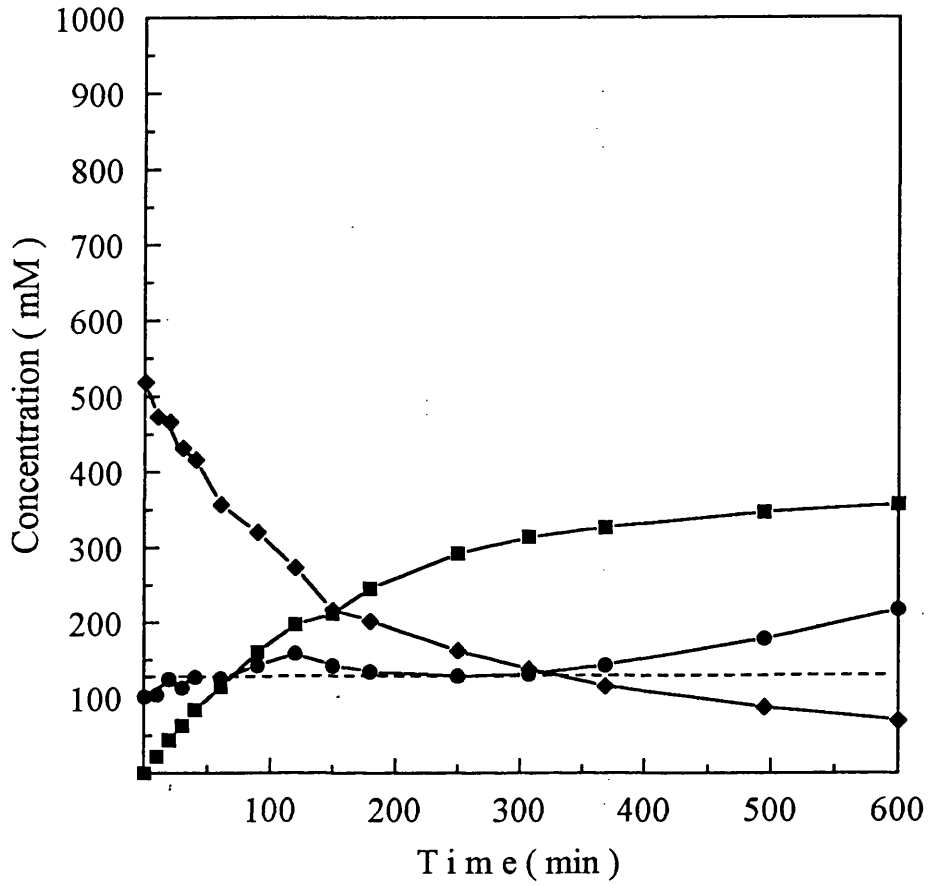
Figure 5.16   Neu5Ac synthesis continuous fed-batch biotransformation, constant Pyr regime. Neu5Ac (■), Pyr (●) and ManNAc (◆) profiles monitored with time. Pyruvate feed: constant 130 mM.

Table 5.4   Experiments for 19.5 mL plug flow reactor.

experiment	flowrate (mL/min)	initial concentrations (mM)			Neu5Ac (mM)	% equilibrium conversion[1]
		GlcNAc	ManNAc	Pyr		
1	0.15	-	520.0	820.0	120.7	35
2		-	458.7	181.8	152.9	91
3	0.2	-	458.7	181.8	132.2	77
4		170	465.0	210.0	64.2	30
5	0.5	-	458.7	181.8	62.1	37
6		170	465.0	210.0	25.1	12
7	1.0	-	462.0	187.2	38.5	23

1- % equilibrium conversion = conversion on limiting substrate / maximum conversion achievable due to equilibrium. Based on Keq = 29.4 M^{-1} (Kragl, 1992).

in the reactor. However, when 520 mM ManNAc were fed with 1.6 Pyr molar excess under the same conditions, only 35 % equilibrium conversion was achieved. The % equilibrium conversion was found inversely proportional to the flowrate (standardised according to bed volume, Figure 5.17, Rosen *et al.* in press) through the column. The conversion dropped from 91 % at 0.15 mL / min to 23 % at 1 mL / min, as expected (Table 5.4, experiments 2, 3, 5, 7).

5.3.2.3.2 Reactor operational conditions

In order to evaluate the reactor kinetics under conditions that would mimic actual production conditions, the plug flow reactor was tested with enriched ManNAc solution. ManNAc was prepared by GlcNAc alkaline epimerisation, according to the protocol of Dawson *et al.*, 1994 (Appendix 3). Since the epimerisation equilibrium imposed a ManNAc / GlcNAc ratio of 1 / 4 (Comb and Roseman, 1960), the mixture was enriched by isopropyl alcohol GlcNAc crystallisation and Methanol extraction, yielding a solution with ManNAc / GlcNAc ratio of 2.8 / 1 (Appendix 3). GlcNAc is a competitive inhibitor (Kragl, 1992, Mahmoudian *et al.*, in press) and this effect is shown in Figure 5.17 for a plug flow reactor. At 0.2 and 0.5 mL / min (Table 5.4, experiments 4,6) flowrates, a decrease of 25-47 % on equilibrium conversions was observed when the enriched mixture with 170 mM GlcNAc was pumped onto the 19.5 mL reactor. The PFR was scaled-up to 104 mL column and the operating flowrate was increased proportionally to the column unit volume (Rosen *et al.*, in press). 465 mM ManNAc, 210 mM Pyr and 170 mM GlcNAc were passed through the PFR at 0.5 and 1 mL /min (Figure 5.17) to mimic production conditions. The equilibrium conversions achieved were 66 % and 54 % respectively. The 104 mL column was then operated continuously for 21 hours at 0.5 mL / min (Figure 5.18). The conversion of Neu5Ac first achieved 140 mM and then decreased to 100 mM at 21 hours. The mass balance across the column indicated that product degradation did not occur. However, the observed decrease in conversion could be caused by either column channelling or loss of enzyme activity, even though the enzyme was found stable for at least 10 hours during batch reactions (Figure 5.12). The effect of GlcNAc and the operating parameters of the plug flow reactors will be discussed in Section 5.4.2.
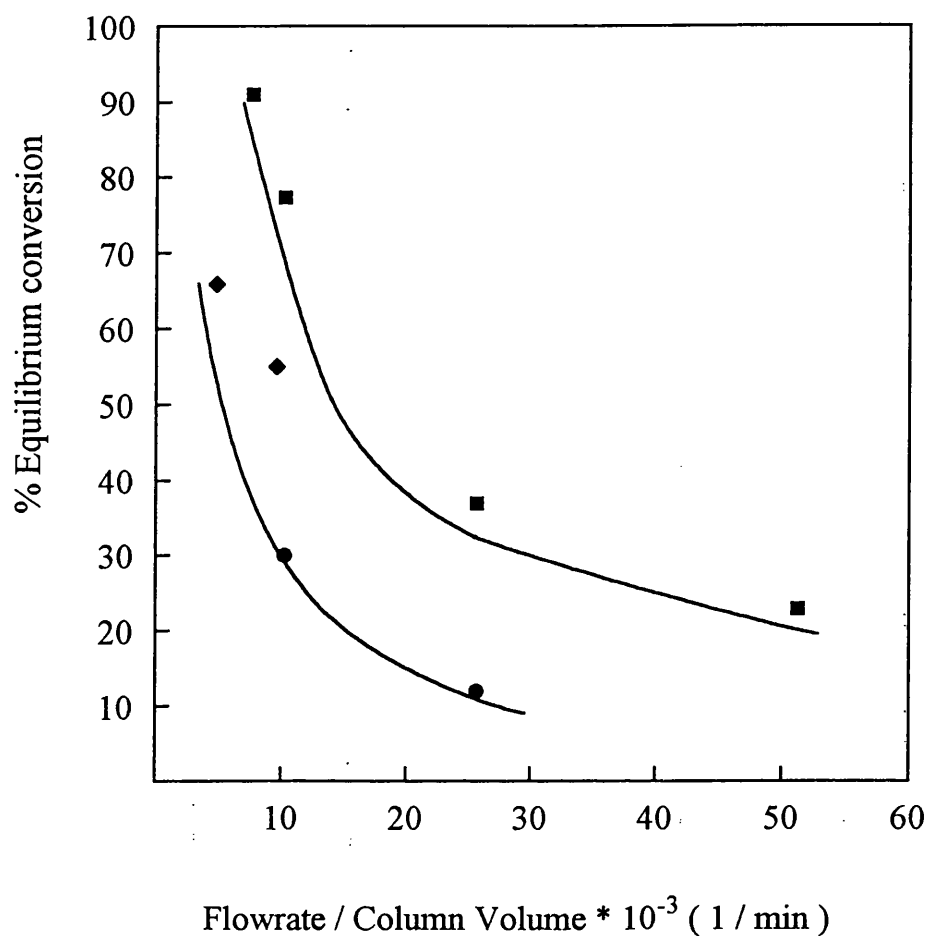
Figure 5.17   Plug flow reactor biotransformations. Conversion against standardised flowrate for 19.5 mL column with 460 mM ManNAc and 180 mM Pyr (■), with 465 mM ManNAc, 210 mM Pyr and 170 mM GlcNAc (●) and for 104 mL column with 465 mM ManNAc, 210 mM Pyr and 170 mM GlcNAc (◆).
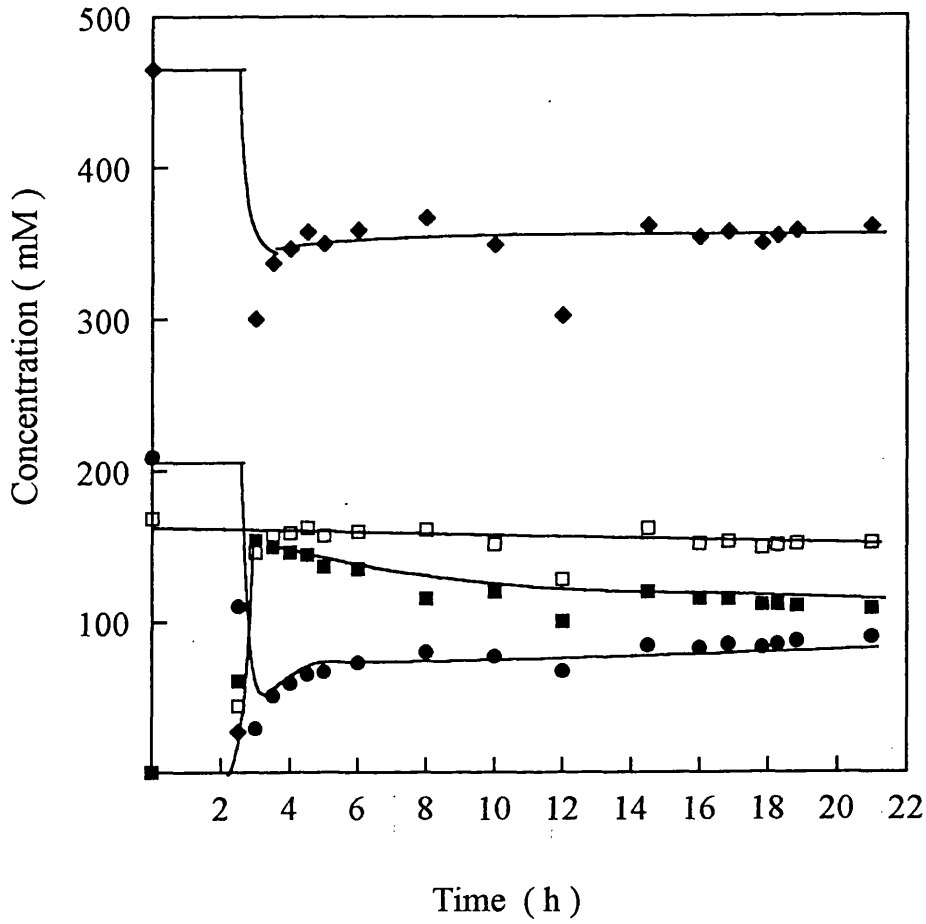
Figure 5.18   Production simulation with plug flow reactor. Neu5Ac (■), Pyr (●), ManNAc (◆) and GlcNAc (□) profiles were monitored with time.

5.3.2.4 *IN SITU* PRODUCT REMOVAL REACTOR

In situ product removal (ISPR) in the synthesis of Neu5Ac has the potential to drive the thermodynamics towards complete stoichiometric conversion (i.e. yield improvement) and avoid the detrimental effects of Neu5Ac accumulation on reaction rate (i.e. productivity improvement). Different solutions can be implemented via reactor choice and separation options. Ion exchange chromatography has been selected as a means of non-specific ISPR (Section 5.3, Figure 5.10). The breakthrough capacity of the resin was selected to minimise the circulation of Neu5Ac in the reaction. In order to evaluate the process advantages of ISPR under one of the many possible conditions, 2 Dowex 1x2 400 columns in the bicarbonate form were connected in parallel to a 104 mL enzyme column during the previous experiment (Figure 5.18). At 8 hours, the stream leaving the reactor (130 mM Neu5Ac, 80 mM Pyr, 170 mM GlcNAc, 335 mM ManNAc) was switched to the first ion exchanger until the resin was saturated to its breakthrough capacity and then diverted to the second column. Both pyruvate and Neu5Ac were adsorbed on the ion exchangers (Figure 5.19). The stream containing GlcNAc and ManNAc was collected and titrated to pH 7.5 with pyruvic acid to eliminate excess bicarbonate ions. ManNAc and Pyr were added to compensate the conversion to Neu5Ac and the solution was recycled to the substrate reservoir. After 114 minutes the resin columns were disconnected, washed with water, eluted and regenerated with 2M bicarbonate. 4.2 mmoles Neu5Ac were recovered. The mass balance across the reactor-separation unit with recycle stream has shown that the conversion based on ManNAc was complete while the yield across the reactor was 28 %. In this particular case, the kinetic advantage was expressed by the use of a plug flow reactor, exposing the enzyme to inhibiting Neu5Ac concentrations only towards the end of the column. Moreover, since this example was chosen to evaluate possible process condition, a ManNAc-GlcNAc mixture was employed. the GlcNAc inhibition then imposed an additional constraint to the enzyme kinetics. This reactor design has the potential to be implemented in continuous operations and would become beneficial for those reactions which equilibrium values do not favour product formation.
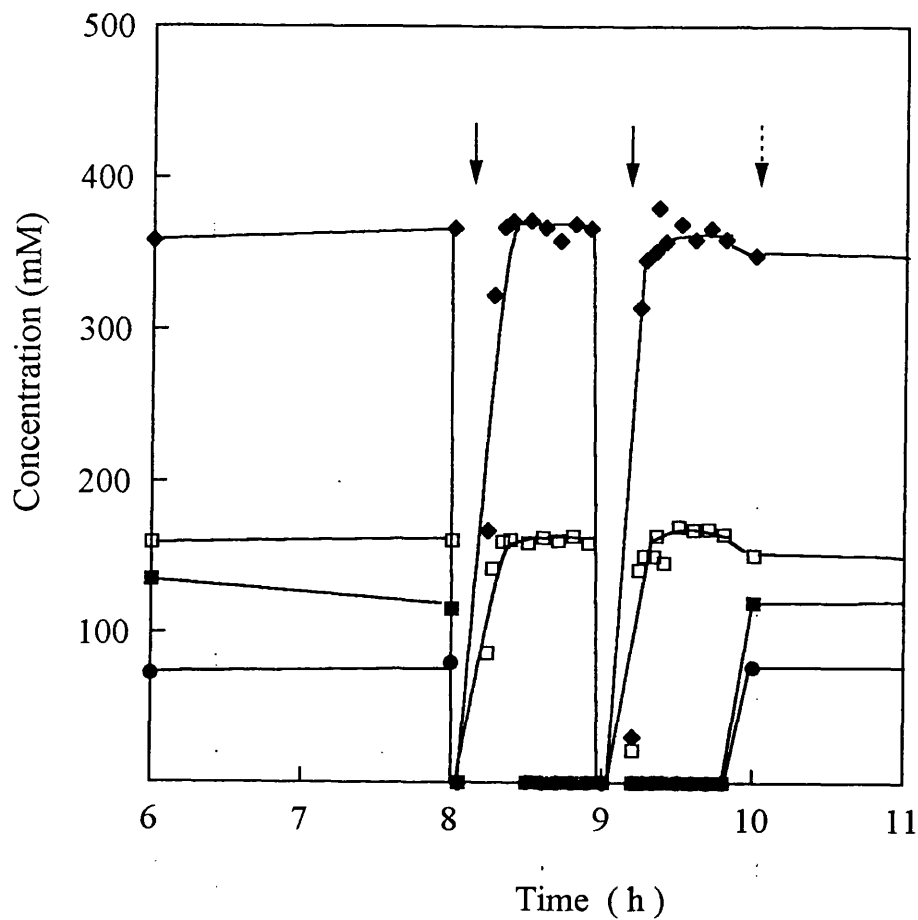
Figure 5.19   *In-situ* product removal biotransformation. Neu5Ac (■), Pyr (●), ManNAc

(◆) and GlcNAc (□) leaving the reactor were monitored with time (please refer to Figure

5.18). Arrows indicate switch to ion exchange columns. Dotted arrow: columns taken off-

line.

5.4 DISCUSSION

5.4.1 *IN SITU* PRODUCT REMOVAL CHARACTERISATION

ISPR reactor design according to Freeman *et al.* (1993) is justified in order to implement processes that would otherwise not be possible to achieve, because of product decomposition and deleterious effects of product on the enzyme (toxicity and inhibition). In this work, Neu5Ac synthesis presents a case of complex kinetics (product inhibition, substrates inhibition at high molarities) and thermodynamic limits on product concentration. An evaluation of ISPR is given to the synthesis of Neu5Ac in order to compare this reactor design with other production possibilities. In the characterisation of a suitable ISPR technique, an anion exchanger step was chosen in order to assess the constraints on reactor design based on non-specific separation means. More specific separation techniques could have allowed more flexibility in reaction conditions. The characterisation was able to rule out at early stages unsuitable conditions for ISPR and to propose an initial reactor flow sheeting. Several designs could have originated from the array of reactor options and separation techniques. One possible design was then implemented under possible production conditions. The reactor selection and operation gave beneficial kinetic conditions to the enzyme while the ISPR and substrate recycling overcame the thermodynamic limitation of the system.

5.4.2 COMPARISON OF OPERATING STRATEGIES

Batch, fed-batch, plug flow and plug flow with *in situ* product removal reactors have been evaluated as possible production methods for the synthesis of Neu5Ac. The effect of GlcNAc competitive inhibition has been investigated by Kragl (1992) and Mahmoudian *et al.* (in press) on batch reaction kinetics. In this work, the effect of substrate concentrations and presence of GlcNAc was investigated in a PFR kinetics mode. The effect of substrate inhibition could be overcome by operating the reactor at low flowrates (Wang *et al.*, 1979) beyond the investigated experimental range. Moreover, to overcome the constraint of low productivity, an ISPR reactor was implemented, increasing conversion yield through a recycle loop. Various production methods together with the enzyme membrane reactor developed by Kragl and co-workers (1992) are compared in Table 5.5 for different values upon which reactor choice should be implemented. The parameters are:

- SPACE TIME YIELD (g / L / d): this value expresses the productivity of a reactor system per unit volume of reactor. However, this is not an indication whether the system is operating under favourable kinetic conditions.

- SPECIFIC ENZYME PRODUCTIVITY (g / d / g catalyst): this value represents how effectively the catalyst is used. Kinetics limitations from reactor design and enzyme loading influence this figure. Values based on Neu5Ac aldolase immobilised on Eupergit C ™ (Appendix 3).

- PRODUCT CONCENTRATION (g / L): factor affecting the DSP sizing and timing, as well as possible process options.

- PRODUCT / ENZYME (g / g catalyst): indication of the operational stability of the catalyst. Assumptions were made in order to compare different reactor times and configurations. Values based on Neu5Ac aldolase immobilised on Eupergit C ™ (Appendix 3).

- EQUILIBRIUM YIELD (%): indication about the thermodynamics of the system and whether the reaction achieved full conversion.

- Neu5Ac / (Neu5Ac+Pyr) (%): in case of ion exchange separation, this value measures the effective utilisation of ion exchanger capacity.

In this thesis, distinct manufacturing scenarios are considered to originate from costs and availability restrictions, caused either by enzyme limitations, downstream processing, substrate and products or time constraints (Table 3.1). Three production examples are here developed further. In Figure 5.20, the case is elaborated for product limitations, when final product quantity is the most important parameter. In this example, space time yield and %

Table 5.5 Reactor characteristics for Neu5Ac biotransformation.

Reactor	Space Time Yield g / L / d	Specific Enzyme Productivity g / d / g catalyst	Neu5Ac g / L	Product/ Enzyme[3] g / g catalyst	Equilibrium Conversion[5] %	Neu5Ac/ (Neu5Ac+Pyr) %
1. batch. 1M Pyr, 0.5 M ManNAc, Eupergit: 10.13 g, volume: 23 mL.	374.2[2]	0.85	126.8	0.29	94.8	44.4
2. enzyme membrane reactor[1]. 0.3 M ManNAc, 0.6 M Pyr, 240 mL, 0.06 L/h flowrate, 6 g / L aldolase	470.0	0.04	76.1	0.46	90.1	40.8
3. fed batch. 530 mM ManNAc, feed 3 M Pyr: 2.81 μL / min, Eupergit: 10.1 g, volume: 23.62mL	175.2[2]	0.41	73.0	0.17	99.0[6]	83.6
4. fed batch. 516 mM ManNAc, feed 3.03 M Pyr: 5.62 μL/min, Eupergit: 10.55 g, volume: 24.01mL	341.5[2]	0.77	85.4	0.19	70.4[6]	70.1
5. fed batch. 519 mM ManNAc, feed 3.03 M Pyr: 130 mM, Eupergit: 10.35 g, volume: 20.23 mL.	394.6[2]	0.77	100.9	0.20	80.5[6]	73.7
6. PFR 19.5 mL. 181 mM Pyr, 454 mM ManNAc, Eupergit: 10.67 g, flowrate: 0.15 mL/min	523.6	0.96	47.3	0.96[4]	91.0	84.4
7. PFR 19.5 mL. 181 mM Pyr, 454 mM ManNAc. Eupergit: 10.67 g, flowrate: 1.0 mL/min	879.4	1.61	11.9	1.61[4]	22.9	21.3
8. PFR 104 mL. 210 mM Pyr, 465 ManNAc, 170 mM GlcNAc, Eupergit: 56.95 g, flowrate: 0.5 mL/min	286.9	0.52	41.4	0.52	66.1	85.7

1-Kragl *et al.* (1992) 2- no downtime considered 3-based on complete denaturation of enzyme after reaction 4-based on 21 hours operations 5-based on Keq = 29.4 M^{-1} (Kragl. 1992) 6- based on batch mass balance.

Figure 5.20   Reactors evaluation with product constraints. The arrow indicates the optimisation direction. 1- batch, 2- enzyme membrane reactor, 3- fed-batch, feeding limitations, 4- fed-batch, enzyme limitations, 5- fed-batch, constant Pyr, 6- plug flow reactor, high conversion, 7- plug flow reactor, low conversion , 8- plug flow reactor with GlcNAc.

equilibrium conversion have been related to each other and various reactors have been assessed. Under these condition, the enzyme membrane reactor (Kragl et al., 1992) and PFR operated at low flowrate and high conversion would be the most beneficial designs. In case of the enzyme limitations (Figure 5.21), design should focus on the most effective use of enzyme (specific enzyme productivity) and overall enzyme stability (product to enzyme ratio). In order to compare enzyme operating stabilities for different reactor designs, complete denaturation after one reaction cycle has been assumed. In this case, a PFR with high throughput would be advantageous despite the requirement for large volumes and low yields. Under these assumptions, the effect of GlcNAc on reactor performance (point 8, Figure 5.21) suggests further separation between upstream processing and the biotransformation step. Downstream processing limitations (Figure 5.22) would impose high product concentration to minimise processing volumes and high product purity (here, % Neu5Ac / Neu5Ac + Pyr). Under these limitations, fed-batch reactor would be the most appropriate design for Neu5Ac synthesis. A clear benefit could arise by the development of specific ISPR since the product stream would be purified and concentrated at the same time. The description of different qualitative scenarios established the design selection on appropriate process variables. Process economics would be then required in order to quantify the importance of each parameter and advance the process synthesis towards detailed design, reactor optimisation and scale-up.

5.5 SUMMARY

In this chapter,

- *In situ* product removal reactor design for Neu5Ac synthesis was characterised (Section 5.3.5).
- Dowex 1x2 400 was chosen as a model for non-specific separation. Resin characterisation, evaluation of interferences and dynamic interactions led to the selection of a reactor with low outlet pyruvate concentration, bicarbonate anion exchanger form and titration to pH 7.5 to eliminate counter-ions.
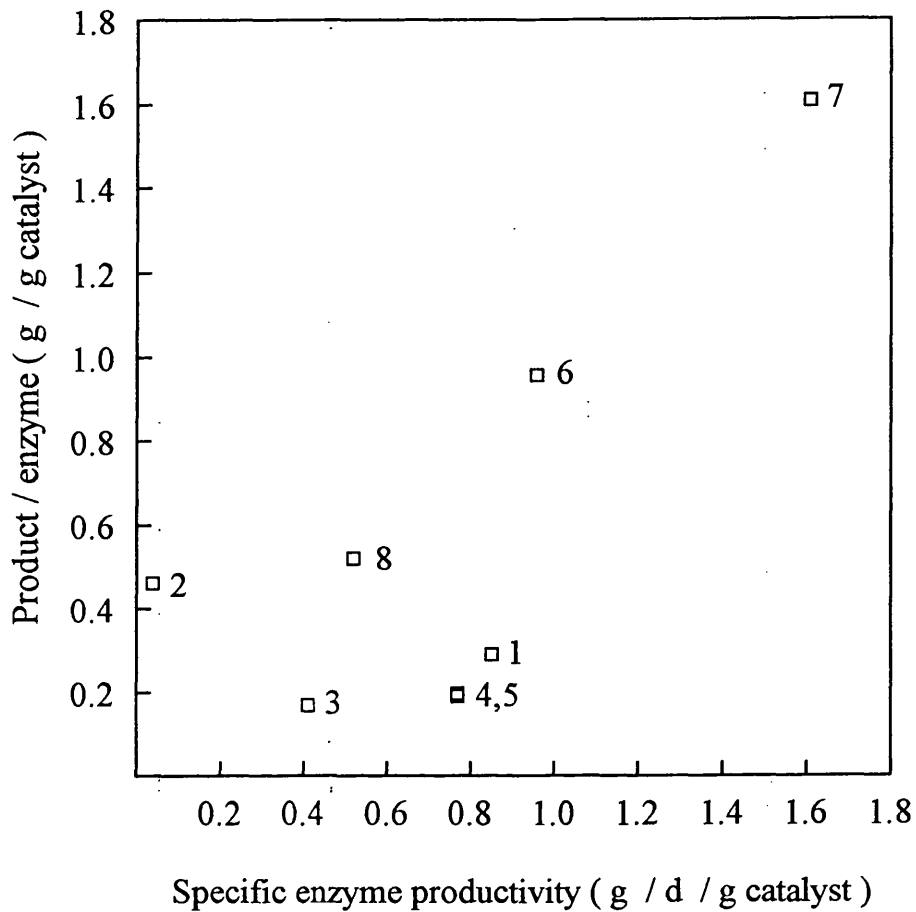
Figure 5.21 Reactors evaluation with enzyme constraints. 1- batch, 2- enzyme membrane reactor, 3- fed-batch, feeding limitations, 4- fed-batch, enzyme limitations, 5- fed-batch, constant Pyr, 6- plug flow reactor, high conversion , 7- plug flow reactor, low conversion , 8- plug flow reactor with GlcNAc.
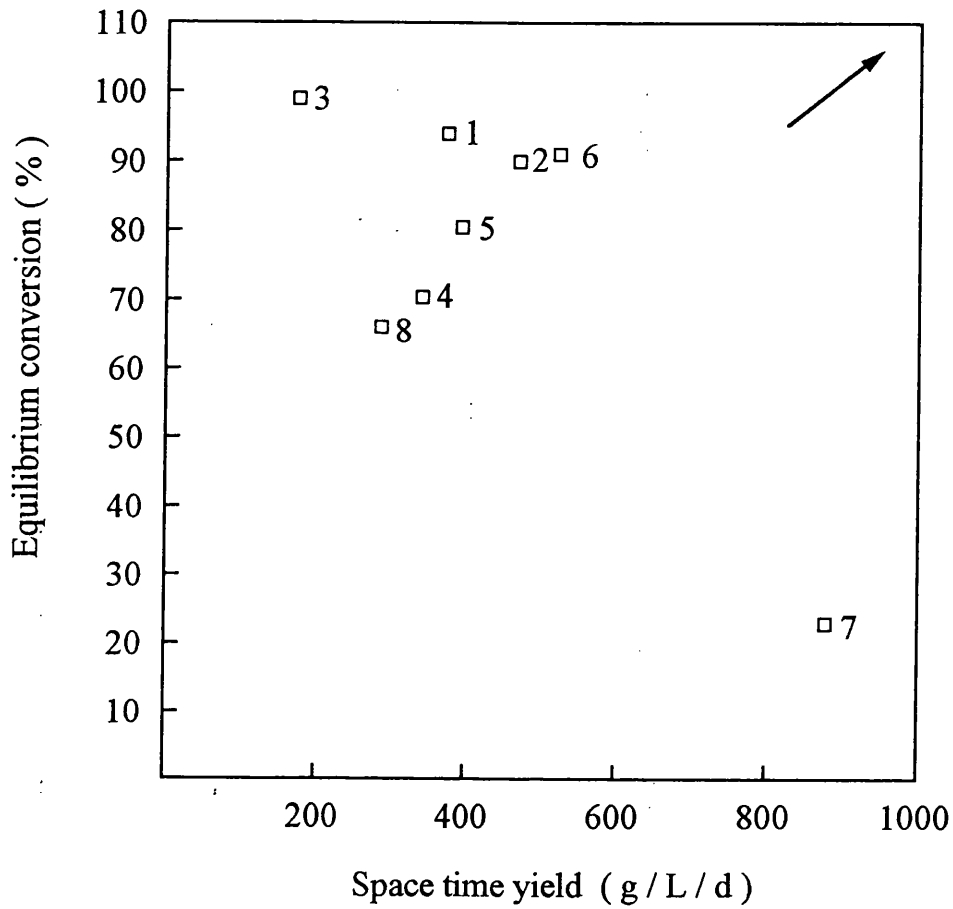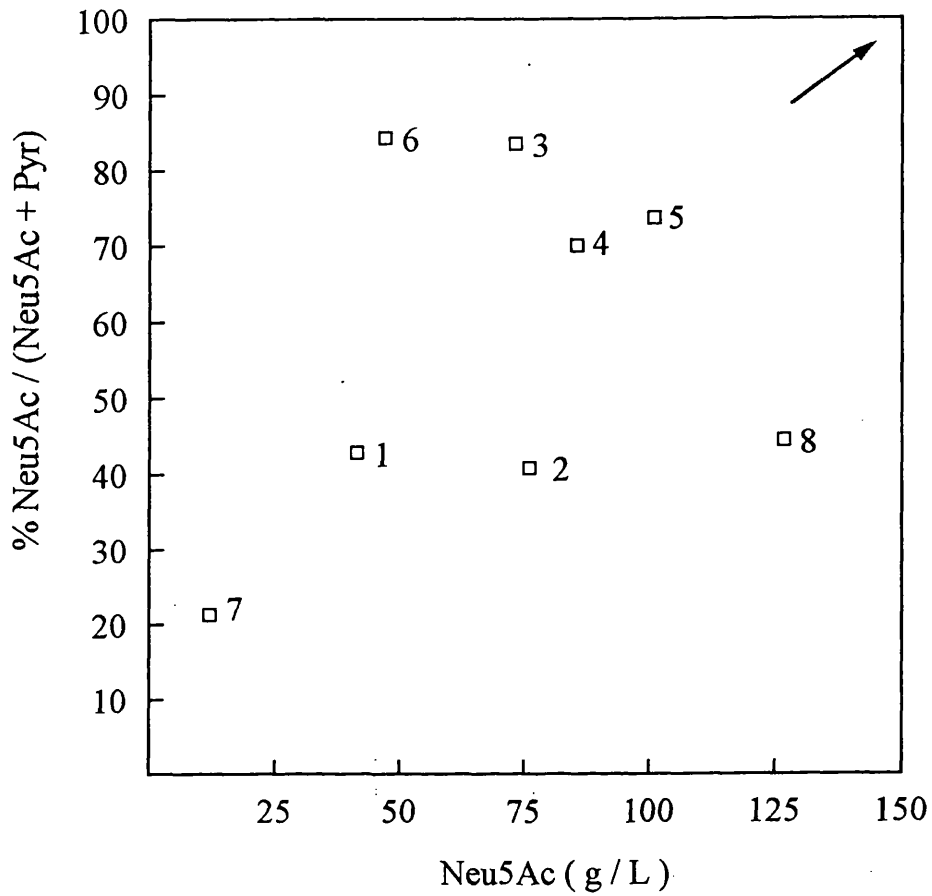
Figure 5.22   Reactors evaluation with DSP constraints. The arrow indicates the optimisation direction. 1- batch, 2- enzyme membrane reactor, 3- fed-batch, feeding limitations, 4- fed-batch, enzyme limitations, high conversion, 5- fed-batch, constant Pyr, 6-plug flow reactor, 7- plug flow reactor, low conversion , 8- plug flow reactor with GlcNAc.

- Pyruvate pulse fed-batch biotransformation (at 200 m Pyr and 500 mM ManNAc) achieved 34 % increase in initial reaction rate compared to a batch reaction with the same amount of substrates starting at 2.17 Pyr molar excess. Double substrate pulse fed reaction achieved the same amount of Neu5Ac as the batch with a decrease of 90 % residual Pyr.

- Continuous fed-batch at a constant concentration of 130 mM Pyr achieved a 59 % increase over the batch initial reaction rate together with a Neu5Ac / Pyr ratio of 2.7 at the end of reaction.

- A 19.5 mL plug low reactor was operated with 459 mM ManNAc and 182 mM Pyr, achieving up to 91 % equilibrium conversion at 0.3 bv / h. Under the same conditions, only 35 % conversion took place when 520 mM ManNAc with 1.6 Pyr molar excess was used.

- In order to mimic production conditions, 25-47 % decrease in equilibrium conversions was observed when a substrate solution with 170 mM GlcNAc was pumped onto the 19.5 mL reactor at 0.6-1.5 bv / h. A 104 mL column was operated for 21 h with an equilibrium conversion of 66 % at 0.29 bv / h.

- An example of ISPR was evaluated with a PFR in the presence of 170 mM GlcNAc. The reactor-separation unit achieved complete ManNAc conversion, while the reactor yield was 28 %. This reactor design would be favourable for reactions which equilibrium does not favour product formation.

- Different reactor designs have been evaluated for processing scenarios based on product, enzyme and DSP constraints. The enzyme membrane reactor (Kragl et al., 1992) and PFR at low flowrate were beneficial for product limitations, PFR with high throughput was advantageous for enzyme constraints and fed-batch reactor was appropriate for DSP constraints.

The reactor evaluation concluded the experimental work in the rational approach to biotransformation process design. The overall experimental methodology and hierarchy of information in process characterisation, evaluation and selection will be discussed in Chapter 6.

6

GENERAL DISCUSSION

6.1 Neu5Ac MODEL FOR BIOTRANSFORMATION PROCESS DESIGN

The structured approach to biotransformation process design is composed of the characterisation step, the identification of constraints, the definition of process options and the process evaluation (Chapter 1, Woodley and Lilly, 1996). In this thesis, this approach has been undertaken for the chemo-enzymatic synthesis of Neu5Ac. The reaction has been taken as a model since it is an example of equilibrium controlled reaction that takes place in a single phase, Neu5Ac aldolase has been proved a very broad spectrum catalyst for aldol condensations of hexoses, pentoses and even tetroses (Fitz *et al.*, 1995), and eventually this case presented the challenge of observing the benefits and drawbacks of integrating the reaction step with either the chemical epimerisation step upstream and the product separation downstream of the reaction. Within this framework, this thesis contributes to the general design approach of process design with two main aspects. First, the characterisation step has been divided further in two sets of experiments, so that beneficial reaction environments are scanned quickly and unfeasible options are ruled out, accelerating the design procedure. Second, economic considerations in the form of different processing scenarios have been introduced in the identification of constraints, in the discussion of different USP-biotransformation integrated reactions and in the evaluation of limited options in the reactor design. This introduction is likely to shift the importance of the structured approach away from the enzyme towards other elements of the process. So far, the methodology was set up to select the best conditions for the biocatalyst (Lilly, 1992, Woodley and Lilly, 1996). But with time, as enzymes become a commodity due to recombinant DNA technology, other parameters (substrate costs, DSP, operation times) should be taken in consideration when selecting a reactor design. Constraints are shifting, it is important to address these issues and identify the boundaries while the process technology is advancing.

6.2 BENEFITS OF THE STRUCTURED APPROACH

The structured approach to process design is able to bring several advantages to the overall process design and scale-up to manufacturing. This approach can identify bottlenecks in the process scale-up, with the use of operating windows. A correct process research enhances ramp-up of production and transfer to manufacturing, avoiding plant capacity expansion as the only option available in case of market requirements (Pisano and Wheelright, 1995). The characteristics of the process and its financial optimisation would dictate the flexible limits that would be set according to the most severe constraint. For example, in the case of Neu5Ac aldolase (Figure 3.1), enhancement in protein engineering or decrease in enzyme cost would shift the activity limit towards higher ManNAc and Pyr concentrations. Protein synthetic evolution, (Moore and Arnold, 1996) can overcome additional constraints on the availability of specific catalysts.

This approach is able to rule out unfeasible process options in the early stage of process research. The characterisation step is composed by two blocks of experimental work. The first block of experiments should be aimed to identify the characteristics of the reaction components and restrict the number of conditions within which the biotransformation is advantageous. The second set of experiments identifies the interactions between the various components and provides together with the first set of experiments the constraints of the reaction. The respective hierarchy of these two sets of data is here discussed.

This thesis has developed as described in Figure 6.1. From the characterisation results (Chapter 2) a first consideration of process options evaluation (Chapter 3) has given rise to two separate directions. One part of the investigation referred to process integration (Chapter 4), specifically between the chemical and the biochemical step. The other research work has focused on reactor designs for the synthesis of Neu5Ac, where work was undertaken to assess the feasibility of an ISPR reactor (Chapter 5). Retrospectively, only part of the experiments would be essential (Figure 6.1) for the assessment of the appropriate reaction system. In the characterisation block, both components and interactions characterisations should be undertaken to obtain an initial scenario of the reaction environment. While the characterisation proceeds towards the identification of constraints and further experimental work, the importance of interactions characterisation increases. This is also due to the possibility of gathering data on all reaction components during biotransformation runs. The interactions characterisation learning curve could be enhanced

Characterisation

**Constraints
Identification**

Components
● Reactants and product solubility
● Reactants and product stability
● pH Enzyme activity-stability
● Equilibrium
Interactions
● Reaction at selected conditions
● Enzyme Km, Ki at selected conditions

**Process Option
Evaluation**

**Epimerisation
Biotransformation
Integration**

**Reactor
Configurations
for Neu5Ac Synthesis**

ISPR

Components
● Epimerisation Equilibrium
Interactions
● Epimerisation kinetics and limits
● Integrated reactions at various pH

Interactions
● Binding, Elution
● Interferences
● Capacity

Reactors

Interactions
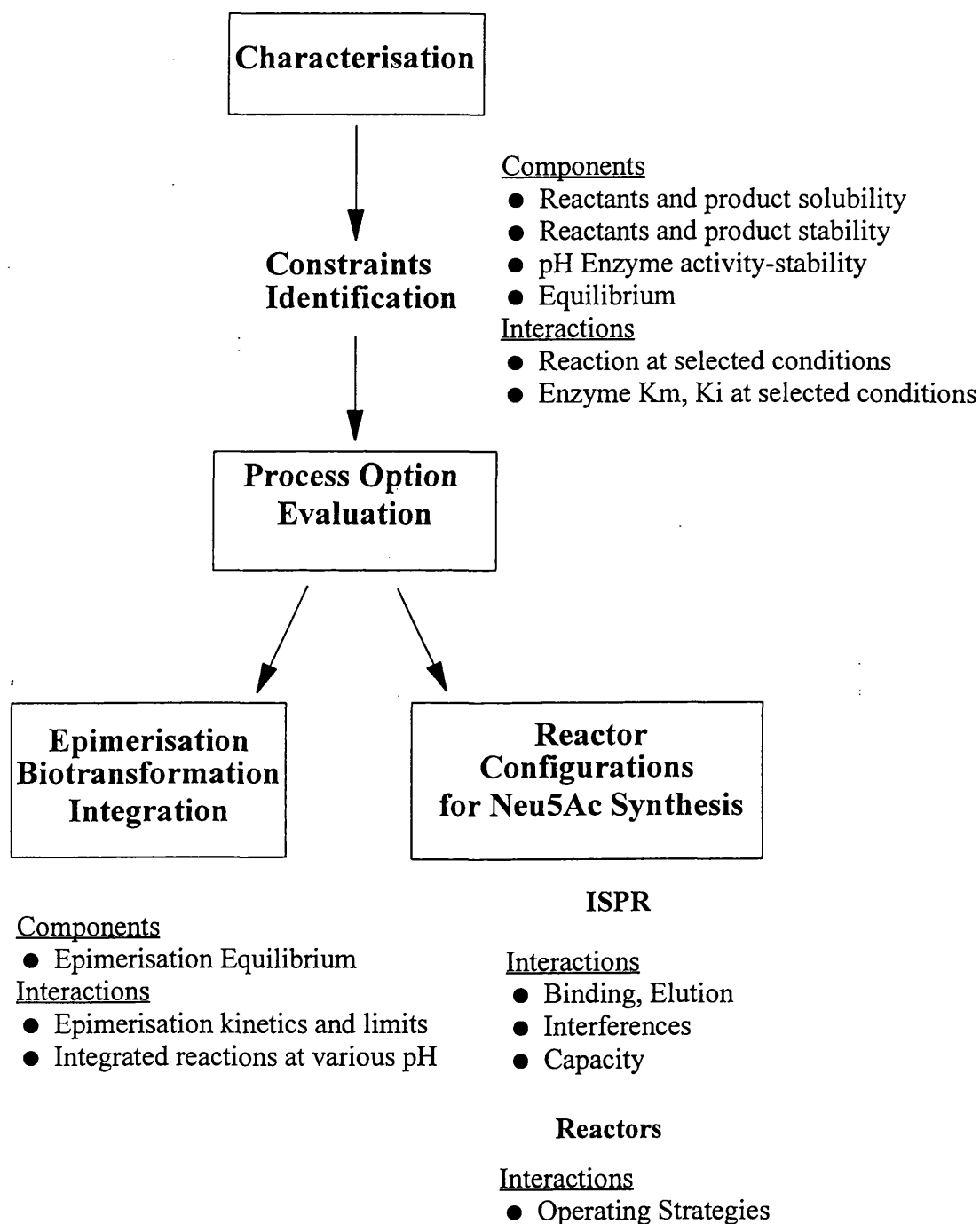● Operating Strategies

Figure 6.1   Essential experiments for the process design of Neu5Ac chemo-enzymatic synthesis.

further by rational design of experiments, such as full or partial factorial design of multivariable design of experiments (Haaland, 1989). In the characterisation of ISPR (Chapter 5), the choice of non-specific separation method has imposed further experimental work based on ion exchange chromatography. In order to minimise future experimental work in this area, the interactions of the binding process should be approached first, followed by evaluation of interferences and finally by capacity determination under the selected conditions. Future work may be directed towards the evaluation of more specific ISPR methodology. This structured approach could be then utilised as a knowledge-based system for the whole range of aqueous based enzymatic equilibrium reactions for which only a minimal number of experiments will be required to make the appropriate selection for reactor design and operation. Other future work should focus on the validation of these experiments with the characterisation of another reaction catalysed by Neu5Ac aldolase, as discussed in Section 6.3. Eventually, this rational approach gives the framework for process research that could be implemented alongside with basic research in order to collect the relevant data for process synthesis of new chemical entities not yet researched.

6.3 FURTHER CONSIDERATIONS AND FUTURE WORK

The specific findings of this thesis for the chemo-enzymatic synthesis of Neu5Ac are presented in Chapter 7. Among various aspects of biotransformation research, future work should focus to continue the following aspects of this project:

- The enantiomeric specificity and selectivity of Neu5Ac aldolase has been extensively employed in carbohydrate chemistry (Fitz *et al.*, 1995). Future work could be focused on the validation of this design approach by the investigation of ManNAc derivatives. The use of a poorly soluble or toxic substrate could provide a test for the benefits and drawbacks of introduction of a second organic phase in the reaction system (Dervakos *et al.*, 1989).

- The development of a specific method for Neu5Ac ISPR could provide the basis for an evaluation of the benefits of specific product removal in equilibrium systems.

The constraints dictated by environmental regulations, regulatory requirements (for example with respect to enantiomeric excess, Stinson, 1995), safety and process economics would dictate additional constraints on the process. These specifications are required early in the process synthesis to reduce the number of process options and therefore speed-up the design procedure. Eventually, this work would then find its application as a part of a larger knowledge system in the manufacturing of novel compounds.

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CONCLUSIONS

In the framework of a rational approach to biotransformation process design, discussed in Chapter 6, three main specific objectives for the chemo-enzymatic synthesis of Neu5Ac have been accomplished in this thesis. They are:

- To characterise the chemo-enzymatic synthesis of Neu5Ac and assess its constraints for process design (Woodley and Lilly, 1992, 1996).

- To evaluate the integration of the biotransformation step with either upstream (the epimerisation) and downstream operations.

- To determine the appropriate reactor design and operation for Neu5Ac synthesis.

In light of these objectives, the following has been concluded:

- The solubility limits of ManNAc (1.6 M) and pyruvate (3.6 M) at pH 7.5 restricted the maximum substrate concentrations in the biotransformation.

- The degradation of reaction components in alkaline environment imposed a constraint on the integrated biotransformation at high pH. While ManNAc and GlcNAc were found stable below pH 12 at 25 $^\circ$C, Pyruvate was degraded above pH 10.5 and Neu5Ac aldolase has been proved beneficial to pyruvate stability, while degradation was not related to the presence of air (oxygen).

- Neu5Ac, GlcNAc, ManNAc and Pyr remained stable at pH 2. However, the integration of acid crystallisation and biocatalysis was unfeasible (Uchida et al., 1984).

- The enzyme was stable at pH 7.15- 7.5. At alkaline pH there was a loss of 30% activity at pH 10.5 and 75% at pH 11.3 after 8 hours. GlcNAc and ManNAc were shown to increase

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aldolase deactivation at pH 10.5, while Pyr or pyruvate degradation products increased enzyme stability.

- The double substrate steady-state model for Neu5Ac aldolase developed by Kragl and co-workers (1992) was not found to be valid for concentrations beyond 305 mM ManNAc and 562 mM Pyr. Pyruvate had a strong inhibitory effect on initial rates of reaction above 500 mM up to 3.6 M (saturation concentration) and ManNAc was found inhibitory above 750 mM.

- An operating window was defined, relating the data for solubilities of the substrates, the enzymatic activity and the final obtainable product concentration (arbitrarily fixed at 0.2 M Neu5Ac at equilibrium) as process boundaries for a batch reaction.

- GlcNAc-ManNAc epimerisation took place above pH 9.0 and the approximate specific reaction rate increased exponentially with pH to $39.88 * 10^{-3}$ hours at pH 12. The equilibrium constant was 0.249 (25 °C, pH 10.7).

- 31.3 % conversion on Pyr was achieved in an integrated biotransformation at pH 10.5. 30 % pyruvate degradation was measured besides the conversion to Neu5Ac. The amount of active enzyme also decreased to 41 %. The "pseudo" steady state at the end of reaction ("pseudo" Keq = 6.728) was comparable to the equilibrium achieved with enzymatic synthesis (Kragl et $al.$, 1991).

- Dowex 1x2 400 was evaluated for ISPR. The hydroxide form of the resin degraded Neu5Ac. Acetate ions inhibited enzyme activity (78 % inhibition at 1 M concentration). Both chloride and formate ions depleted resin bound Neu5Ac and formate competed for Neu5Ac binding. This led to the selection of a reactor with low outlet pyruvate concentration, bicarbonate anion exchanger form and titration to pH 7.5 to eliminate counter-ions.

- Pyruvate pulse fed-batch biotransformation with 500 mM ManNAc achieved 34 % increase in initial reaction rate compared to a batch with the same amount of substrates

starting with 2.17 Pyr molar excess. Double substrate pulse fed reaction achieved the same amount of Neu5Ac as the batch with a decrease of 90 % residual Pyr.

- Continuous fed-batch at constant 130 mM Pyr achieved a 59 % increase over the batch initial reaction rate and a Neu5Ac / Pyr ratio of 2.7 at the end of reaction.

- A 19.5 mL PFR achieved 91 % equilibrium conversion at 0.3 bv / h with 459 mM ManNAc and 182 mM Pyr while only 35 % conversion took place when 520 mM ManNAc with 1.6 Pyr molar excess was used.

- 170 mM GlcNAc caused 25-47 % decrease in equilibrium conversions in a 19.5 mL PFR at 0.6-1.5 bv / h with 465 mM ManNAc and 210 mM Pyr. A 104 mL column was operated for 21 h with an equilibrium conversion of 66 % on Pyr at 0.29 bv / h.

- An example of ISPR was evaluated with a PFR in the presence of 170 mM GlcNAc. The reactor-separation unit achieved complete ManNAc conversion, yielding 4.2 mmoles Neu5Ac. This reactor design would be favourable for reactions in which equilibrium does not favour product formation.

- The enzyme membrane reactor (Kragl *et al.*, 1992) and PFR at low flowrate were appropriate for both high space time yield and percent equilibrium conversion. PFR with high throughput was advantageous for high specific enzyme productivity and high product to enzyme ratio. Fed-batch reactor was appropriate for both high product concentration and high product purity.

APPENDIX 1
NAME AND ADDRESSES OF EQUIPMENT
AND CHEMICAL SUPPLIERS

Beckman Instruments (UK.) Ltd
Progress Road
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Kontron Instruments,
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Mettler-Toledo Ltd.
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Radiometer Copenhagen
Manor Court
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Perkin Elmer Ltd.
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Buckinghamshire HP9 19A,
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Pharmacia LKB Biotechnology AB
Björkgatan 30
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Philips

Pye umicam Ltd.

Cambridge

UK.

Röhm GmbH

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Poole

Dorset BH12 4QH

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Toyobo Co., Ltd.

2-8 Dojima Hama 2-chome,

Kita-ku, Osaka 530,

Japan.

APPENDIX 2

ANALYTICAL TECHNIQUES
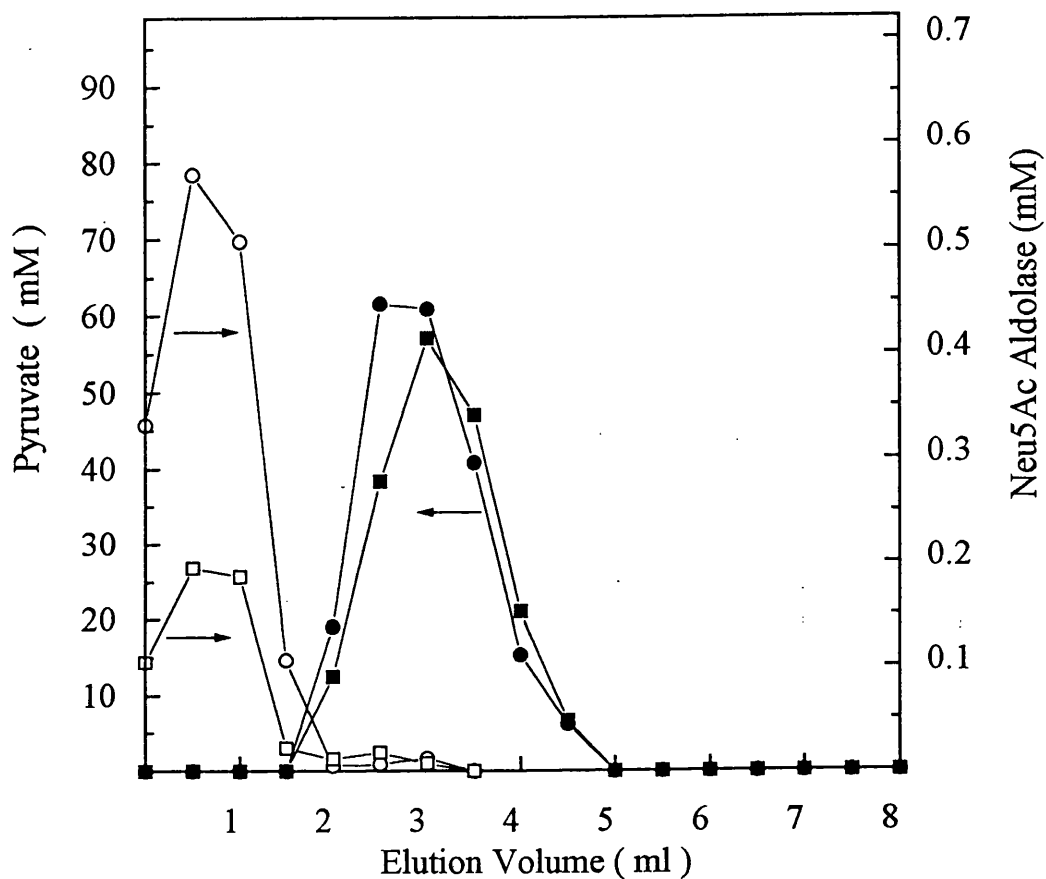
Figure A2.1   Neu5Ac aldolase gel filtration. The profiles of Pyruvate (● pH 11, ■ pH 3.5) and Neu5Ac Aldolase (O, pH 11, □ pH 3.5) eluting from a Pharmacia Hi-Trap ™ column (5 mL bed volume) were followed respectively by HPLC and Bio-Rad protein assay. Incubation of Neu5Ac Aldolase with Pyruvate was interrupted by this gel filtration. 1.5 mL were collected for subsequent spectrophotometry and protein assays (Section 2.3).
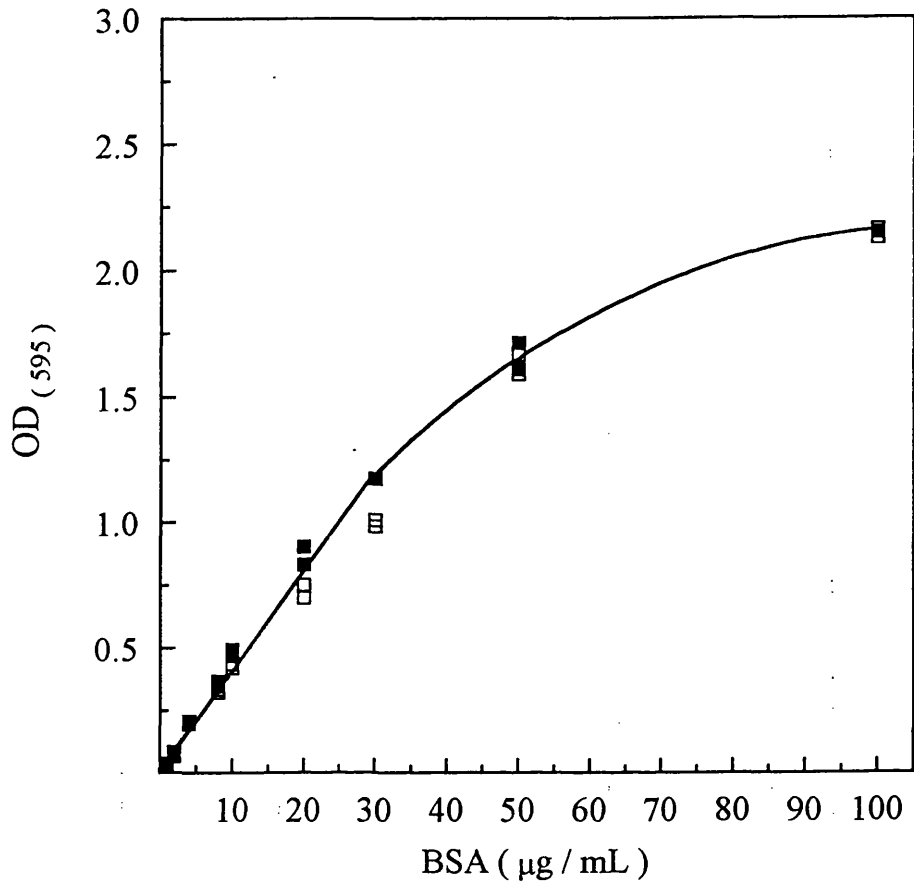
Figure A2.2   Bio-Rad protein assay. 800 μL of BSA sample in 100 mM Tris-HCl buffer (pH 7.9) were incubated with 200μL of BioRad dye for 10 min (■) and 60 min (□). Optical density (OD) at 595 nm was recorded against Tris-HCl buffer. A linear relation up to 35 μg / mL BSA was found.
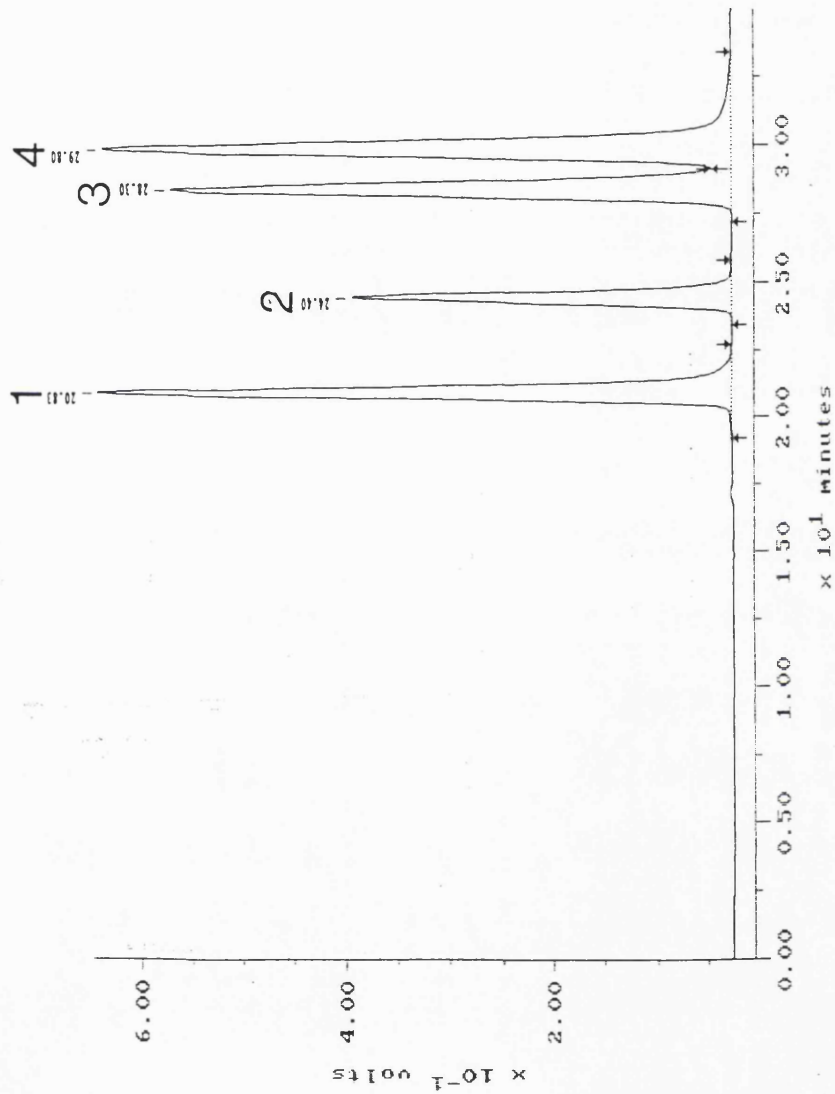
Figure A2.3   Pyr, Neu5Ac, ManNAc and GlcNAc chromatograph. Assay of 3.63 mM Pyr (1), 2.12 mM Neu5Ac (2), 3.34 mM ManNAc (3) and 3.56 mM GlcNAc (4). Conditions: flowrate 0.45 mL / min, isocratic 6 mM H_2SO_4 running phase, 65 $^\circ$C, separation by 2 Aminex HPX-87H columns (Bio-Rad). Elution times (in minutes) are reported on the chromatograph.

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APPENDIX 3

PLUG FLOW AND *IN SITU* PRODUCT REMOVAL REACTORS

A3.1 ACTIVITY DETERMINATION OF CRUDE Neu5Ac ALDOLASE IMMOBILISED ON EUPERGIT-C™

150 mL of Eupergit-C™ with immobilised cell lysate of *E. coli* NAN-24 F (Dawson *et al.*, 1994) were kindly provided by Glaxo Group Research. The cell lysate was immobilised according to the procedure described in Mahmoudian and co-workers (1993). It was then necessary to measure the enzymatic activity of the beads and compare it to free enzyme.

Assay development

Free enzyme:	Immobilised enzyme:
0.5 g / L Neu5Ac aldolase (Toyobo)	33.33 g / L Eupergit C
40 mM ManNAc	40 mM ManNAc
80 mM Pyr	80 mM Pyr
in 50 mM Phosphate buffer (Kragl, 1992)	in 50 mM Phosphate buffer (Kragl, 1992)
10 mL volume	15 mL volume

The biotransformations have been followed by HPLC (2.2.3.2).

The initial reaction rate was measured from linear regression (Figure A3.1) on duplicates average of the reaction profile:

Free enzyme activity = 0.7825 mM /min = 1.565 µmol / min / mg

Immobilised enzyme activity = 0.0266 mM / min = 0.798 µmol / min / g

Therefore for calculation purposes: 1g immobilised enzyme = 0.51 mg free enzyme.
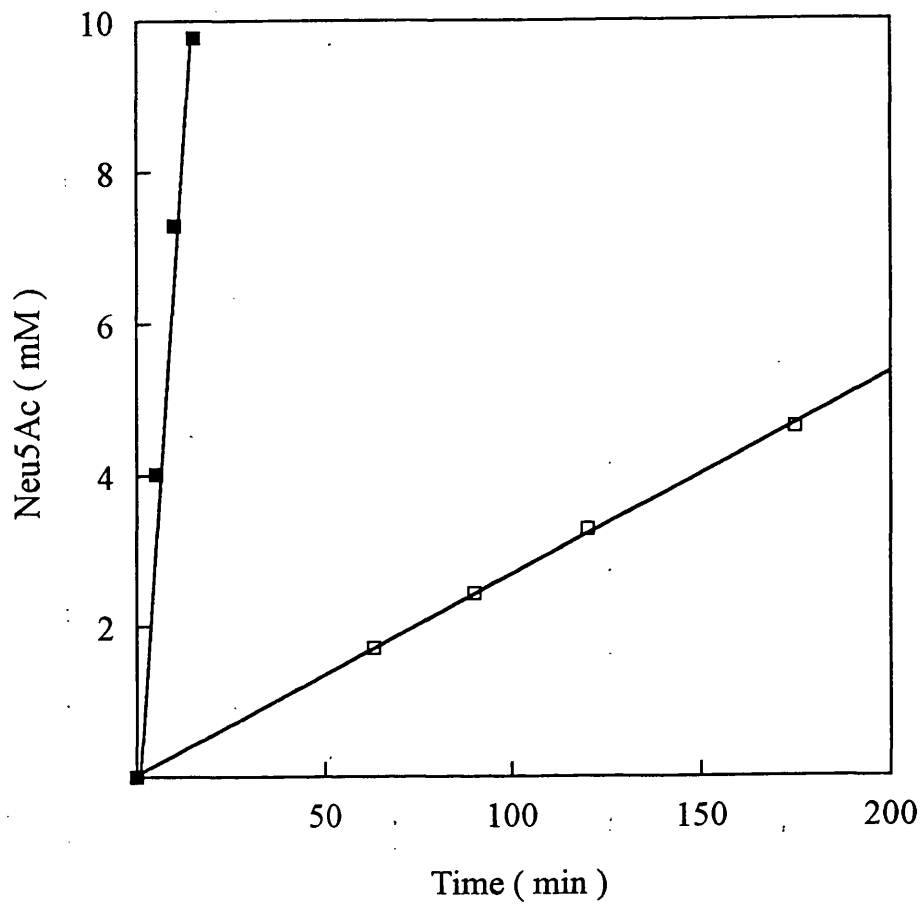
Figure A3.1   Initial reaction rate with free (■) and immobilised (□) Neu5Ac aldolase.

A3.2 PLUG FLOW REACTOR DESIGN

Conditions

- Substrate reservoir with 500 mM ManNAc, 200 mM Pyr, pH 7.5.

Assumptions

- Ideal plug flow
- Michaelis-Menten kinetics

  Since the enzyme $Km = 0.136$ mM for pyruvate (Kragl, 1992),

  $S_O \gg Km$,

  zero-order reaction for Pyr,

  single ManNAc Michaelis Menten kinetics under these conditions
- Conversion on ManNAc: 30 % (150 mM Neu5Ac)
- 1g Eupergit immobilised enzyme = 0.51 mg free enzyme
- 20 mL column volume (19.11 g Eupergit)

The equation describing an ideal plug flow reactor (Wang *et al.*, 1979):

$$X \, S_O - Km \ln (1 - X) = K \, E \, / \, q$$

The plug flow equation could be resolved for q:

Based on Kragl (1992):

$K = $ 13.8 μmoles / min / mg

$Km = $ 0.402 M

$$0.3 \times 0.5 - 0.402 \ln (1-0.3) = (13.8 \times 10^{-6} \times 9.746) / q$$

$q = $ 13.8 $\times$ 10^{-6} $\times$ 9.746 / 0.29

$q = $ 0.46 mL /min

Based on Kim *et al.* (1988):

$K = $ 25 μmoles / min / mg

$Km = $ 0.7 M

$0.3 \times 0.5 - 0.7 \ln(1-0.3) = (25 \times 10^{-6} \times 9.746) / q$

$q = 25 \times 10^{-6} \times 9.746 / 0.64$

$q = 0.38$ mL /min

where:

X	-	Fractional conversion
K	mol / min / mg	Maximal activity
S_O	mol / L	Initial substrate molarity
E	mg	Enzyme quantity
Km	mol / L	Michaelis-Menten Constant
q	mL / min	Flowrate

It was possible to calculate q from batch records (Figure 5.12):

With 10.42 g Eupergit beads in 25 mL volume, Neu5Ac formation rate was measured:

Neu5Ac formation rate   = 1.957 mM / min   = 48.925 μmol / min

This rate could be maintained in a CSTR. For 30 % conversion (this case) the enzyme required in a CSTR is equal to that for a PFR (Wang *et al.*, 1979). Accounting for enzyme differences and feeding 500 μmol / mL ManNAc:

Neu5Ac formation rate   = 48.925 x 19.11 / 10.42   = 89.70 μmol / min

q   = 89.70 / 500   = 0.18 mL /min.

Therefore for calculation purposes: average q   = 0.34 mL /min.

A3.3 BICARBONATE ION EXCHANGER COLUMN DESIGN

Assumptions

- 15 mL (10 g) Dowex 1x2 400 in bicarbonate form
- Inlet stream composition: 350 mM ManNAc, 50 mM Pyr, 150 mM Neu5Ac
- Inlet flowrate: 0.34 mL / min

- Breakthrough capacity for bicarbonate form (5.3.1.2.1):   = 340 μmol / g

Ion mass balance (base: 1 min) = 0.34 x (50 + 150)   = 68 μmol

Saturation time: = 10 x 340 / 68   = 50 min

68 μmol / min bicarbonate ions would be displaced in the outlet stream (outlet concentration: 200 mM bicarbonate). For every saturated column taken off-line, 2550 μmol ManNAc and Pyr should be added to the reservoir.

A3.4 TITRATION UNIT DESIGN

In order to eliminate excess bicarbonate ions, titration to pH 7.5 was required.

Titration with pure pyruvic acid is used in order not to introduce other anions in the system. A titration experiment was performed. 50 mL of 200 mM bicarbonate was titrated with 1 M glacial pyruvic acid. From Figure A3.2 it was possible to calculate the ratio between Pyr and bicarbonate required to maintain pH 7.5:

Titration ratio: = 0.71 mmoles / 10 mmoles

Therefore 7.1 % of Pyr consumed should be provided back in pure acid form.

For the scale of the reactor concerned, this amount was neglected since the titrated solution would be recycled to the substrate reservoir.

A3.5 ManNAc ENRICHMENT

2 Kg of GlcNAc were epimerised at pH 10.9 for 88 hours in 8.05 L solution. The reaction was monitored by HPLC (5.2.2.10) and achieved equilibrium at 715 mM GlcNAc and 182 mM ManNAc. The substrate solution was enriched in the content of ManNAc by selective crystallisation of GlcNAc, as described by Mahmoudian *et al.* (in press). All operations were performed at Glaxo Group Research pilot plant.
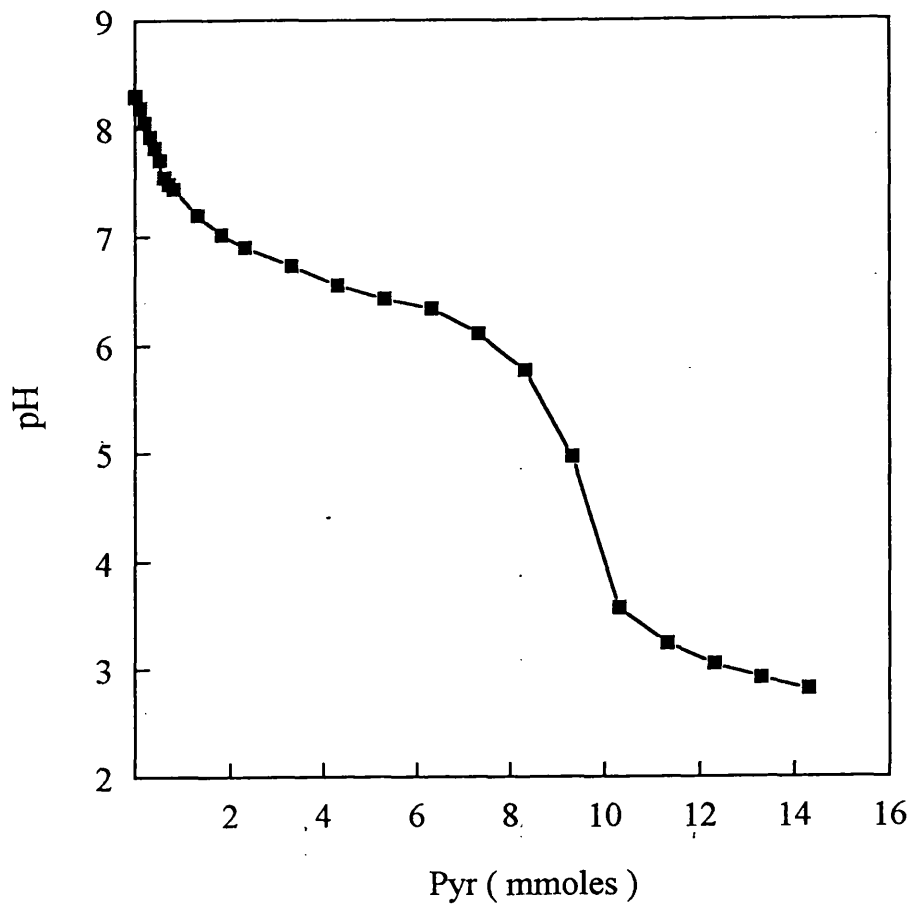
Figure A3.2   Sodium bicarbonate titration with pyruvic acid.

<u>Enrichment protocol</u>

1. Epimerised mixture (8.05 L)

   pH adjusted to pH 4.3 with 50 mL phosphoric acid. 40.5 L Propan-2-ol was added and the solution cooled to 8 $^{\circ}$C overnight. Solid removed by Whatman 54 paper filtration, 5L Propan-2-ol wash and vacuum drying. Yield: 1.041 Kg solid GlcNAc.

2. Propan-2-ol solution (47 L)

   Rotary evaporation to 8 L (50 $^{\circ}$C bath temperature), solidification at 4 $^{\circ}$C overnight. Solid removal by filtration and drying as above. Yield: 377 g solid GlcNAc

3. Propan-2-ol solution (9 L)

   Evaporation to dryness as in previous step. Solid extraction by methanol (5 L) at 50 $^{\circ}$C. Solid removal by filtration and re-extraction with 3 L methanol. Yield: 111 g solid GlcNAc.

4. Bulk methanol extract (9.1 L)

   Extract evaporated to dryness. Solid dissolved in water.

5. Aqueous substrate solution (1.5 L)

   Concentrations: 930 mM ManNAc, 340 mM GlcNAc (by HPLC, 5.2.2.10).

The substrate solution was diluted two-fold and used in plug-flow reactor experiments (5.3.2.3, 5.3.2.4).

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