Optimisation of active recombinant protein production, exploring the impact of small heat-shock proteins of *Escherichia coli*, IbpA and IbpB, on in vivo reactivation of *inclusion bodies*

Dissertation

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Abbreviation

APS	ammonium persulfate
ATP	3', 5'-adenosine triphosphate
BSA	bovine serum albumin
CHO	chinese hamster ovary
DCW	dry cell weight
DDT	1, 4-dithiothreitol
DOT	dissolved oxygen tension
EDTA	ethylenediaminetetraacetic acid
HDF	defined medium
HSP	heat shock protein
IBs	inclusion bodies
IPTG	isopropyl-ß-D-thiogalactopyranoside
Ibp	inclusion bodies protein
kbp	kilo base pairs
kDa	kilo Dalton
LB	Luria-Bertani
mRNA	matrix RNA
MDa	mega Dalton
OD_{600}	optical density at 600 nm
PAGE	polyacrylamide gel electrophoresis
p-NPG	p-nitrophenyl-α-D-glucopyranoside
PVDF	polyvinylidene fluoride
rpm	round per minutes
RNA	ribonucleic acid
SDS	sodium dodecyl sulphate
sHSP	small heat shock protein
TF	trigger factor
Tris	Tris-(hydroxymethyl)-Amino Methane
v/v	volume per volume
w/v	weight per volume
U	unit

Nomenclature

μ_{max}	maximum specific growth rate, h^{-1}
μ_{set}	set point of the specific growth rate, h^{-1}
X	dry cell mass, g L^{-1}
X_{ind}	dry cell mass at induction time, g L^{-1}
q_P	specific product formation rate, g g^{-1} h^{-1}
t_{ind}	time after induction, h
$Y_{X/S}$	yield coefficient for biomass per substrate, g g^{-1}

CONTENTS

1. INTRODUCTION

1.1 Production of recombinant proteins in *Escherichia coli*

Genetic engineering techniques were discovered first by Cohen et al. (1973), who could „cut and paste" a segment of DNA and reproduce (clone) the new DNA in bacteria. Up to now, those techniques have been developed from the transfer of genetic information between prokaryotic organisms to a technology of producing proteins in foreign hosts. The technology of recombinant protein production enables a large amounts of high value proteins (e.g. for therapeutic use), which are often limited by their low natural availability, to be produced. About 30 recombinant products have been approved for therapeutic use and are industrially produced. Examples for commercial therapeutic proteins are compiled in Table 1.

Table 1. Example of therapeutic agents, produced by recombinant DNA technology

Protein	Company	Organism
Human Insulin	Berlin-Chemie	*E. coli*
	Lilly	
Somatropin	Pharmacie & Upjohn	*E. coli*
	Lilly	
	Novo Nordisk	
tPA	Boehringer Ingelheim	CHO cells
	Boehringer Mannheim	*E. coli*
Hepatitis B antigen	Smith Kline Beecham	*Saccharomyces cerevisiae*
Epoetin alpha	Janssen-Cilag/Amgen	CHO cells
Epoetin beta	Boehringer Mannheim	

From Zündorf and Dingermann (2000)

Different host systems are available for production of recombinant proteins: eukaryotic cells such as cells from yeasts, filamentous fungi, insects, plants, mammals, and prokaryotes including different bacteria such as *Escherichia*, *Bacillus* and *Staphylococcus*. Each cell type has its advantages and drawbacks. The amount and quality of the produced recombinant proteins are influenced by factors such as gene copy number, transcription and translation efficiency, mRNA stability, stability and solubility of the proteins as well as post-translation modification. Eukaryotic cells can perform certain post-translational modifications of proteins like glycosylations, amidations, and methylations, which are not carried out by prokaryotic cells. Yeast cells can export proteins into the medium and can glycosate proteins, but normally produce a pattern of glycosylation different from animal cells. For large proteins with complex glycolysation pattern and other post-translational modifications, animal cells need to be used. They produce proteins of high quality but have several limitations such as sensitivity to infections, need for expensive complex medium and low yields. Moreover, little is known about the interrelation between physiology and cultivation parameters.

Usually, production of recombinant proteins in microbial cells represents the most convenient and cost-effective method. *Escherichia coli* has become the most extensively used bacterial host, since it is a genetically and physiologically well-characterised organism that grows fast and requires simple medium. Common problems during overproduction of recombinant proteins in *E. coli* are formation of *inclusion bodies* (IBs) or degradation. Foreign proteins expressed in *E. coli* sometimes contain regions that are recognised by specific host proteases, leading to cleavage and sometimes to subsequent degradation (Baneyx and Georgiou, 1990; Enfors, 1992). Recombinant proteins might aggregate to form IBs. IBs are very dense particle, exhibit amorphous structures (Bowden et al., 1991) and contain almost exclusively overexpressed proteins (e.g. Rinas and Bailey, 1992; Valax and Georgiou, 1993; Carrió et al., 1998). Proteins aggregated in IBs are normally in an inactive, unfolded or incorrectly folded form, requiring refolding in the functional active form (Buchner and Rudolph, 1991). Nevertheless proteins with secondary structure and also native-like conformations have been found in IBs (Oberg et al., 1994).

Formation of IBs can be beneficial, if the protein product is not stable to host proteases or in its active form is toxic to the cells. In this case, IBs are desired and strategies for increasing IB formation have been developed. Furthermore, IB formation can simplify some steps of the downstream processing. IBs are enriched in the protein of interest; the purity after IB

purifications can be up to 90% (Lilie et al., 1998). IBs can be easily removed from host cell proteins and debris by low speed centrifugation (Taylor et al., 1986). To obtain product in its native structure, however, a renaturation strategy is required (Rudolph, 1996; Rudolph and Lilie, 1996; Clark, 2001). General strategies for recovering native proteins from IBs include three steps: (1) isolation of IBs including washing several times to remove undesired coprecipitation proteins, (2) solubilisation of IBs in a strong denaturant such as urea or guanidinium chloride to break intermolecular interactions, (3) renaturation of solubilised proteins by dialysis or dilution to remove the denaturant. For renaturation of proteins containing disulfide bonds, the supplementation of redox systems to the renaturation buffer is needed (Creighton, 1986). Redox systems provide the appropriate redox potential to allow formation and reshuffling of disulfide bonds.

This strategy is possible only for small proteins. Large multidomain proteins, in contrast, normally fail to fold properly, often leading to kinetically trapped intermediates that aggregate, even in diluted solution and at low temperature (Jaenicke, 1998). Moreover, the folding process *in vitro* can take very long, up to several days. Due to competition between aggregation and folding *in vitro* (Kiefhaber et al., 1991), the protein concentrations must be kept low. Thus, to get a given amount of native protein, large volumes of buffers are needed. The yield of protein refolding *in vitro* can be improved by coupling denaturated proteins to a matrix (Stempfer et al., 1996), adding low molecular weights additives (Rudolph and Lilie, 1996), genetically engineering hydrophobic patches (Wetzel, 1994), shifting the temperature rapidly (Betts and King, 1998), or adding molecular chaperones (as reviewed by Thomas et al., 1997). But the results are still modest, especially for large multidomain proteins. Moreover, refolding *in vitro* is an expensive and time consuming process that sometimes is not easy to scale up. To simplify the downstream processing, maximisation of the yield of soluble active product already during production *in vivo* is an attractive alternative.

1.2 The problem of protein aggregation *in vivo*

Protein aggregation *in vivo* results in the formation of IBs or amyloid fibrils. It causes major economic and technical problems in biotechnology and pharmaceutical industries. Its effect can be lethal in patients, who suffer from a disease caused by protein aggregation such as amyloidoses or prion diseases (Sipe, 1992; Carrell and Lomas, 1997). Figure 1 presents a model of protein aggregation, suggested by Hartl and Hayer-Hartl (2002), according to which

protein aggregation can occur by intermolecular interaction between unfolded proteins or partially folded intermediates.

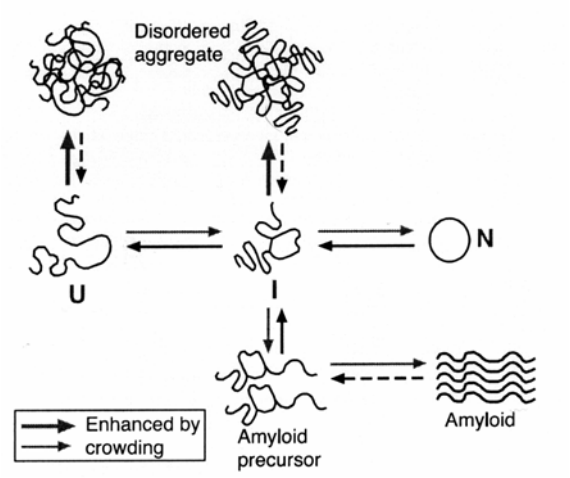

Fig. 1: Model of protein aggregation *in vivo*. Aggregation of non-native protein chains occurs as a side-reaction of productive folding in the crowded environment of the cell (Hartl and Hayer-Hartl, 2002). Crowding is predicted to enhance the formation of aggregates, but this effect has not yet been demonstrated experimentally. Disordered aggregates mean IBs

Partially folded intermediates are key precursors in protein aggregation (Wetzel, 1994; Kim and Yu, 1996; Speed et al., 1996). This corresponds to the observation of secondary structure of ß-lactamase in IBs (Przybycien et al., 1994) or native-like secondary structure of interleukin-1ß in IBs (Oberg et al., 1994). Fink (1998 and 1999) suggested a model, according to which aggregation could principally occur only by intermolecular interactions between partially folded intermediates.

Formation of IBs has been found upon expression of recombinant proteins (Marston, 1986; Schein, 1989; Rudolph, 1996). IBs form not only from heterologous proteins, but also from homologous proteins (Mitraki and King, 1989), indicating that the formation is not specific for “foreign proteins“. The formation of IBs is not host-specific, since they have been found in other bacteria like *Bacillus* (Wang et al., 1989), in yeast such as *Saccharomyces* (Binder et al., 1991) and mammalian cells (e.g. Broido et al., 1991). IBs form also from proteins secreted to the periplasm like ß-lactamase (Chalmers et al., 1990).

Reasons leading to formation of IBs include: heterologous nature of the protein (especially for proteins that require post-translational modifications), high protein synthesis rate, protein with

high hydrophobicity which aggregates intermolecularly as a result of non-covalent association, and lack of available chaperones (Mukhopadhyay, 1997).

In vivo aggregation of ß-lactamase is only observed when the expression rate exceeds 2.5% of the total protein synthesis rate (Bowden and Georgiou, 1990). In *in vitro* experiments, aggregation increases with increasing protein concentration (Lilie et al., 1998).

Point mutations, which change the hydrophobicity of a protein, can alter stability and solubility of the protein (Luck et al., 1992; Schein, 1993) and change the amount of protein deposited in IBs considerably (Wetzel et al., 1991; Mitraki and King, 1992).

Lower amount of available molecular chaperones due to either the rapid rate of recombinant protein synthesis or the formation of long-lived folding intermediates which bind the molecular chaperone has been proposed to cause IBs formation (Rinas and Baley, 1993; Thomas and Baneyx, 1996b; Kedzierska et al., 2001).

1.3 The quality control network

Elevated temperatures denature proteins and induce the heat-shock response, which features chaperones and proteases that frame the quality control network and determine the fate of non-native protein conformations, i.e. proper folding, degradation or aggregation (Wickner et al., 1999). Especially during recombinant overproduction, proteins may escape the quality control network and aggregate to form IBs.

Molecular chaperones and proteases are key components of the quality control network; both recognise hydrophobic regions exposed by non-native proteins. While chaperones prevent aggregation and promote correct folding, energy-dependent proteases eliminate aberrant proteins, and thereby protect cells from damage. Some chaperones can function also as proteases (Gottesman et al., 1997; Spiess et al., 1999).

Protein folding *in vivo* is enabled by molecular chaperones, which do not contribute conformational information to protein folding, but protect proteins from aggregation by binding to unfolded or partially folded intermediates. Therefore, under conditions of macromolecular crowding in the cell and at ambient temperature of 37°C, under which hydrophobic effects are expected to be strong, proteins can still properly fold, and the time frame for successful folding is short.

Molecular chaperones are essential for the correct folding of proteins in the cell under physiological and stress condition. According to molecular weight, chaperones are divided into several families: Hsp100, Hsp90, Hsp70, Hsp60, and small heat-shock proteins (sHsp) of 12 to 43 kDa. Proteins from the same family normally have sequence homologies and are functionally and structurally related, whereas there are hardly any homologies between chaperones from different families (Walter and Buchner, 2002).

There are two extensively studied molecular chaperones in *E. coli*, the Hsp70 system, including DnaK and its cofactors DnaJ and GrpE, and the Hsp60 system of GroEL and the cofactor GroES. Their structural and mechanistic features were extensively summarised by Bukau and Horwich (1998). Both Hsp70 and Hsp60 complexes can prevent protein aggregation at high temperature (Höll-Neugebauer et al., 1991; Schröder et al., 1993) and promote folding (Mendoza et al., 1991; Schröder et al., 1993; Buchberger et al., 1996; Thomas and Baneyx, 1996a). They can hydrolyse ATP for their activities (Liberek et al., 1991; Martin et al., 1991). These two chaperone systems can function independently (Goloubinoff et al., 1989; Ziemienowicz et al., 1993; Szabo et al., 1994) or together (Langer et al., 1992; Kedzierska et al., 2001).

The Hsp70 and Hsp60 systems, together with the ribosome bound chaperone trigger factor (TF), contribute to *de novo* folding. Nascent proteins generally interact first with TF, which can support proper folding of 65-80% of total proteins (Hartl and Hayer-Hartl, 2002). 5-18% of newly synthesized proteins interact with Hsp70 (Deuerling et al., 1999; Teter et al., 1999) and 5-15% with Hsp60 (Houry et al., 1999).

Proteins that have escaped from the protein quality control network aggregate. Aggregated proteins, however, are not a dead end of the protein quality network. They can be disaggregated and refolded into the biological active form (Carrió et al., 1999; Carrió and Villaverde, 2000). The heat-shock chaperones DnaK (Hsp70) and ClpB (Hsp100) have been identified as the major participants in resolubilisation and refolding of protein aggregates (Laskowska et al., 1996a; Goloubinoff et al., 1999; Kędzierska et al, 1999; Mogk et al., 1999; Zolkiewski, 1999). While small aggregates can be solubilised *in vitro* by DnaK alone (Diamant et al., 2000), the larger aggregates need the participation of ClpB (Hsp100) for resolubilisation (Goloubinoff et al., 1999; Mogk et al., 1999; Diamant et al., 2000). Two potential mechanisms of cooperative action of ClpB and DnaK in resolubilisation were proposed recently (Fig. 2). According to the first mechanism, the aggregates interact first with

ClpB and afterwards with DnaK (Goloubinoff et al., 1999; Ben-Zvi and Goloubinoff, 2001). The mechanism has been suggested from two observations. First, when aggregated proteins were incubated with ClpB and DnaK/DnaJ/GrpE, the turbidity increased 30-45 min before activity appeared, indicating that large aggregates were decomposed to smaller ones before the native structure of the aggregated enzyme was reconstituted. Second, ClpB interacted directly with aggregates and altered the structure of aggregates, whereby the aggregates expose more hydrophobic surfaces (Goloubinoff et al., 1999). The weak complex of ClpB with DnaK with nucleotide-dependent interaction has been detected by affinity chromatography (Schlee et al., 2004). It was proposed that this complex facilitates the transfer of intermediates between ClpB and DnaK during refolding of substrates from aggregates. Mogk and Bukau (2004) suggested another mechanism, according to which the ClpB and DnaK act on the aggregates in parallel. This mechanism has been proposed based on the evidence that the DnaK mediated solubilisation is still stimulated by ClpB (Diamant et al., 2000).

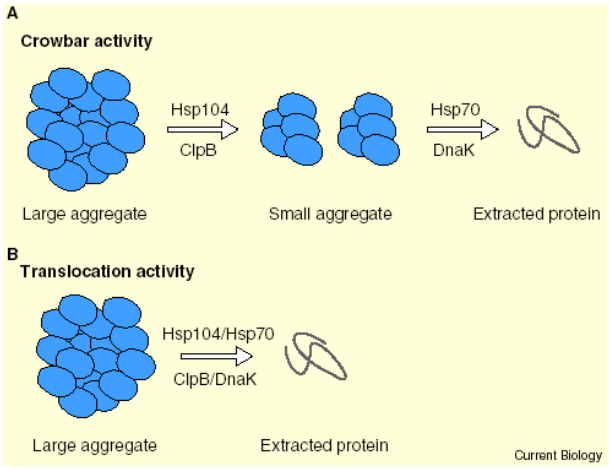

Fig. 2: Potential mechanism of protein disaggregation by ClpB. (A) Sequential mechanism: ClpB may break large aggregates into smaller species via a crowbar activity. Small sized aggregates served as substrate for DnaK binding. (B) Parallel mechanism: ClpB may extract unfolded proteins from aggregates via a translocation activity. DnaK may prevent reaggregation of the extracted protein (Mogk and Bukau, 2004).

A latest model postulate the existence of an additional step in the chaperone dependent disaggregation process, namely the binding of DnaK and DnaJ to the aggregates prior to ClpB action, which is also the rate limiting step in the initiation of the disaggregation reaction (Zietkiewicz et al., 2004).

1.4 Regulation of the *Escherichia coli* heat-shock response

The inducing signal of the heat-shock response is the accumulation of misfolded proteins (Goff and Goldberg, 1985). There are at least 119 heat-shock proteins (Richmond et al., 1999); the synthesis of most of them relies on transcription factor sigma 32 (σ^{32}), a product of the *rpoH* gene (Neidhardt and VanBogelen, 1981) and a subunit of the RNA polymerase (RNAP). Only the interaction of RNAP core protein with σ^{32}, one specificity factor of RNAP, allows the heat-shock promoters to be transcribed (Cowing et al., 1985). The cellular concentration of σ^{32} is limiting for heat-shock genes transcription (Craig and Gross, 1991). The induction of the heat-shock response is achieved by elevated concentrations of σ^{32}, primary by increased stability of σ^{32} (Straus et al., 1987) and by enhanced translation of *rpoH* mRNA (Grossman et al., 1987; Straus et al., 1987). Whereas elevated temperatures induce the heat-shock response by both pathways (Straus et al., 1987), misfolded proteins induce the heat-shock response only by stabilisation of σ^{32} (Kanemori et al., 1994).
Stress-dependent modulation of the heat-shock response is mediated by the antagonistic action between the positive transcriptional activator σ^{32} and the negative modulator DnaK (Bukau, 1993; Tomoyasu et al., 1998). DnaK directly associates with σ^{32} and prevents its interaction with the RNAP core protein (activity control) (Tatsuta et al., 1998), deliver σ^{32} to the proteolytic machinery (Tilly et al., 1989; Straus et al., 1990) and also might repress the translation of *rpoH* mRNA (Grossman et al., 1987; Straus et al., 1990). The central key of homeostatic control is that misfolded proteins induced by stress sequester DnaK away from σ^{32}, leave it stable and active (Bukau, 1993). Consequently, the transcription of σ^{32}-dependent heat-shock genes including *dnaK* is enhanced, until a sufficient amount of DnaK is synthesised to bind both misfolded proteins and σ^{32}. Since most DnaK molecules are bound at every instant (Tomoyasu et al., 1998), already small changes of the protein-folding status promptly induce the heat-shock response.

1.5 The small heat-shock proteins of *Escherichia coli*, IbpA and IbpB

The small heat-shock proteins IbpA and IbpB of *E. coli* have been found associated with recombinant proteins in IBs (Allen et al., 1992) and with heat-aggregated proteins (Laskowska et al., 1996a). IbpA and IbpB are two sequence-related small heat-shock proteins (sHsps) of a molecular mass of 16 kDa (Allen et al., 1992). Similar to sHsp from other

organisms, IbpA and IbpB form multimeres, whose state may vary depending on temperatures (Shearstone and Baneyx, 1999; Kitagawa et al., 2002). *In vitro*, IbpB exhibits pronounced size heterogeneity, forming amorphous, soluble loose aggregates up to micrometer size (Shearstone and Baneyx, 1999). Upon prolonged incubation at 50°C, the multimeres of 2-3 MDa reversibly dissociate to monomers in the case of IbpA and to oligomers of 650-700 kDa in the case of IbpB (Kitagawa et al., 2002). During this dissociation, more hydrophobic surfaces are exposed, providing extended binding sides for non-native proteins (Shearstone and Baneyx, 1999). These multimeres are re-constituted after lowering the temperature (Kitagawa et al., 2002), and secondary structure changes of IbpB were perfectly reversible in the temperature range between 32°C and 50°C, but not between 10 and 32°C (Shearstone and Baneyx, 1999).

Small heat-shock proteins IbpA and IbpB *in vitro* bind unfolded proteins and prevent their aggregation, and suppress inactivation of enzymes in an ATP-independent manner (Shearstone and Baneyx, 1999; Kitagawa et al., 2002). For refolding, however, transfer to the DnaK-DnaJ-GrpE system is required (Veinger et al., 1998). Also *in vivo*, a delivery to DnaK is required for reactivation (Kędzierska et al., 2001). IbpA/IbpB hence are supposed to serve as reservoir of folding-competent intermediates for further folding by the Hsp70 system, as small heat-shock proteins from other organism do (Haslbeck et al., 1999; Ehrnsperger et al., 1997; Lee et al., 1997).

In vivo, IbpA/IbpB seem to protect the cells during harsh heat treatment. Cultures of *ibpAB* deletion mutants recovering at 46°C from incubation at 50°C grew with lower rates than wild-type cultures, and lower final cell densities were reached with a double *ibpAB dnaK756* mutant compared to the single *dnaK756* mutant (Thomas and Baneyx, 1998). After prolonged incubation at 50°C, a higher percentage of cellular proteins aggregate in an *ibpAB* mutant than in isogenic wildtype cells (Kuczyńska-Wiśnik et al., 2002). A double mutant *ΔclpB ΔibpAB* exhibits strongly increased protein aggregation at 42°C compared to the single mutants (Mogk et al., 2003b).

IbpA/IbpB are induced under control of σ^{32} (Cheng et al., 1993), but some evidences indicate the existence of additional levels of regulation (Jürgen et al., 2000; Hoffmann and Rinas, 2000). IbpA/IbpB are induced by some recombinant proteins (Allen et al., 1992; Jürgen et al., 2000) but not by others (Valax and Georgiou, 1993; Hoffmann and Rinas, 2000).

Although IbpA and IbpB share more than 50% of homology in their sequence (Allen et al., 1992) and are both involve in resistances to heat and superoxidave stresses (Kitagawa et al., 2002), they show distinct features. Whereas overexpression of *ibpB* alone results in accumulation of IbpB mainly in the soluble cellular fraction, overexpression of *ibpA* alone or together with *ibpB* lead to their migration into insoluble cellular fraction (Shearstone and Baneyx, 1999; Kuczyńska-Wiśniket al., 2002), and IbpA but not IbpB stabilizes pre-β-lactamase in the insoluble cellular fraction. Thus IbpA was supposed to transfer bound proteins to the insoluble cellular fraction (Kuczyńska-Wiśniket al., 2002). Overexpression of either *ibpA* or *ibpB* alone leads to retardation of removal of heat-aggregates compared to the wildtype strain, although the removal was faster with *ibpB* overexpression than *ibpA* or *ibpAB* together (Kuczyńska-Wiśnik et al., 2002).

The precise role of IbpA/IbpB in IBs or heat aggregates remains unclear. IbpA/IbpB have been suggested to participate in removal of the heat aggregates (Laskowska et al., 1996b; Mogk et al., 2003b), mediate a transfer of proteins into the insoluble cellular fraction (Carrió and Villaverde, 2002; Kuczyńska-Wiśnik et al., 2002), and to sequester bound proteins from attack of proteases or to prevent aggregation of these proteins (Kitagawa et al., 2002; Laskowska et al., 2004; Han et al., 2004).

The IbpA/IbpB were not essential for IB dissolution *in vivo* (Carrió and Villaverde, 2001). However, *in vitro* experiments indicated that dissociation and refolding of sHsp bound proteins relies on the disaggregation action of DnaK and ClpB (Mogk et al., 2003a). Also *in vivo*, IbpA/IbpB seem to cooperate with DnaK and ClpB (Mogk et al., 2003b). A function of IbpA/IbpB in promoting rapid resolubilisation of heat-aggregated proteins by DnaK or DnaK/ClpB was suggested (Mogk et al., 2003a, b).

1.6 Strategies for improving the yield of native proteins *in vivo*

Approaches employed to improve the production of recombinant protein in native form include optimisation of cultivation conditions and coexpression of chaperones.

Cultivations parameters are important factors to achieve the native conformations of recombinant proteins. For example, the enzyme α-glucosidase PI from *Saccharomyces cerevisiae* is a soluble, monomeric, cytoplasmic enzyme with molecular mass of 67,500 Dalton. It is not glycolysed and contains four unlinked cysteine residues. Overexpression of

α-glucosidase PI leads, as of most recombinant proteins, to formation of IBs under optimal growth condition of 37°C in complex medium, but was folded properly at the low temperature of 24°C, a low pH value of 5 and with partial induction (Kopetzki et al., 1989).

It has been proposed that the yield of native protein depends on the rates of folding, aggregation and protein synthesis (Kiefhaber et al., 1991; Rudolph, 1996). Hence, an increase of folding rate and a decrease of protein synthesis rate lead to increased amounts of correctly folded protein. Under growth conditions such as low temperature or low gene expression rate by using weaker promoter or partial induction, formation of IBs can be reduced or avoided (Kopetzki et al., 1989; Schein, 1989; Bowden and Georgiou, 1990; Chalmers et al., 1990; Shatzman, 1990; Strandberg and Enfors, 1991). Low temperature results on higher soluble product concentration and lower amount of IBs (Schein and Noteborn, 1988; Kopetzki et al., 1989). Altering cultivation parameters including pH value or medium composition can also result on higher concentration of soluble product (Kopetzki et al., 1989; Baneyx et al., 1991).

Shake flask cultivations are characterised by insufficient oxygen supply, unregulated pH and progressive accumulation of by-products. For production of larger amount of product, cultures are grown in bioreactors, in which pH value and dissolve oxygen tension can be controlled. Batch, continuous and fed-batch are modes of cultivation operation commonly used in bioreactors.

In the batch process, exponentially growing cells have an increasing demand for oxygen. As the growth rate is not limited, this demand will exceed the oxygen transfer capacity of most bioreactors. Moreover, during rapid aerobic growth, when energy sources (e.g. glucose) are present in excess, acetate is formed (Andersen and von Meyenburg, 1980; Seeger et al., 1995). Acetate inhibits growth and affects recombinant protein production, when its level increases above 1.0 to 1.5 g L^{-1} (Shimizu et al., 1988; Jensen and Carlsen, 1990; Luli and Strohl, 1990; San et al., 1994).

Fed-batch cultivation is a useful technique for production of recombinant protein. The fed-batch technique is based on limitation of growth rate of the cultures by controlling feeding rate of medium. Therefore, overflow metabolism, accumulation of toxic by-products and insufficient oxygen availability can be avoided. The fed-batch process is usually started with a batch culture prior to the fed-batch phase. Fed-batch culture with constant feeding rate of the growth limiting carbon source are characterised by progressive nutrient limitation and

gradually declining specific growth rate values. In fed-batch cultures with exponential increasing feeding rate, the feeding rate is calculated in proportion to the exponential increase in cell mass. This is a very efficient way to reach high cell densities, which enhances the product yields in bioprocesses. Futhermore, fed-batch cultivations with exponential increasing feeding rate can control the growth rate of the cell, thus can be used to optimise the solubility and specific concentration of product.

The concentration of ribosomes is higher at higher specific growth rate (Herbert 1961; Koch, 1970; Bremen and Deniss, 1987). Therefore, higher capacity of the protein-synthesising system at higher growth rate allows faster synthesis of cellular protein to maintain the growth, and also leads to faster synthesis of recombinant protein. However, plasmid contents are lower at higher growth rate (Seo and Bailey, 1986; Lim et al., 1998; Saraswat et al., 1999). Different pattern of correlation of the specific concentrations of recombinant proteins with specific growth rate have been reported. The specific concentrations of recombinant proteins were higher at higher specific growth rate (Shin et al., 1998; Sandén et al., 2002) or at lower specific growth rate (Kim et al., 1996; Riesenberg et al., 1990). Turner et al. (1994) found maximum specific protein concentration at a middle value of specific growth rate. Bech Jensen and Carlsen (1990) or Zabrieskie et al. (1987) found no correlation between specific product concentration and specific growth rate.

One of the important reasons for these conflicting results is the stress caused by additional synthesis of recombinant proteins. The stress induced by high synthesis rate of recombinant proteins may affect the cells that leads to sooner stop of net accumulation of product and thereby results in lower specific product concentration. The overexpression of recombinant proteins in *E. coli* was found to impair growth and cause ribosome destruction (Dong et al., 1995), induce the heat-shock response (Parsell and Sauer, 1989; Rinas, 1996) and alter the expression rates of components of the protein synthesis system (Rinas, 1996). Similar to numerous recombinant proteins, overexpression of recombinant α-glucosidase in *E. coli* also inhibits cell growth and chromosomal replication, affects the glucose uptake capacity, and causes cell segregation into viable-but-not-culturable and plasmid-free cells (Teich et al., 1998; Jürgen et al., 2000; Lin et al., 2001). It has been assumed that there is a competition between genes of housekeeping and recombinant proteins at the level of transcription as well as at the level of translation for the ribosomes (Vind et al., 1993; Lin, 2000). Consequently, a

higher level of recombinant protein synthesis causes more severe damage to the cell due to stronger competition.

There were very few reports about the correlation of specific growth rate and product quality. Hellmuth et al. (1994) found a maximum specific activity of aprotinin::ß-galactosidase fusion protein activity at a specific growth rate of the 0.13 h^{-1}. Higher or lower of specific growth rate in the range studied from 0.07 h^{-1} to 0.2 h^{-1} resulted in lower specific activity of the protein. The specific protein concentration was, in contrast, not affected by specific growth rate. The tendency of formation of recombinant protein isoforms, containing N^{ε}-modified lysine residues, was higher at higher specific growth rate (Ryan et al., 1996).

Aside from optimisation of cultivation parameters, coexpression of chaperones is another good possibility to improve the yield of native protein. From the first coexpression of *groELS,* which could increase the yield of active Rubisco up to tenfold (Goloubinoff et al., 1989), plenty of attemps have been undertaken so far (for review see Georgiou and Valax, 1996; Thomas et al., 1997). However, there is no one general folding chaperone for all heterologous proteins, rather individual chaperone systems work for individual target proteins (Thomas et al., 1997). Trial-and-error of screening of suitable chaperones for overexpression sometimes results only in faster degradation of the product (Thomas et al., 1997). In addition, coproduction of chaperones must normally be accompanied by optimisation of growth conditions (Lawson et al., 1993; Thomas and Baney, 1996). Coproduction of chaperones can lead to phenotype changes (Blum et al., 1992), which can be detrimental for cells viability or cause growth impairments.

Occasionally, multi-component chaperone systems are required to overexpress. The simultaneous overexpression of several chaperone genes is, however, often difficult to achieve (Blum et al., 1992; Nishihara et al., 1998; Nishihara et al., 2000).

Since chaperones are heat-shock proteins, their synthesis can be simultaneously induced by elevated temperatures (Goloubinoff et al., 1989). But as heat-shock response involves both molecular chaperones and proteases, the results are unpredictable.

1.7 Objectives

For the production of recombinant proteins, the major challenge lays in maximising the yield of biologically functional proteins. In this thesis, the production of active α-glucosidase from the yeast *Saccharomyces cerevisiae* was optimised in two aspects. First the cultivation parameters that optimise the productivity were determined and their influence on the cultivation process was analysed. Second the new approach of reactivating the *inclusion bodies* inside the living cell was studied. The processes implementing this approach were developed in bench-top and pilot plant scale for production of α-glucosidase. Inclusively, the roles of the small heat shock proteins of *E. coli*, IbpA and IbpB, in cell protection and in the metabolism of *inclusion bodies* metabolism were investigated. The small heat shock proteins IbpA and IbpB have been found tightly associated with *inclusion bodies* of numerous recombinant proteins and were implicated to promote the rapid accelerate *inclusion bodies* disaggregation.

The enzyme α-glucosidase PI from *Saccharomyces cerevisiae* was chosen as a model protein. Its native conformation can be easily monitored by an enzyme assay.

Cultivation parameters influencing the yield of active α- glucosidase were investigated. In this work, fed-batch cultivations with exponential increasing feeding rate to enable constant growth rate were used. Whereas the effect of temperature and growth rate was interdependent in shake flasks, they could be controlled independently in fed-batch cultivations. In that way the impact of either specific growth rate alone or temperature on production of active α-glucosidase were investigated. Furthermore, from obtained data, specific growth rate and temperature were optimised to maximise the active α-glucosidase production.

The disintegration of IBs and subsequent reactivation of disaggregated proteins opens a new possibility to rescue the proteins that have been aggregated. There were, however, no reports about its application for production of soluble proteins *in vivo*. Therefore, a new protocol of production by *in vivo* reactivation was investigated in shake flasks and in fed-batch cultivations. Parameters such as temperature during IBs production or temperature during disaggregation and reactivation, the influence of cultivations medium and impact of feeding rate F (μ_{set}) were investigated to optimise the yield of active product.

The role of the small heat-shock proteins from *E. coli*, IbpA and IbpB, in cell protection as well as their effect on product quality and inclusion bodies metabolism was studied. α-Glucosidase production in *E. coli* interferes with host vitality, e.g. impairs glucose uptake (Lin et al., 2001), affects the protein synthesis system (Teich et al., 1998; Lin et al., 2001) and induces the heat-shock response (Jürgen et al., 2000). Therefore, the response of cells with or without *ibpAB* overexpression or with deletion of *ibpAB* to the production of α-glucosidase was analysed. Parameters such as cell growth, product accumulation and heat-shock response at various temperatures were evaluated. In addition, the IBs were produced in these three strains with different levels of IbpA/IbpB and the impact of IbpA/IbpB levels on disaggregation, reactivation, resolubilisation and degradation at different temperatures were investigated.

2. MATERIALS AND METHODS

2.1 Strains and plasmids

The strains used in this work are listed in Table 2. They are derivatives of *Escherichia coli* MC4100 (Casadaban, 1976).

Table 2. *E. coli* strains used in this work

Strains	Genotype and/or description	Source or reference
MC4100	*araD139 Δ(argF-lac)U169 rpsL150 relA1 flbB5301 deoC1 ptsF25 rbsR*	Laboratory stock
JGT17	MC4100 *Δibp1::kan*	Thomas and Baneyx, 1998
BB1553	MC4100 *dnaK::kan*	Mogk et al., 1999
BB4561	MC4100 *clpB::kan*	Mogk et al., 1999

The plasmids used in this work are listed in Table 3. The plasmid pKK177-3/GLUCP1 (Kopetzki et al., 1989) contains the α-glucosidase gene of yeast *Saccharomyces cerevisiae* under the control of the *tac*-hybrid promoter. The plasmid pUBS520 (Brinkmann et al., 1989) was co-transformed to improve the production of α-glucosidase (Lin, 2000). The sequence of the plasmid pIbp (Thomas and Baneyx, 1998) between the two *Hin*dIII restriction sites, coding for *ibpA* and *ibpB* including their native promoter, was cloned into the *Hin*dIII site immediately downstreams of the α-glucosidase gene in pKK177-3/GLUCP1. This gave a new plasmid named pKK177-3/GLUCP1_ibpAB, in which the *ibpAB* operon is under the control of its native promotor and of the *tac*-promoter upstream of the α-glucosidase gene.

Table 3. Plasmids used in this work

Plasmids	Description	Source or reference
pKK177-3/GLUCP1	pBR322 derivative encoding the α-glucosidase gene	Kopetzki et al., 1989
pUBS520	pACYC177 derivative encoding the *dnaY* gene and *lacI* repressor gene	Brinkmann et al.,1989
pIbp	pTG10 derivative encoding the *ibpAB* gene	Thomas and Baneyx, 1998
pKK177-3/GLUCP1_ibpAB	pKK177-3/GLUCP1 derivative with inserted *ibpAB* gene	This work

2.2 Molecular biological methods

2.2.1 Plasmid isolation

Plasmid isolation

Plasmids were isolated from *E. coli* cells (5 mL overnight culture) using the QIAprep Spin Miniprep kit (Quiagen, Hilden) according to the protocol recommended by the manufacturer.

Agarose gel electrophoresis

The test for plasmid presence as well as the separation of DNA fragments took place on 0.8-1% (w/v) agarose gels in TAE buffer (40 mM Tris/acetate, pH 8.0, 1 mM ethylenediaminetetraacetic acid [EDTA]). The DNA samples were mixed with loading buffer (40% (w/v) glycerol, 0.1 % (w/v) sodium dodecyl sulphate [SDS], 0.1 M EDTA, 0.2% (w/w) bromphenolblue) and applied on the gel. The gels were run in a horizontal mini-electrophoresis chamber (Roth, Karlsruhe) under voltages between 50 V and 80 V. The 1 kb DNA ladder (New England Biolabs, Frankfurt am Main) served as standard. The gel was stained for 20 min with an ethidium bromide solution (1 µg mL^{-1}), and the DNA was visualised on a transilluminator.

2.2.2 Enzymatic modification of DNA

Extraction of DNA fragments from agarose gels

DNA fragments were extracted from agarose gels and purified by spin column using QIAquick Gel extraction kit (QIAGEN, Hilden). DNA fragments were eluted in water.

DNA cleavage

The plasmids were cleaved overnight in a total reaction volume of 100 µL with restriction nuclease *Hin*dIII, *Bam*HI, or *Ear*I at 37°C. The amount of restriction nuclease and the buffer for the individual nucleases were according to the protocol recommended by the manufacturer (Biolabs). The cleavage process was arrested by heating at 65°C for 20 min.

Dephosphorylation of plasmid vectors

Alkaline phosphatase, i.e. calf intestinal phosphatase CIP (Biolabs, Frankfurt am Main), was used to catalyse the removal of 5‘-phosphate groups from DNA to avoid self ligation of the vector. 0.5 unit of enzyme per microgram vector DNA was added to the DNA solution after cleavage and the mixture was incubated at 37°C for one hour. The enzyme was deactivated by incubation at 65°C.

Ligation

The plasmid pKK177-3/GLUCP1 was ligated with the *ibpAB* fragment using T4-Ligase (New England Biolabs, Frankfurt am Main). The amount of fragment was used in fourfold molar excess relative to the amount of plasmid.

The concentration of plasmids or DNA fragments was estimated by quantitative comparison of band intensity of plasmids or DNA fragments with the band intensity of the ladder on the agarose gels (1 kb ladder, Biolabs, Frankfurt am Main).

For subsequent transformation, the ligation solution was dialysed against distilled water, using a nitrocellulose membrane (0.025 µm pore diameter, Millipore, Eschborn) for about one hour to remove salt.

2.2.3 Transformation of *E. coli* cells

Preparation of electrocompetent cells

Luria-Bertani (LB) medium was inoculated with 1 % of overnight preculture. The culture was incubated at 37°C to an OD_{600} of about 0.5-0.8, chilled on ice for 10-20 min, centrifuged at 4000×*g*, 4°C for 10 min and washed 3 times with ice cold glycerol 10 % (v/v). The volume of glycerol was reduced each time from 100 % to 50 % to 10 % of the culture volume. Afterwards, the cells were resuspended in 0.5 % volume of 10 % (v/v) ice cold glycerol. Aliquots of 40 µL were transferred into sterile Eppendorf tubes, shock frozen in liquid nitrogen and stored cold at -80°C.

Transformation via electroporation

The electroporation was carried out in Gene Pulser II (Bio-Rad, München). About 1-10 ng plasmid or 10-100 ng DNA from ligation was applied to a 40 µL aliquot of electrocompetent cells, mixed and transferred in the previously chilled cuvette (0.2 cm gap). The electroporation was carried out with a 25 µF capacitor, a resistor of 200 Ω under a voltage of 2.5 kV. After pulsation, the cells were transferred into 1 mL LB medium and were incubated at 37°C and 650 rpm on a thermomixer (Eppendorf) for 30-60 min. 100 µL of incubated cells were directly plated on LB plates. The remaining volume was centrifuged at 3000 rpm for 2 min and about 750 µL supernatant was removed. The remaining cells were resuspended and plated on LB agar plates. Recombinant *E. coli* clones were selected using appropriate antibiotics.

2.3 Cultivation media and conditions

2.3.1 Cultivation media

Complex medium

For Luria-Bertani (LB) medium, following components were dissolved in distilled water:

- bacto trypton (pancretic digest of casein) 10.0 $g\ L^{-1}$
- yeast extract 5.0 $g\ L^{-1}$
- NaCl 5.0 $g\ L^{-1}$

Defined medium

The composition of the defined medium is given in Table 4. The initial concentration of glucose in batch medium was 5 g L^{-1} or 10 g L^{-1}. For preparation of 8 L batch medium, $(NH_4)_2HPO_4$, KH_2PO_4, citric acid, and trace elements were dissolved in 6.33 L distilled water. After adjustment of pH to 6.3 with 5 N NaOH, the medium was filled in the bioreactor. Ferric citrate was added directly to the bioreactor and dissolved during sterilisation. The medium was sterilised at 121°C for 30 min. Glucose and $MgSO_4$ solutions were sterilised each separately in an autoclave also at 121°C for 30 min and were transferred aseptically into the bioreactor afterwards. Before inoculation, the pH value was adjusted to 6.8 with aqueous NH_3 (25 % (w/v)). Glucose, $MgSO_4$ and trace element solutions for the feeding solution (Table 4) were each sterilised separately and mixed together afterwards. The trace element solution was prepared from stock solutions with the concentrations listed in Table 5.

Antibiotics were sterilised by filtration and supplemented aseptically to all media from the agar plates to shake flask and bioreactor after sterilisation. Antibiotics should not be added at high temperatures to avoid their inactivation. The final concentrations of the antibiotics were: ampicilline 100 µg mL^{-1}, chloramphenicol 50 µg mL^{-1}, kanamycine 35 µg mL^{-1}.

Table 4. Composition of the defined medium "HDF" (Korz et al., 1995)

Component	Batch medium	Feeding solution
Glucose[a]	5 or 10 g L^{-1}	875 g L^{-1}
$MgSO_4$*7 H_2O	1.2 g L^{-1}	20 g L^{-1}
KH_2PO_4	13.3 g L^{-1}	-
$(NH_4)_2HPO_4$	4.0 g L^{-1}	-
Citric acid *H_2O	1.7 g L^{-1}	-
Ferric citrate	100.8 mg L^{-1}	40.0 mg L^{-1}
$CoCl_2$*6 H_2O	2.5 mg L^{-1}	4.0 mg L^{-1}
$MnCl_2$*4 H_2O	15.0 mg L^{-1}	23.5 mg L^{-1}
$CuCl_2$*2 H_2O	1.5 mg L^{-1}	2.3 mg L^{-1}
H_3BO_3	3.0 mg L^{-1}	4.7 mg L^{-1}
Na_2MoO_4*2 H_2O	2.1 mg L^{-1}	4.0 mg L^{-1}
$Zn(CH_3COO)_2$*2 H_2O	33.8 mg L^{-1}	16.0 mg L^{-1}
EDTA	14.1 mg L^{-1}	13.0 mg L^{-1}

[a] Initial concentration of glucose for preculture and shake fask experiments was 20 g L^{-1}

Table 5. Trace elements stock solutions

Component	Concentration / (g L^{-1})
Ferric citrate	12
$CoCl_2$*6 H_2O	2.5
$MnCl_2$*4 H_2O	15
$CuCl_2$*2 H_2O	1.5
H_3BO_3	3.0
Na_2MoO_4*2 H_2O	2.5
$Zn(CH_3COO)_2$*2 H_2O	13.0
EDTA	8.4

2.3.2 Cultivation conditions

For shake flask experiments, preculture and main culture were grown on complex medium Luria-Bertani (LB), supplemented with the appropriate antibiotics. For the preculture, 10 mL complex medium medium in a 100 mL shake flask was inoculated with a single colony from complex medium agar plate and incubated on a rotary shaker at 37°C, 140 rpm. For the main culture, 200 mL complex medium medium in a 1 L shake flask with baffles was inoculated with 1 % (v/v) of the overnight preculture and was incubated at 37°C to an OD_{600} of about 0.5. Then α-glucosidase synthesis was induced by 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG), and the culture was transferred to the temperatures indicated in the result section.

For shake flask experiments in defined medium, the first preculture was prepared as described above. The second preculture was inoculated with 1 % of the first precultures and was grown in 100 mL shake flask containing 10 mL defined medium under the same condition as the first preculture. The main culture was grown as in case of complex medium, except that 200 mL complex medium medium was replaced by defined medium.

For cultivation in bioreactor, the first preculture was grown in complex medium as above. The second preculture was grown in 200 mL defined medium under the same condition overnight. The main cultivation was carried out in a 10 L bioreactor BIOSTAT C equipped with a digital measurement and control unit (DCU) to control agitation, pH value, temperature and dissolved oxygen tension and connected to the process control system MFCSwin (B. Braun Biotech, Melsungen). The cultivation was started as batch cultures with an initial volume of 8 L, a glucose concentration of 5 or 10 g L^{-1} at a temperature of 37°C. Air flow rate (4 to 16 vvm) and stirrer speed (300 to 1250 rpm) were controlled in cascade manner to maintain a constant dissolved oxygen tension above 40 %. The pH value was maintained at 6.8 by automatic addition of aqueous NH_3 (25 % (w/v)). The foam formation was suppressed by antifoam agent polypropylenglycol (PEG, 50 %, Roth, Karlsruhe). After depletion of the glucose, the stirrer speed controlled by the DCU decreased, because the cells stopped to consume oxygen. The decrease of the stirrer speed was the signal for process control system to activate the flow controller at the DCU. The DCU operated a peristaltic pump (type 101U/R, Watson Marlow Ltd, Rommerskirchen) to pump the feeding solution. The feeding solution was placed on a balance connected to the DCU so the exact feeding rate could be feed-back controlled.

The fed-batch process is the most widely used technique in the industrial production of recombinant proteins. A feeding rate that increases exponentially over time allows the specific growth rate to be kept constant. The feeding rate was calculated continuously throughout the cultivation according to equation (1):

$$F = \frac{1}{S_0} \cdot q_S \cdot X \cdot V = \frac{1}{S_0} \cdot \left(\frac{\mu_{set}}{Y_{X/S}} + m_E \right) \cdot X_f \cdot V_f \cdot \exp\left[\mu_{set} \cdot (t - t_f)\right] \quad (1)$$

With

F : feeding rate, (g h^{-1})

S_0 : substrate concentration in the feeding solution, (g g^{-1})

q_S : specific substrate consumption rate, (g g^{-1} h^{-1})

X : cell density, (g L^{-1})

V : volume, L

μ_{set} : set point of the specific growth rate, h^{-1}

$Y_{X/S}$: yield coefficient for biomass per substrate utilised excl. maintenance, (g g^{-1})

m_E : maintenance coefficient, (g g^{-1} h^{-1})

X_f : dry cell weight at the time of feeding start, (g L^{-1})

V_f : volume at the time of feeding start, L

t : cultivation time, h^{-1}

t_f : time of feeding start, h^{-1}

2.3.3 Reactivation experiments

For reactivation experiments, the cultures were grown under the conditions described above. At the times after induction indicated in the result section, the cultures were transferred to the indicated temperatures with or without prior arrest of protein synthesis by addition of tetracycline to the final concentration of 25 µg mL^{-1}.

2.4 Cell disruption methods

2.4.1 Cell mill

The frozen pellets corresponding to the sample volume of $V_{culture}$ = 4/OD mL were resuspended in $V_{cell\ extract}$ = 350 µL of 10 mM potassium phosphate buffer pH 7, 1 mM EDTA.

Then 0.5 g glass beads were added (diameter 0.25-0.5 mm). The cells were disrupted at 4°C using a cell mill MM 2000 (Retsch, Haan) with an amplitude of 80 % for 5 min. After centrifugation at 13000 rpm for 10 min, the soluble cell fraction in supernatant was transferred to a new tube and used for enzymatic assays (see 2.5.3)

2.4.2 Ultrasonication

The frozen pellets corresponding to the sample volume of $V_{culture} = 4/OD$ mL were resuspended in 580 µL 10 mM potassium phosphate buffer pH 7, 1 mM EDTA. 20 µL of lysozyme (concentration of 1 g L^{-1}) was added and the cells suspension was incubated on ice for about 30 min. Afterwards cells were disrupted by continuous ultrasonication with a sonicator (UP 200S Ultraschallprozessor, Dr. Hielscher GmbH, Teltow) on ice, at an amplitude of 50 % for 20 sec. The soluble and insoluble cell fractions were separated by centrifugation at 13000 rpm for 20 min. The insoluble proteins were washed twice with the 10 mM potassium phosphate buffer pH 7, 1 mM EDTA and stored at –20°C.

2.4.3 French press

French press was used to get a larger amount of purified inclusion body (IBs). About 5 g wet biomass was resuspended in 25 mL 0.1 M Tris/HCl pH 7, 1 mM EDTA using the homogeniser Ultraturrax T25 (IKA-Labortechnik, Staufen). Lysozyme at a final concentration of 0.3 mg mL^{-1} was added to the cell suspension and the latter was incubated for 30 min on ice. After that, the cells were disrupted by means of high-pressure homogenisation in the homogeniser Gaulin Micron Lab 40 (APV homogeniser GmbH, Lübeck), in 3 passages. After centrifugation for 10 min at 1200×*g* for removing unbroken cells, DNAse (at a final concentration of 3 mM) and $MgCl_2$ (at a final concentration of 10 µg mL^{-1}) were added to the cell lysate and the latter was incubated at room temperature for 20-30 min to digest the DNA. Half a volume of 60 mM EDTA, 6 % Triton X100, 1.5 M NaCl pH 7 was added and the lysate was incubated for further 30 min on ice. The pellet obtained after centrifugation was washed 3-5 times with buffer (0.1 M Tris/HCl pH 7, 20 mM EDTA). The purified IBs were immediately used or stored at –20°C.

2.5 Analytical methods

2.5.1 Cell density

Culture growth was monitored by optical density and cell dry weight measurements.

- The optical density at a wavelength of 600 nm (OD_{600}) was measured in a spectrophotometer (Ultrospec 3000, Pharmacie, Sweden). Fresh medium was used as a reference. The cell suspension was diluted to keep the correlation between absorption and cell density linearly, which was below 0.5.

- For dry cell weight determination (CDW), 2 mL of culture suspension was transferred into a preweighed Eppendorf tube and was centrifuged at 13000 rpm at 4°C for 3 min. The pellet was washed once with 0.9 % (w/v) NaCl and was dried at 60°C until the weight remains constant (at least 24 h). CDW was determined in quatruplicate.

2.5.2 Glucose concentration

The glucose concentration was determined by the hexokinase/glucose-6-phosphatedehydrogenase method (Kit No. 139106, Boehringer Mannheim GmbH, Mannheim). Culture supernatant obtained after centrifugation was used for glucose determination. After incubation at 80°C for 20 min to deactivate enzymes interfering with the determination, each sample was applied in quatruplicate on a microtiter plate with 96 wells.

Principle of the methods is based on following reactions: first glucose is phosphorylated to glucose-6-phosphate in the presence of the enzyme hexokinase and adenosine-5'-triphosphate (ATP). Second in the presence of the enzyme glucose-6-phosphate dehydrogenase, glucose-6-phosphate is oxidized by nicotinamide-adenine dinucleotide phosphate (NADP) to gluconate-6-phosphate with the formation of reduced nicotinamideadenine dinucleotide phosphate (NADPH). The amount of NADPH formed in this reaction is stoichiometric to the amount of D-glucose. The increase in NADPH is measured by means of its absorption at 340 nm.

Thus, the absorption of the sample before and after addition of hexokinase/glucose-6-phosphatedehydrogenase with subsequent incubation was measured on the DIAS (Dynatech Immunoassay system) reader (Dynatech Laboratories, Denkendorf). Glucose concentration

was calculated based on a calibration curve, obtained from standard solutions, which were included on the same microtiter plate.

2.5.3 Glucosidase activity

Cells were disrupted with the cell mill (see 2.4.1) and the supernatant obtained after centrifugation was used for α-glucosidase activity measurement. The activity assay is based on the increase of absorbance at 405 nm due to enzymatic cleavage of *p*-nitropenyl-α-D-glucopyranoside (pNPG) by α-glucosidase. The absorption was measured on a Spectrophotometer DU 7400 (Beckman, München). Activity of α-glucosidase was assayed at 30°C in 0.1 M phosphate buffer (pH 6.8) using 2 mM pNPG (Sigma) as substrate (Kopetzki et al., 1989a). The reaction volume was V_{test} = 1 mL, including 100 µL of substrate pNPG (stock solution of 20 mM), and sample volume V_{sample} and filled up to 900 µL with phosphate buffer. The volume of the sample V_{sample} was varied from 1 to 100 µL depending on the activity of α-glucosidase in the sample. For calculation of the specific activity, the protein concentration C in the sample was determined by Bradford assay (s. 2.5.5.1).

Enzymatic activity was calculated according to the equation

$$\text{volumetric activity} / (\text{U mL}^{-1}) = \frac{V_{test} * \Delta E_{405}/\Delta t}{\varepsilon_{405nm} * d * V_{sample}} * \frac{V_{cell\,extract}}{V_{culture}}$$

$$\text{specific activity} / (\text{U mg}^{-1}) = \text{volumetric activity} / \text{C}$$

$\Delta E_{405}/\Delta t$: slope of absorbance at 405 nm vs. time

V_{test} : total reaction volume in the cuvette (1 mL)

ε_{405nm} : absorbance coefficient, $\varepsilon_{p\text{-nitrophenol}}$ = 7.8 µmol $^{-1}$ cm^{-1} (pH 6.8)

d : length of the cuvette (1 cm)

V_{sample} : volume of sample applied for measurement (µL), from 1 to 100 µL

$V_{cell\ extract}$: cell extract volume (350 µL)

$V_{culture}$: sample volume ($4/OD_{600}$ mL culture)

C : protein concentration (in mg mL^{-1})

2.5.4 Polyacrylamide gel electrophoresis (PAGE)

2.5.4.1 One-dimensional gel electrophoresis

The one dimensional gel electrophoresis was carried out on the Criterion system (Bio-Rad Laboratories, München), using precast Tris/HCl 15 % resolving gels (Bio-Rad), which were produced according to Laemmli (1970). The gel size was 13.3 × 8.7 cm.

Protein samples, either total cell fractions (total pellet) from a culture volume of 1/OD mL or insoluble cell fractions, prepared from a culture volume of 4/OD mL^{-1} after ultrasonication as described in section 2.4.2, were resuspended in 100 µL loading buffer (0.125 M Tris/HCL, pH 6.8, 20 % (v/v) glycerol, 4 % (w/v) SDS, 5 % (v/v) 2-mercaptoethanol, 0.02 % (w/v) bromphenolblue); soluble proteins were diluted with one volume loading buffer. After incubation at 95°C for 10 min, the samples were loaded on the gels and were run at 200 V for about 50-55 min in running buffer (0.025 M Tris, 0.192 M glycine, 0.1 % (w/v) SDS).

The low molecular weight calibration kit for electrophoresis (Amersham Biosciences, Freiburg), with molecular weight in the range from 14.4 to 94 kDa served as molecular weight standard.

2.5.4.2 Two-dimensional gel electrophoresis

The frozen samples were dissolved in lysis buffer (8 M urea, 4 % of 3-((2-cholamidopropyl)-di-methylammonio)-1-propane-sulfonate [CHAPS], 60 mM dithiothreitol [DDT], 2% Pharmalyte 3-10, 0.002 % bromphenolblue). After one hour incubation at room temperature, 50 µL of the dissolved sample was diluted in 450 µL of rehydration solution (8 M urea, 0.5 % (w/v) CHAPS, 0.2 % (w/v) DDT, 0.5 % (v/v) Pharmalyte 3-10, 0.002 % (w/v) bromphenolblue) and then applied into one strip holder. The first dimension isoelectric focusing was carried out on IPGphor Isoelectric Focusing system (Pharmacie Biotech), using Immobiline Dry strip (24 cm, pH 3-10). The Immobilisation pH gradients (IPG) strips (ready-made) were lowered with the gel side down onto the solution inside the strip holder. Afterwards, 2 mL cover fluid was applied onto the ends of the IPG strip to minimise evaporation and urea crystallisation. The isoelectric focusing process was managed according to the recommended protocol by the manufacturer, with voltage gradient divided in 4 steps, taken together 8 h 30 min (Step 1: 200 V for 1 h, Step 2: 500 V for 1 h, Step 3: 1000 V for 1 h, Step 4: 8000 V for 5 h 30 min). The maximum current was set to 50 µA per IPG strip.

The second dimension SDS-PAGE electrophoresis was performed on Ettan Dalt six electrophoresis system (Amersham Biosciences, Freiburg). The homogeneous gel solution, containing 188 mL of ready–to-use acrylamid/bisacrylamid solution Rotiphorese Gel 30 (Roth, Karlsruhe) that contains 30 % (w/v) acrylamide and 0.8 % (w/v) bisacrylamide, 113 mL of 4× resolving buffer (1.5 M Tris/HCl, pH 8.8), 93 mL of distilled water, 4.5 mL of 10 % (w/v) SDS, 4.5 mL of 10 % (w/v) ammoniumperoxodisulfate [APS] and 0.62 mL of 10 % tetramethylethylenediamine [TEMED, diluted from 99 % TEMED] was poured onto the gel caster and let polymerise for at least one hour.

The IPG strips from the first dimension were equilibrated two times for 15 min in SDS equilibration buffer (50 mM Tris/HCl pH 8.8, 6 M urea, 30 % (v/v) glycerol, 2 % (w/v) SDS and 0.002 % (w/v) bromphenolblue), the first additionally containing 1 % (w/v) DDT and the second 2.5 % (w/v) iodoacetamide [IAA]. DDT and IAA were freshly prepared directly before use. Equilibrated IPG strips were loaded onto the gel cassette and sealed by agarose sealing solution (25 mM Tris, 192 mM glycine, 0.1 % (w/v) SDS, 0.002% bromphenolblue, 0.5 % (w/v) agarose). The gels were run in running buffer (0.025 M Tris, 0.192 M glycin, 0.1 % (w/v) SDS) at 10°C and 2.5 W per gel for 30 min and afterwards at 100 W for six gels until the run were completed, which normally took 4 to 5 h.

2.5.4.3 Gel staining

Coomassie blue- and silver-staining was used in this work. Coomassie blue staining was used for quantitative analysis of protein concentration, and silver staining was used for visualisation of proteins of low concentration.

Coomassie-blue staining. For staining, gels were rinsed in Coomassie-blue-solution for two hours. Gels were destained using destaining solution, containing 40% (v/v) methanol and 10% (v/v) acetic acid, until the background became colourless.

Staining solution:

- Coomasie Brilliant Blue G250 (Roth) 1 % (w/v)
- Methanol 40 % (v/v)
- Acetic acid 10 % (v/v)
- Water 50 % (v/v)

Silver staining. Gels were first fixed in acetone solution, containing 2.5 % (v/v) trichloroacetic acid (TCA) and 0.04 % (v/v) formalin solution, for 5 min. Then they were rinsed in acetone solution for 5 min, later in 0.17 % (v/v) aqueous sodiumthiosulphate ($Na_2S_2O_3$) solution for 1 min, and then in aqueous solution containing 1.3 % (v/v) silver nitrate ($AgNO_3$) and 1 % (v/v) formalin for 8 min. Every time before transferring from one solution to the next, gels were washed thoroughly with deionised water. The gels were developed in aqueous solution containing 2 % (w/v) sodiumcarbonate (Na_2CO_3), 0.04 % (v/v) $Na_2S_2O_3$ and 0.04 % (v/v) formalin solution. The development of the gels was stopped by transfer the gels to1 % acetic acid solution.

Concentration of the stock solutions used for silver staining were

- Acetone 50 % (v/v)
- Formalin 37 %
- Sodiumthiosulphate ($Na_2S_2O_3$) 10 % (w/v)
- Acetic acid 1 % (v/v)
- Trichloroacetic acid (TCA) 50 % (w/v)
- Silver nitrate ($AgNO_3$) 20 % (w/v)

For storing, gels were dried or alternatively kept wet in ethanol solution.

2.5.5 Protein concentration determination

2.5.5.1 Protein concentration determination according to Bradford

Protein concentrations were estimated according to the protocol recommended by Bio-Rad (Bio-Rad Laboratories, München) for protein concentrations below 25 µg mL^{-1}. The samples must first be diluted with 10 mM potassium phosphate buffer pH 7, 1 mM EDTA to get a protein concentration less than 25 µg mL^{-1}. 800 µL of diluted sample was mixed with 200 µL of dye reagent concentrate (Bio-Rad). After 5 min, the absorption at 595 nm was measured versus blank, representing of 800 µL 10 mM potassium phosphate buffer pH 7, 1 mM EDTA and 200 µL dye reagent concentrate. Bovine serum albumin (BSA) from Bio-Rad was used as standard.

2.5.5.2 Protein quantification on SDS-PAGE by densitometry

After destaining, the gels were scanned by an image scanner (Amersham Pharmacia Biotech). The volume of protein bands on the one-dimensional SDS-PAGE was quantified in arbitrary units using the Phoretix 1D program (Phoretix, England). Membrane proteins, which should be constant in all samples (Mogk et al., 1999), were used as internal reference (cf. Fig. 20 and Fig. 30). The quota of the reference protein in the total cell protein was calculated. The concentration of the protein of interest was calculated using the following equation, considering that 1 g dry cell mass contains 500 mg protein (Ingraham et al., 1983).

$$\text{Amount of protein} / (\text{mg g}^{-1}) = \frac{\text{Volume of protein of interest}}{\text{Volume of reference protein}} * \frac{\text{Quota of reference (in \%)}}{100\%} * 500$$

For two-dimensional gel electrophoresis, the proteins spots were identified by comparison with the *E. coli* 2D database (http://www.expasy.org/cgi-bin/map2/def?ECOLI) (Wilkins et al., 1998). The identity of individual protein spots was verified by N-terminal sequencing at the Max-Planck Institute in Halle. The volume of the protein of interest was analysed with the software Phoretix 2D (Phoretix, England). Three spots, which appeared in all gels and whose volumes seemed not to be affected by the genotyp of the used strains under the experimental conditions were used as reference (cf. Fig. 24). The relative volume of individual spots was thus calculated by dividing the spot volume by the sum of the volumes of the three references.

2.5.5.3 Blotting proteins from gel to PVDF membrane

For sequencing, proteins were transferred from gel to polyvinylidene fluoride (PVDF) membrane by blotting. The PVDF membrane was first soaked with methanol for 10 sec, then was transferred to transfer buffer (20 mM Tris, 150 mM glycine, 20 % (v/v) methanol and 0.02 % (w/v) SDS) for 15 min. Whatman papers were soaked with transfer buffer. The assembly was arranged from the bottom to the top in the following sequence: 3 Whatman papers, gel, PVDF membrane and again 3 Whatman papers. For a successful blot, air bubbles were avoided. For this purpose, a glas stick was rolled over the membrane and Whatman papers to make it tightly stick together. The protein was botted for 1 hour at 40 V, using blotting apparatus SEMI-PHOR (Hoefer Scientific Instruments, San Francisco, USA).

After blotting, the PVDF membrane was stained with Coomassie blue solution (10 % (v/v) acetic acid, 30 % (v/v) methanol, 0.006 % (w/v) Coomassie G250) for 5-30 sec and destained

with destainer (10 % (v/v) acetic acid and 30 % (v/v) methanol) for 1-5 min. After drying for a whole day at room temperature, the membrane was used for sequencing.

3. RESULTS

3.1 Impact of cultivation parameters on the production of active α-glucosidase

3.1.1 Direct production of α-glucosidase

3.1.1.1 Production in shake flasks

For production of α-glucosidase PI of the yeast *Saccharomyces cerevisiae* in recombinant *E. coli*, two production methods were used in both shake flask and fed-batch cultivations in this work: the direct production, in which the temperature is kept constant throughout the production phase, and the production with subsequent reactivation, in which the temperature was reduced at indicated times after induction to initiate the reactivation of previously aggregated α-glucosidase. These experiments were carried out with the wildtype strain MC4100:pKK177-3/GLUCP1:pUBS520.

To study the effect of temperature on α-glucosidase production, cultures were grown on complex medium at 37°C to an optical density OD_{600} of about 0.5, were induced with 1 mM IPTG and subsequently incubated at 24°C, 30°C, 37°C or 42°C.

Growth was faster at higher temperatures in the range 24°C to 37°C. The growth of the culture at 42°C was as fast as at 37°C within two hours after induction but slown down afterwards (Fig. 3 A).

The total amount of α-glucosidase four hours after induction was highest at the optimum growth temperature of 37°C and reached 60 mg g^{-1}, which was about 50 % higher than at 24°C, as estimated from Coomassie-stained SDS-PAGE (Fig. 3 D). Despite the specific product concentration increased faster at higher temperature, it also sooner reached stationary level; the product accumulation stopped two hours after induction at 37°C, while it continually increased until the end of cultivation at 24°C (four hours after induction) (Fig. 3D). It seems that the specific product concentration at low temperature can surpass that at high temperature after long cultivation. At 42°C, the product accumulation reached a same value as at 37C one hour after induction, but as it stopped to increase afterwards, the final

product concentration reached similar value as at 24°C (Fig. 3 D). The concentration of soluble α-glucosidase was very low at temperature above 37°C; nearly all the produced α-glucosidase was deposited in insoluble cell fraction (Fig. 3 E, F). The soluble product was accumulated already at 30°C, but 30% higher final concentration of soluble product was found at lower temperature of 24°C (Fig. 3 E) accounting for 80 % of total α-glucosidase (Fig. 3 F).

No plasmid loss occurred in these experiments (Fig. 20), so the slow production was not a consequence of plasmid instability; rather, degradation may be enhanced in response to the dual stress of high temperature and production of an aggregation-prone protein.

The specific α-glucosidase activity (activity per mg cell proteins) obtained was highest at 24°C, and reached 3 U mg^{-1}, which was nearly twofold higher than at 30°C. At higher temperatures, the maximum specific activity was only 0.2-0.5 U mg^{-1} (Fig. 3 B).

As cultures grew very slowly at 24°C, the volumetric activity (activity per culture volume, thus depends also on cell density) increased with slower rate at 24°C than at 30°C (Fig. 3 C), giving better yield of active product at the intermediate temperature of 30°C, despite higher specific activity at 24°C. The volumetric α-glucosidase activity was very low at high temperature (37°C - 42°C) independent of fast growth at temperature of 37°C or slowing growth at 42°C (Fig. 3 C). These high temperatures hence are not appropriate for production of active proteins.

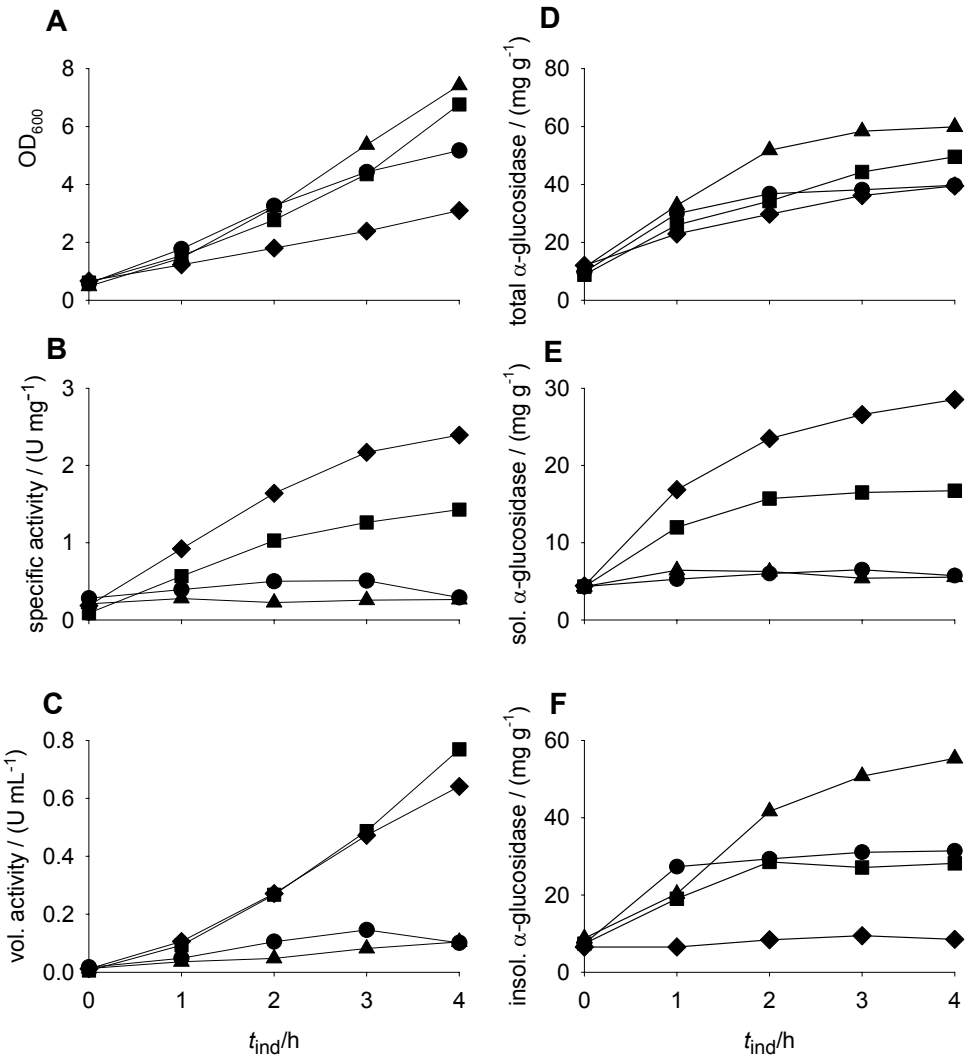

Fig. 3: Production of α-glucosidase at different temperatures. Cultures of the wild-type strain MC4100:pKK177-3/GLUCP1:pUBS520 were incubated at 37°C to an OD_{600} about 0.5, then induced with 1 mM IPTG and shifted to various temperatures (24°C - diamant, 30°C - square, 37°C - triangle, 42°C - circle). Time is given relative to the time of induction. (A) Optical density OD_{600}, (B) specific α-glucosidase activity in units per milligram of total protein, (C) volumetric α-glucosidase activity in units per millilitre of the culture. α-Glucosidase concentrations in milligram per gram dry cell weight were estimated by densitometry of Coomassie-stained SDS-PAGE of (D) total cell protein, (E) soluble cell protein and (F) insoluble cell protein, separated by centrifugation for 20 min at 13 000 rpm after cells disruption by ultrasonication.

For investigation the effect of pH value at different temperatures, α-glucosidase was produced on complex medium at 37°C or 30°C with a pH value buffered at 5 or 7. Low pH values slowed down growth (Fig. 4 A); the final OD_{600} four hours after induction at pH value of 5 was twofold lower than at pH value of 7 independent of temperatures 30°C or 37°C. Concomitant to twofold lower OD_{600}, the specific α-glucosidase activities were two times higher at both temperatures (Fig. 4 B). Consequently, the pH value did not affect the volumetric activity (Fig. 4 C).

Low temperature of 30°C slightly slown down the growth relative to 37°C at both pH values (Fig. 4 A). The fivefold higher specific and volumetric activity was obtained at 30°C than at 37°C independent of pH values (Fig. 4 C).

Since the changes of specific activity or cell densities were similar at both pH values independent of temperatures on one hane, and those changes were similar at both temperatures independent of pH values on other hand (Table 6), the effect of temperature and pH value was found to be independent from each other.

Table 6. Effect of medium pH value on growth and specific α-glucosidase activity four hours after induction

Temperature	Specific activity / (U mg^{-1})			OD_{600}		
	pH 5	pH 7	Change[a]	pH 5	pH 7	Change[a]
30°C	3.37	1.43	2.3	3.20	6.8	0.5
37°C	0.60	0.26	2.3	3.78	7.4	0.5
Change[b]	5.6	5.5		0.85	0.91	

[a] Specific activity or optical densities at pH 5 relative to that at pH 7

[b] Specific activity or optical density at temperature 30°C relative to that at 37°C

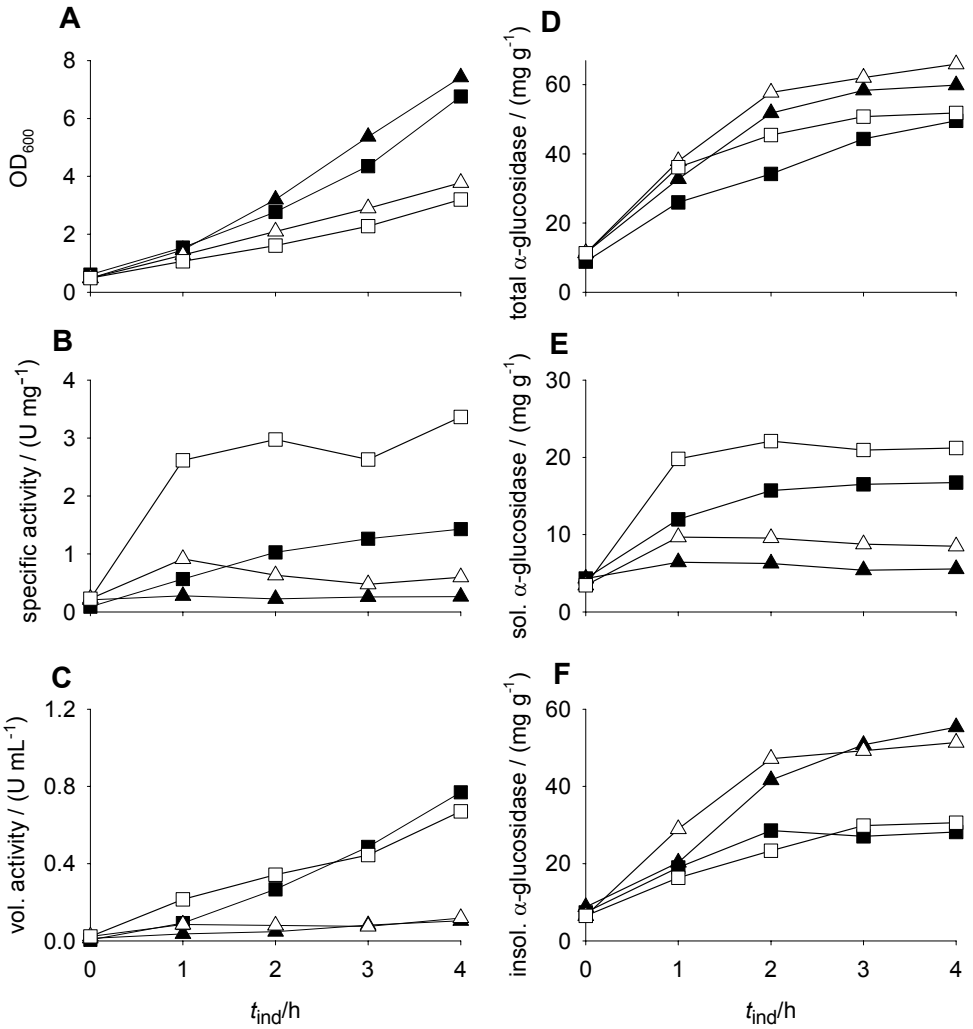

Fig. 4: Production of α-glucosidase at diverse temperatures and pH values. Cultures were incubated at 37°C in complex medium adjusted to a pH value of 5 (open symbol) or 7 (solid symbol) to an OD_{600} of 0.5, induced with 1 mM IPTG and kept at 37°C (triangle) or shifted to 30°C (square). Time is given relative to the time of induction. (A) Optical density OD_{600}, (B) specific α-glucosidase activity, (C) volumetric α-glucosidase activity, and α-glucosidase concentrations in (D) total, (E) soluble and (F) insoluble cell fractions.

Concomitant to higher specific α-glucosidase activity, higher concentrations of soluble product were obtained at low pH value than at high pH value (Fig. 4E), which resulted in higher specific product concentration at low pH value. The higher concentration of soluble product was observed at both temperature values (Fig. 4D). But the amount of insoluble α-glucosidase was similar (Fig. 4 F). Thus, production at low pH values resulted in a higher ratio of soluble to insoluble α-glucosidase. In contrast, a pronounced increase of IBs formation of a fusion protein consisting of protein A from *Staphylococcus aureus* and β-

galactosidase from *E. coli* (SpA-ßgal) at low pH value at 39°C and 42°C in bioreactor was found (Strandberg and Enfors, 1991).

The concentration of soluble α-glucosidase reached a stationary level earlier at low pH value than at high pH value (Fig. 4E). The maximum concentration of 22 mg g^{-1} at low pH value was obtained already before one hour after induction at 30°C (Fig. 4 E) and maintained afterwards despite continued increase of the concentration of total α-glucosidase up to three hours after induction (Fig. 4 D).

The impact of full *versus* partial induction was examined at two IPTG concentrations of 1 and 0.05 mM both in complex and defined medium. The most prominent effect of low inducer concentration was a reduction of amount of insoluble α-glucosidase; in both media about 40-50% lower concentrations of insoluble product at lower IPTG concentration was obtained (Table 7). Lower concentration of IPTG resulted in 25% lower concentrations of total α-glucosidase in complex medium, but did not affect the concentration of total α-glucosidase in defined medium (Table 7).

Table 7. Effect of inducer concentration in complex and defined medium

Medium	Complex medium (4 hours induction)			Defined medium (22 hours induction)		
IPTG concentration	1 mM	0.05 mM	Change[a]	1 mM	0.05 mM	Change[a]
OD_{600}	6.76	7.2	7 %	5.24	7.12	36 %
Specific activity / (U mg^{-1})	1.44	1.64	14 %	5.66	7.27	28 %
Volumetric activity / (U mL^{-1})	0.7	1	43 %	1.72	4.38	155 %
Soluble α-glucosidase / (mg g^{-1})	17	19	12 %	35	43	23 %
Insoluble α-glucosidase / (mg g^{-1})	28	14	-50 %	33	19	-42 %
Total α-glucosidase / (mg g^{-1})	45	33	-27 %	68	63	-7 %

[a] calculated as difference of the value at partial induction relative to full induction

Another effect of low inducer concentration was an increase of active α-glucosidase, which more pronounced in defined medium. The specific and volumetric α-glucosidase activities were 14 % and 33 % higher than with full induction on complex medium, and 28 % and 155

% higher on defined medium (Table7).

Similar final optical densities at both inducer concentrations were obtained on complex medium (Table 7). In contrast, the growth of the cultures after induction on defined medium was significant impaired at full induction, resulting in the two times lower final optical densities relative to partial induction after 22 hours cultivation (Table 7).

Thus, althought low inducer concentration has a similar effect on both media such as reduction of IBs formation and increase the α-glucosidase activities, the effect was different on growth. Furthermore, the changes in active yield such as specific and volumetric activities and soluble product were intensified in defined medium (Table 7). This observation was inaccordance with the changes of specific growth rate of induced and uninduced cells in both medium. The specific growth rate was not affected after induction on complex medium; the specific growth rate of (0.67±0.04) h^{-1} at 30°C were obtained in induced and uninduced cells. In contrast on defined medium, the specific growth rate is considerable lower in induced cells than uninduced cells; it was only (0.25±0.01) h^{-1} and (0.34±0.01) h^{-1} with full and partial induction respectively in induced cells relative to (0.37±0.01) h^{-1} in uninduced cells.

Those distinct behaviours were probably due to different role of stress induced by overexpression of aggregation-prone recombinant proteins in different medium. It seems that the stress has the more important role on defined medium than on complex medium. The fact that at high temperature of 45°C the cells producing α-glucosidase did not grow after induction on defined medium (not shown) but continued to grow on complex medium (Fig. 3) also supports the stronger stress on defined medium relative to complex medium. Since the production of α-glucosidase on defined medium causes such strong stress; the partial induction produced a significant relief to the cells. Therefore, the growth of the cells on defined medium at partial induction was improved considerably – in contrast to complex medium. Consequently, much higher α-glucosidase activities than with full induction were obtained (Table 7).

3.1.1.2 Production in bioreactors

3.1.1.2.1 Production of α-glucosidase in fed-batch cultivation

The slow growth rate in case of low temperatures or low pH values resulted in higher specific α-glucosidase activity but also caused low cell densities, which can interfere with productivity (Fig. 3 and Fig. 4). Alternatively, fed-batch cultivation can be used since in fed-batch cultivations the growth rate is controlled by the feeding rate. Furthermore, fed-batch cultivations are commonly used to achieve high cell densities of recombinant *E. coli*. In addition, in fed-batch cultivation, the growth rate can be kept constant at various temperatures and thereby the effect of growth rate is isolated from that of temperature.

The fed-batch cultivations with exponentially increasing feeding rate over time to enable a constant growth rate μ_{set} were performed. During the batch phase with initial glucose concentration of 5 g L^{-1} at 37°C, a maximum specific growth rate μ_{max} of (0.73±0.02) h^{-1} and a biomass yield coefficient for glucose of $Y_{X/S}$ = (0.50±0.05) g g^{-1} (dry cell mass obtained per glucose consumed) were obtained. The feeding started automatically after glucose depletion in batch phase. The induction with 0.4 mM IPTG was performed one hour after feeding start.

During the fed-batch phase, the feeding rate, which was calculated by the process control system based on the set specific growth rate μ_{set} = 0.12 h^{-1}, increased exponentially with time (Fig. 5 B). Since culture growth followed the set growth rate (Fig. 5 C) that was lower than the maximum growth rate μ_{max} = 0.73 h^{-1}, the supplied glucose was immediately used by the cells and no glucose accumulated during the feeding phase until ten hours after induction. But afterwards, the cell grew slower than μ_{set} despite continued feeding (Fig. 5 C) and glucose began to accumulate to 1 g L^{-1} in the medium (Fig. 5 A). The glucose concentration in medium increased as a consequence of the decreased cell glucose uptake capacity (Lin et al., 2001; Neubauer et al., 2003). In fed-batch cultivation with a constant feeding rate enabling the cells grow with start specific growth rate of 0.8 h^{-1} and reduce to 0.24 h^{-1} at time of induction (three hours after feeding start), the maximum glucose uptake rate of the cultures producing α-glucosidase decreases 50% after induction compared to cells without induction and glucose accumulated up to 1.5 g L^{-1} already one hour after induction (Lin, 2000).

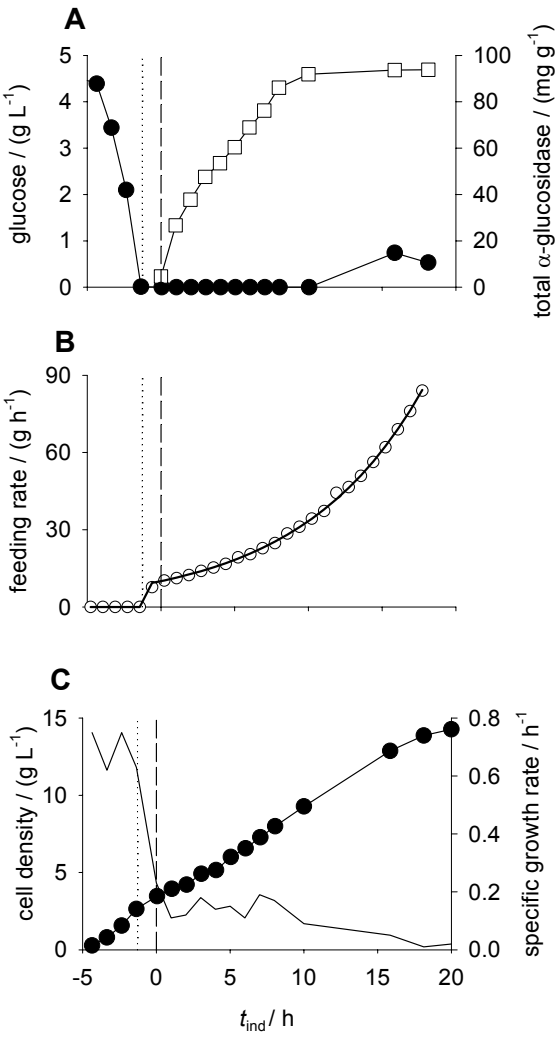

Fig. 5: Fed-batch cultivation for production of recombinant α-glucosidase. *E.coli* strain MC4100: pKK177-3/GLUCP1:pUBS520 was cultivated in a bioreactor in two phase, batch and fed-batch phases. First the culture was grown in batch-phase, characterised by maximum growth rate. After depletion of glucose, the fed-batch phase with limiting glucose feeding started. The glucose feeding rate increased exponentially with time to keep the growth rate constant at a set specific growth rate μ_{set} = 0.12 h^{-1}. One hour after feeding start, 0.4 mM IPTG was added to induce α-glucosidase synthesis. Time is given relative to the time of induction. (A) Specific α-glucosidase concentration (square) and glucose concentration (circle), (B) set point (solid line) and process value (circle) of the glucose feeding rate, (C) dry cell mass (circle) and specific growth rate (solid line). Dotted line indicates time of feeding start. Short dashed line indicates time of induction.

The specific concentration of α-glucosidase reached a stationary level at ten hours after induction, which was 100 mg g^{-1} (Fig. 5 A). The cell densities determined by dry cell mass measurement continuously increased, resulting in further increase of a volumetric product concentration (Fig. 5 C). The constant specific α-glucosidase concentration might a result of

the equilibrium between ongoing synthesised α-glucosidase and dilution of α-glucosidase by cell growth. The degradation as consequence of stress might also contribute to the stop of net accumulation of product.

The fed-batch with employed mode was checked for production of active α-glucosidase. Specific and volumetric activities gradually increased and 18 h after induction reached 9 U mg^{-1} and 25 U mL^{-1}, respectively (Fig. 6 A). Soluble α-glucosidase increased until the end of cultivation and reached 54 mg g^{-1}, accounting for nearly 60 % of total α-glucosidase (Fig. 6 B).

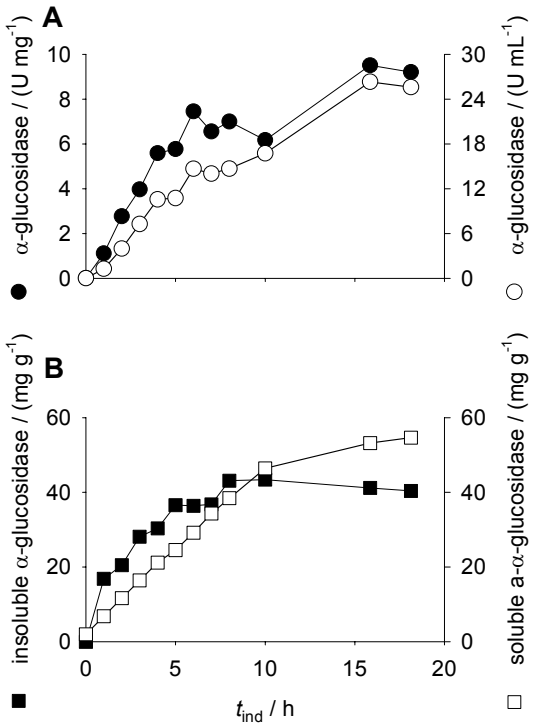

Fig. 6: Production of active α-glucosidase in fed-batch cultivation. Cultivation as described in Fig. 5. (A) Specific activity (solid symbol) and volumetric activity (open symbol) of α-glucosidase; (B) specific concentration of α-glucosidase in insoluble (solid symbol) and soluble (open symbol) cell fraction. Time is given relative to the time of induction.

3.1.1.2.2 Optimum feeding rate and temperature for accumulation of soluble active α-glucosidase

Growth rate and temperature both influence the accumulation of active α-glucosidase. While their effects are interdependent in shake flasks, they can be controlled independently in glucose limited fed-batch cultivation.

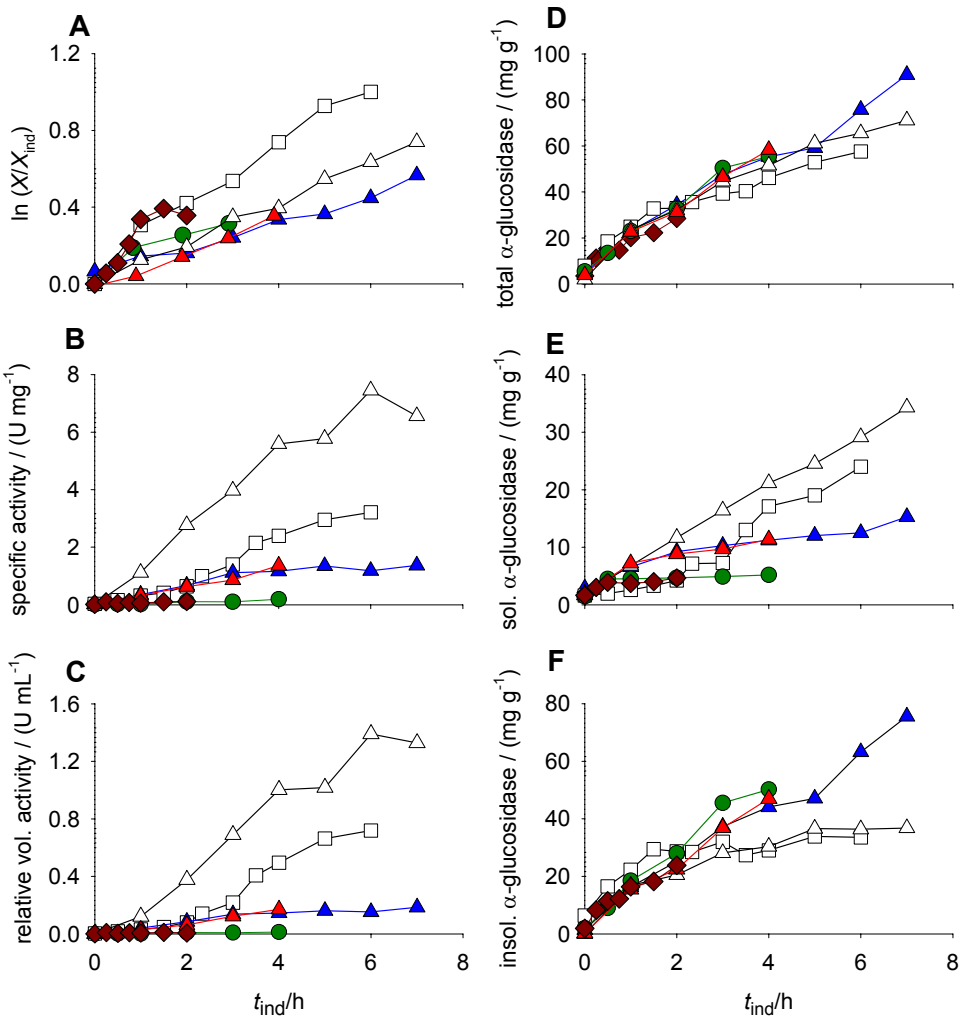

Fig. 7: Optimisation of growth rate and temperature for production of active α-glucosidase. The cultures were grown in batch-phase at 37°C on defined medium with an initial glucose concentration of 5 or 10 g L^{-1}. After glucose depletion, the fed-batch phase was started with a feeding rate to enable growth with μ_{set} = 0.22 h^{-1} (brown diamond), 0.18 h^{-1} (green circle), 0.2 h^{-1} (square), or 0.12 h^{-1} (triangle). IPTG was added to a final concentration of 0.4 mM one hour after feeding start. Temperature was kept at 37°C (solid symbol) or shifted to 30°C (open symbol) directly after induction. (A) Cell density X from dry mass measurement, relative to the cell density at induction, X_{ind}, (B) specific α-glucosidase activity, (C) volumetric α-glucosidase activity, normalized with the optical density at induction, OD_{ind}; specific concentration of α-glucosidase in (D) total, (E) soluble and (F) insoluble cell fractions. Time is given in hours relative to the time of induction. X_{ind} was in the range of 3.2 to 6.6 g L^{-1} and OD_{ind} in the range of 9 to 21 depending on the initial glucose concentration in the batch medium.

The fed-batch cultivations with various growth rates μ_{set} were performed at 37°C or 30°C. At fast feeding rate equal or greater than 0.18 h^{-1}, the actual growth rate μ equalled μ_{set} only in the first two hours after induction. The actual μ decreased afterwards and only four hours after induction similar relative cell densities at $\mu_{set} \geq 0.18$ h^{-1} and $\mu_{set} = 0.12$ h^{-1} at cultivation temperature of 37°C were observed (Fig. 7A). At slow feeding rate, in contrast, the actual

growth rate μ was equal the set point $\mu_{set} = 0.12\ h^{-1}$ until the end of cultivation (seven hours after induction) as caculated from increase of cell densities (Fig. 7A).

Low temperature of 30°C favoured the accumulation of active product; at 30°C, the specific and volumetric α-glucosidase activities four hours after induction were almost 12-fold higher than those at 37°C at a fast growth rate of $0.2\ h^{-1}$ (Fig. 7 B, C). Similarly, at the low growth rate of $0.12\ h^{-1}$, specific and volumetric α-glucosidase activities obtained at 30°C were about 7 and 14 times higher than at 37°C seven hours after induction, respectively (Fig. 7 B, C).

High temperature of 37°C promoted an aggregation; the final concentration of insoluble α-glucosidase at 37°C was about twofold higher than at 30°C (Fig. 7 F). The concentration of soluble α-glucosidase was twofold lower at higher temperature (Fig. 7 E). Since similar values of the specific concentration of total α-glucosidase were obtained at both temperatures (Fig. 7 D), the higher soluble product concentration at lower temperature was not a consequence of higher specific product concentration.

The specific growth rate had a pronounced effect on α-glucosidase activity at both temperatures studied (Fig. 7 B, C). At 37°C, the fast growth rate of $0.2\ h^{-1}$ resulted in very little accumulation of active α-glucosidase, while at the low growth rate of $0.12\ h^{-1}$, specific and volumetric activities were sixfold and tenfold higher, respectively (Fig. 7 B, C). At 30°C, the specific α-glucosidase activity at fast feeding rate was already 12-fold higher than that at 37°C due to temperature effect, but slow growth rate of $0.12\ h^{-1}$ gave further twofold higher specific α-glucosidase activities, resulting on twofold higher volumetric α-glucosidase activities (Fig. 7 B, C).

The higher volumetric α-glucosidase activy during production at slow feeding rate was not a mere consequence of higher specific concentration of α-glucosidase as the feeing rates in the range examined did not alter the specific product concentration (Fig. 7 D). Specific growth rate changed, however, the distribution of soluble and insoluble α-glucosidase (Fig. 7 E, F). The concentration of soluble α-glucosidase was higher at slow growth rate at both 30°C and 37°C (Fig. 7 E).

Thus, both temperature and specific growth rate have an effect on folding of α-glucosidase. Active α-glucosidase can be produced even at 37°C when cultures are grown at low growth

rate. The low growth rate at low pH values hence contributed to higher specific α-glucosidase activity (Fig. 4). On other hand, soluble active α-glucosidase was produced at 30°C in complex medium in shake flask experiments despite high growth rate. High temperatures above 37°C promote aggregation of proteins, leading to low yield of active product. For that reason, production of α-glucosidase at 37°C or 42°C in the shake flask gave only little active α-glucosidase.

Since low growth rates and low temperatures resulted on higher specific α-glucosidase activity, the cultivations were performed with $\mu_{set} = 0.06\ h^{-1}$ and at 24°C.

Cultivation with a $\mu_{set} = 0.06\ h^{-1}$ resulted on 50 % lower specific α-glucosidase concentration than that at $\mu_{set} = 0.12\ h^{-1}$ (Fig. 8 C). At $\mu_{set} = 0.06\ h^{-1}$, the effect of low growth rate on distribution of soluble and insoluble product was still observed; the amount of insoluble α-glucosidase aggregated in IBs was less, accounting for only 5-10% of total α-glucosidase, whereas at $\mu_{set} = 0.12\ h^{-1}$ more than 45% of total α-glucosidase was found in IBs (Fig. 6). The specific activity and concentration of soluble α-glucosidase, however, remained at the same level as in the cultivation with higher growth rate $\mu_{set} = 0.12\ h^{-1}$ (Fig. 8 B).

At the low growth rate of $0.06\ h^{-1}$, twofold lower cell densities than at grow rate $0.12\ h^{-1}$ seven hours after induction were obtained (Fig. 8 A). As specific α-glucosidase activities were similar; the volumetric activity was twofold lower.

The growth rate of the cultures was lower at lower temperatures. Therefore $\mu_{set} = 0.06\ h^{-1}$ can theoretically limited the growth at 30°C but not at 24°C. The similar pattern of growth at 24°C and 30°C (Fig. 8 A) indicated that glucose was still limiting in cultivation at 24°C and the the growth was still limited by set feeding rate at 24°C.

Similar specific α-glucosidase activities were obtained at both temperatures 24°C and 30°C (Fig. 8 B), in contrast to shake flask experiments, in which higher specific α-glucosidase activity at lower temperature was obtained (Fig. 3). This is because of that in shake flask, the growth rate is a function of temperature, whereas the growth rate was kept independent of temperature in employed fed-batch cultivations. Thus, the difference in growth rate but not in temperatures between 30°C and 24°C was the reason of this difference of specific α-glucosidase activities in shake flask.

Under those conditions, little effect of temperature on aggregation could be observed. Similar α-glucosidase concentrations in soluble and insoluble cell fractions were obtained (not shown). Consequently, similar total α-glucosidase was detected independent of temperatures of 24°C or 30°C (Fig. 8 C).

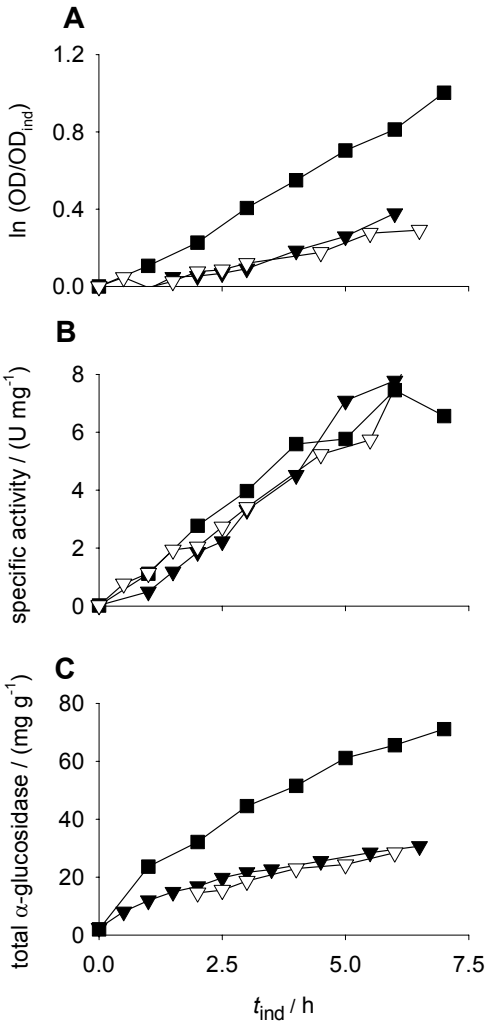

Fig. 8: Lower limit of growth rate and temperature for improvement of production of active α-glucosidase. Cultivation was performed as described in Fig. 7 with μ_{set} = 0.06 h^{-1} (down triangle) and production temperatures of 30°C (solid symbol) and 24°C (open symbol). Data for of μ_{set} = 0.12 h^{-1} (squares) are shown for comparison. Time is given relative to the time of induction. (A) Relative cell density OD/OD$_{ind}$, (B) specific a-glucosidase activity and (C) specific concentration of α-glucosidase in total cell protein.

The data indicated that there is a lower limit of growth rate and temperature for improvement of production of active product. The specific growth rate below 0.12 h^{-1} reduces the amount

of IBs or even prevent IBs formation, thus simplifies the downstream processing, but does not result in higher specific α-glucosidase activity. Furthermore, cultivations at lower specific growth rates reduce the biomasse and thereby interfere with the productivity. The low growth rate may cause additional problems: low specific growth rate caused the induction of some 30 stress-related proteins (Hengge-Aronis, 1996). Low specific growth rate also caused the segregation of population in respiring or nonrespiring cells (Andersson et al., 1996). Little effect on α-glucosidase folding was observed between cultivation temperatures of 24°C or of 30°C, therefore cultivation at temperatures below 30°C is not necessary. Furthermore, low temperature of 15°C has been known to inhibit the translation initation (Farewell and Neidhardt, 1998) or inhibit the chaperonin-assisted folding (Mendoza et al., 1991), thus can also interfere with the productivity.

Fed-batch cultivation with controlled growth rate is an efficient tool for reducing the IBs formation and improving the yield of native protein. Furthermore, higher cell densities can be reached with fed-batch protocol, resulting in higher volumetric activity.

3.1.2 Production of soluble α-glucosidase by *in vivo* reactivation of *inclusion bodies*

3.1.2.1 Reactivation in shake flasks

3.1.2.1.1 Reactivation by arrest of protein synthesis

IBs can disaggregate after arrest of protein synthesis and proteins released from IBs can be active (Carrió et al., 1999; Carrió and Villaverde, 2001). Here, protocols for production of active α-glucosidase are developed based on this observation.

To optimise the *in vivo* reactivation of IBs, the synthesis of α-glucosidase at 37°C in the wildtype strain was arrested two hours after induction with 1 mM IPTG by addition of tetracycline, and the cultures were subsequently incubated at reactivation temperatures of 15°C, 24°C, 30°C or 37° (Fig. 9). Alternatively, the synthesis of α-glucosidase was performed at 42°C and the reactivation at 30°C.

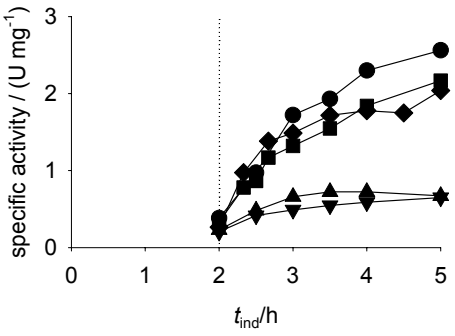

Fig. 9: Optimum temperature profiles for reactivation after arrest of protein synthesis. The cultures were grown at 37°C in complex medium to an OD_{600} of 0.5, were induced with 1 mM IPTG and were kept at 37°C or shifted to 42°C. Two hours after induction, the antibiotic tetracycline was added at a final concentration of 25 µg μL^{-1} to arrest protein synthesis and the temperature was shift from 37°C to 15°C (down triangle), 24°C (diamond), 30°C (square) and 37°C (triangle) or from 42°C to 30°C (circle). Dotted line indicates the time of tetracycline addition. Time is given relative to the time of induction.

Despite *de novo* synthesis of α-glucosidase has stopped, the α-glucosidase activity increased with time. The optimum temperature range for reactivation was 24°C and 30°C (Fig. 9). These temperatures were also optimum for active production (Fig. 3). In this temperature range, the reactivation was very efficient; the specific α-glucosidase activity obtained after five hours (two hours production at 37°C plus three hours reactivation at 24°C) reached more than 2 U mg^{-1} (Fig. 9). Reactivation by isothermal arrest of protein synthesis at 37°C was less successful, giving only half the activity found at 24°C. This could be expected, since the high temperature of 37°C interferes with proper folding also during production. It is, however, remarkable, that some activity is obtained also at this unfavourable temperature. This indicates that the conditions of fast synthesis of the recombinant proteins also contribute to aggregation. A further decrease of the temperature to 15°C was counterproductive and interfered with reactivation (Fig. 9). The reactivation hence seems to be an active process, which might require active metabolism.

The temperature during the formation of the IBs had also an impact on the efficacy of their subsequent reactivation. After production at 42°C, which led to quantitative aggregation of α-glucosidase as at 37°C, 25% higher final reactivation yield was obtained compared to 37°C, reaching 2.5 U mg^{-1} (Fig. 9). When α-glucosidase was produced at 24°C, about 20% of total α-glucosidase was deposited in IBs (Fig. 3), but the hardly any increase of specific activity after tetracycline addition could be seen (Fig. 10). The activity maintained at the similar level

as with unperturbed production without addition of tetracycline (Fig. 10), indicating that with production of α-glucosidase at 24°C the synthesis of active α-glucosidase was essentially completed two hours after induction.

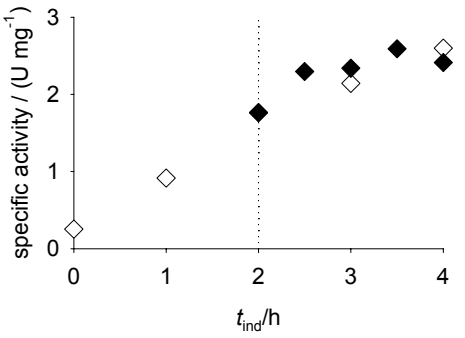

Fig. 10: Isothermal reactivation at 24°C. The culture was grown at 37°C in complex medium to an OD_{600} of 0.5, was induced with 1 mM IPTG and subsequently transferred to 24°C. Two hours after induction, tetracycline was added for arrest of protein synthesis (solid symbol), indicated by dotted line. For comparison, data from a culture of the same strain without addition of tetracycline is shown (open symbol). Time is given relative to the time of induction.

3.1.2.1.2 Reactivation by temperature downshift

In an actual production process, addition of tetracycline causes cell death thus interferes with the productivity. It was tested here whether reactivation can be also initiated by just removing IPTG, which should also cause an arrest of α-glucosidase synthesis without impairing cell growth. After two hours of α-glucosidase production at 37°C, cultures were centrifuged under aseptic condition. The cell pellets were washed twice with sterile complex medium to remove IPTG, then were resuspended in fresh complex medium and incubated further at 24°C.

SDS analysis revealed that no α-glucosidase got lost during washing; the total α-glucosidase concentration was maintained at the same level as before washing (not shown). Reactivation took place in this case (Fig. 11 A); the specific activity increased nearly twofold, but reached a stationary level already about 1.5 hours after resuspension in fresh medium (Fig. 11 A). Dilution of active α-glucosidase by ongoing cell growth prevented a further increase of the specific activity, which was therefore in the end almost 50% lower compared to reactivation by arrest of protein synthesis (Fig. 11 C). The volumetric activity, however, continuously increased after removal of IPTG, resulting in 30% higher volumetric activity after 3 h reactivation than with arrest of protein synthesis (Fig. 11 B). Thus, production of soluble

active α-glucosidase by removal of IPTG was already a clear improvement compared to the initiation of reactivation by arrest of protein synthesis.

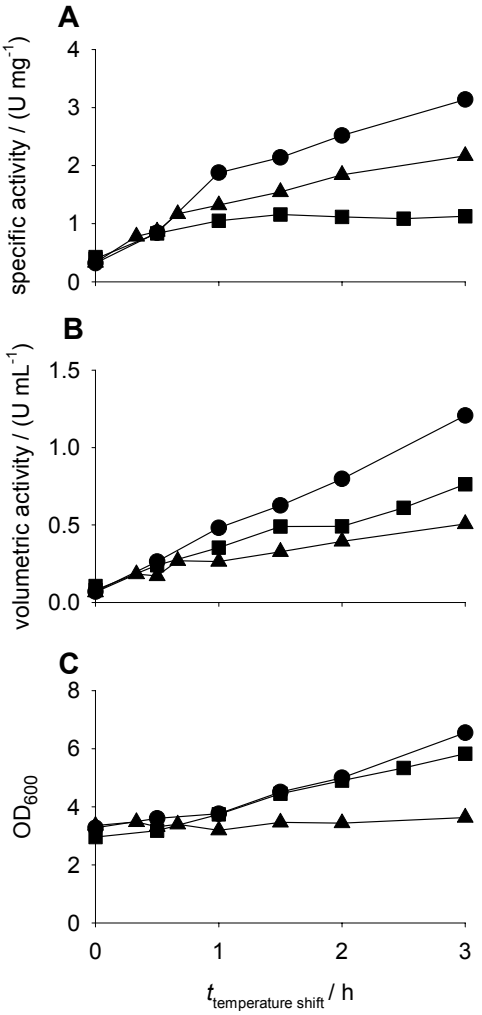

Fig. 11: Production of soluble active α-glucosidase by in vivo reactivation of IBs. The cultures were grown at 37°C in complex medium to an OD_{600} of 0.5 and then were induced with 1 mM IPTG. Two hours later, the reactivation of α-glucosidase from *inclusion bodies* was initiated by a transfer of the cultures to 24°C directly (circle) or with prior arrest of protein synthesis by addition of tetracycline (triangle) or with prior washing twice with fresh medium to remove IPTG (square). (A) Specific activity and (B) volumetric activity of α-glucosidase, (C) optical density OD_{600}. Time is given relative to the time of temperature shift.

As a further alternative, reactivation was started by a temperature downshift without prior addition of tetracycline. The specific α-glucosidase activity rapidly increased within one hour after the temperature downshift and reached three hours after temperature reduction three times higher value than by removing IPTG and was 1.5-fold higher than with prior arrest of

protein synthesis (Fig. 11 A). Additionally, growth monitored by optical density proceeded one hour after the temperature shift, as was seen with removal of IPTG (Fig. 11 C). Altogether, an almost two times higher volumetric activity was obtained than with removal of IPTG (Fig. 11 B). An ongoing synthesis of α-glucosidase at a temperature that is compatible with proper folding of α-glucosidase thus does not interfere with simultaneous reactivation of *inclusion bodies*; rather it contributes to the yield of active α-glucosidase. Thus, this simple scemes has the highest potential for successful production.

3.1.2.1.3 Optimisation of temperature profiles for *in vivo* reactivation

The temperature profile for the production by *in vivo* reactivation was optimised. The cultures were grown at 37°C or 42°C and were shifted to 30°C or 24°C two hours after induction for initiating the reactivation.

The different reactivation temperatures exerted an opposite effect on growth and reactivation – the specific activity obtained at 24°C was higher by the same ratio as the final OD was higher at 30°C (Fig. 12 A, C)– resulting in the same volumetric activities (Fig. 12 D), in accordance with the observations with direct production (Fig. 3). The production at 42°C, however, resulted on highest value of specific α-glucosidase activity, which resulted also in highest volumetric activity (Fig. 12 A, B). Compared to production at 37°C, high production temperature of 42°C resulted in twofold increase of specific α-glucosidase activity.

The specific α-glucosidase activities in case of two hours production plus two hours reactivation at 24°C or 30°C is similar to that in case of four hours direct production at temperature downshift. Thus the active α-glucosidase can (in the reactivation phase) clearly faster accumulate than it did in the direct production. The folding capacity by disaggregation plus production is hence larger than by production alone.

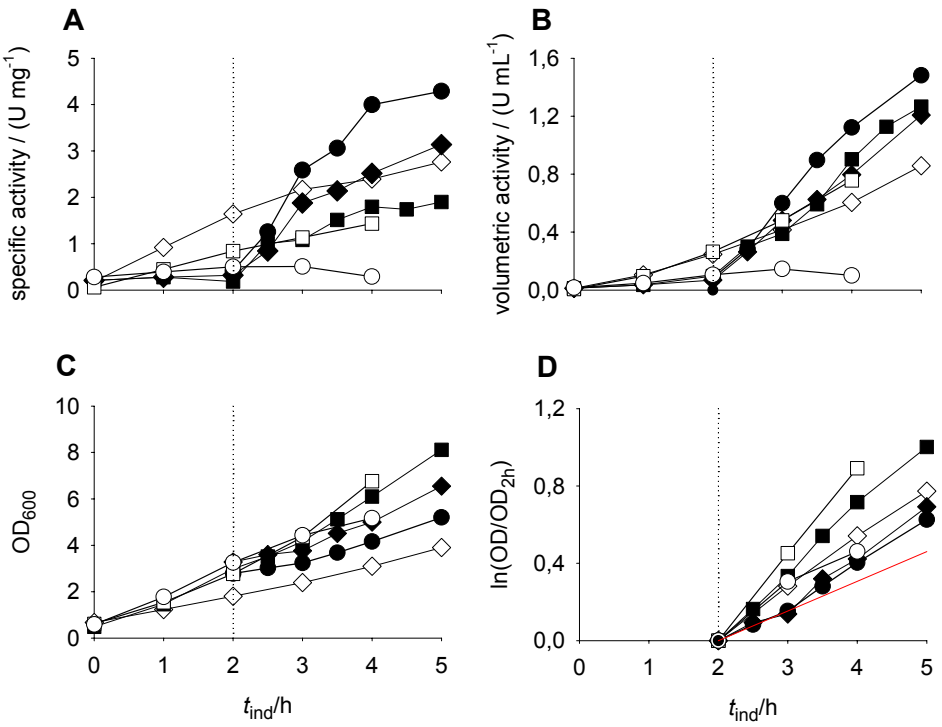

Fig. 12: Production of soluble active α-glucosidase with temperature downshift. The cultures were grown at 37°C in complex medium to an OD_{600} of about 0.5 and then were induced with 1 mM IPTG and kept further at 37°C (◆,■) or 42°C (●). Two hours after induction, cultures were transferred to 30°C (■,●) or 24°C (◆). For comparison, profiles are shown for continuous production (open symbol) at 24°C (◇), 30°C (□) or 42°C (○). (A) Specific activity and (B) volumetric activity of α-glucosidase, (C) optical density OD_{600}, (D) recovery after temperature shift, monitoring by relative OD_{600}; the red line showed the slope of optical density one hour after temperature shift. The dotted line indicates the time of temperature downshift. Time is given relative to the time of induction.

The growth of the culture after the temperature shift was slightly inhibited for about one hour for all cases, specifically when the difference of temperature shift was high (production at 42°C or reactivation at 24°C), as seen from an increase of slope afterwards (Fig. 12 D – red line). The slope increased afterwards to even higher value than in direct production without temperature downshift. Thus growth was completely restored.

As growth at 37°C was faster than at 24°C also concomitant to production of α-glucosidase, the final OD in the reactivation experiments were 30% higher than with permanent production at 24°C (Fig. 12 C), which also resulted in nearly 30% higher volumetric activity three hours after temperature shift (Fig. 12 B).

The specific and volumetric α-glucosidase activities under optimised profiles of temperature downshift, which is 42°C for production and 30°C for reactivation, surpassed the optimum in

direct production already one hour after the temperature downshift, and the margin continued to widen afterwards (Fig. 12 A, B).

3.1.2.1.4 Impact of medium on *in vivo* reactivation

Cultures that had produced α-glucosidase for two hour at 37°C in complex medium or defined medium were transferred to 30°C to initiate the reactivation.

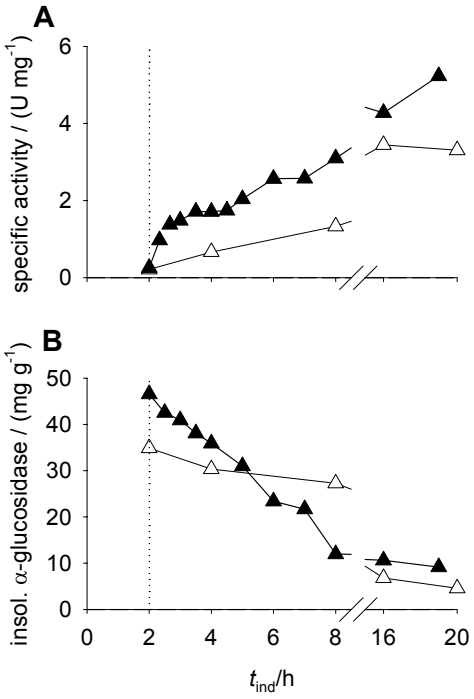

Fig. 13: Impact of medium on in vivo reactivation. The cultures were grown at 37°C in complex (solid symbol) or defined (open symbol) media to an OD_{600} of about 0.5 and then were induced with 1 mM IPTG. Two hours after induction, the cultures were transferred to 30° with prior arrest of protein synthesis by addition of tetracycline. Time is given in hours relative to the time of induction. (**A**) Specific α-glucosidase activity, (**B**) specific concentration of α-glucosidase in the insoluble cell fraction. Dotted line indicates time of tetracycline addition and temperature downshift.

Two hours after arrest of protein synthesis, the specific activity increased from 0.26 U mg^{-1} to 0.54 U mg^{-1} in defined medium compared to 1.8 U mg^{-1} in complex medium. The final yields achieved after over night incubation, however, were similar (Fig. 13 A). Retardation in IBs dissolution in defined medium compared to complex medium was observed; the insoluble α-glucosidase produced on complex medium disaggregated immediately after arrest of protein synthesis, nearly complete already after six hours of reactivation. In contrast, the insoluble α-

glucosidase concentration produced in defined medium decreased only 10 % after the same time of reactivation (Fig. 13 B). The higher specific activity on defined medium (Fig. 13 A) corresponded to complete disaggregation of insoluble α-glucosidase (Fig. 13 B). Thus, it is not only impaired folding or enhanced degradation, which contributed to slow reactivation on defined medium, but mainly the retardation in IB dissolution.

3.1.2.2 Reactivation in bioreactors

3.1.2.2.1 Reactivation of *inclusion bodies* in batch and fed-batch cultivations

In vivo reactivation of α-glucosidase was examined in batch and fed-batch cultivations on defined medium.

In the batch cultivation at 37°C, the cells grew after induction with a maximum specific growth rate of 0.56 h^{-1} (Fig. 14 C). Without induction, the cells grew with maximum growth rate of 0.73 h^{-1} (not shown). Overexpression of recombinant α-glucosidase hence affects the cell growth. In the fed-batch cultivation, the culture growth followed the set point specific growth rate of 0.22 h^{-1} during the two hour production phase (Fig. 14 C). After the temperature downshift to 24°C, the actual specific growth rate was reduced in both cultivations, to 0.09 h^{-1} in fed-batch cultivation and even to 0.05 h^{-1} in batch cultivation (Fig. 14 C). Since the cell grew slower than the set value, glucose accumulated two hours after the temperature shift up to 0.26 g L^{-1}.

The product formation rate in batch cultivation initially was 56 mg g^{-1} h^{-1}, but declined exponentially with a rate constant of 1.21 h^{-1} (corresponding to a half-life of 34 min), resulting in stop of product accumulation two hours after induction. In contrast, the product accumulation in the fed-batch cultivation was initially only half as much as in the batch cultivation, but as it declined with a lower rate constant of 0.17 h^{-1} (half-life four hours), the product accumulation continued to the end of cultivation (four hours after induction) and the specific product concentration surpassed the one obtained in batch cultures from two hours after induction (Fig. 14 A).

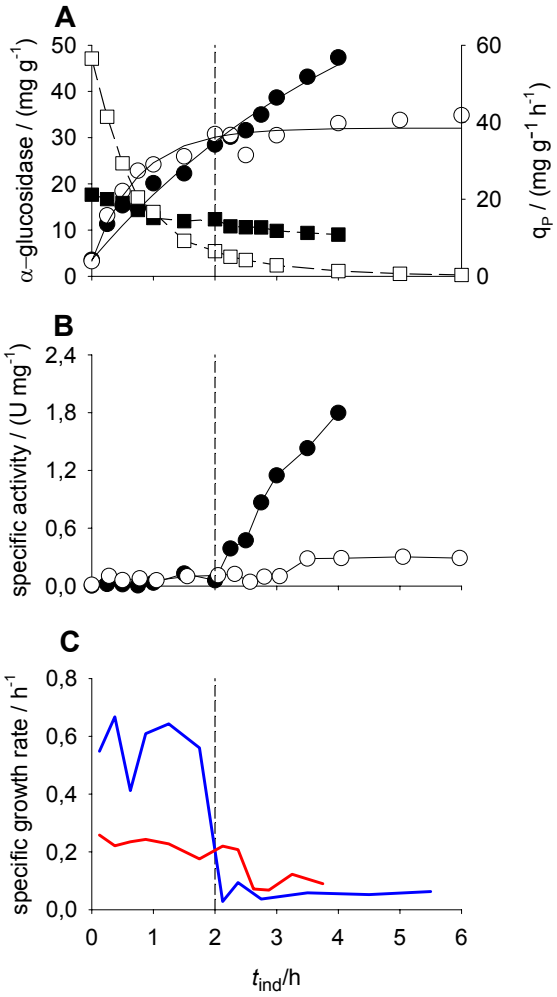

Fig. 14: Production of α-glucosidase in batch and fed-batch cultivation by in vivo reactivation. Batch cultivation (open symbol) performed with an initial glucose concentration of S_0 = 30 g L^{-1}, fed-batch cultivation (solid symbol) with S_0 = 5 g L^{-1} and exponentially increasing feeding rate to keep the growth rate constant at μ_{set} = 0.22 h^{-1}. The cultures were grown at 37°C and were induced with 0.4 mM IPTG four hours after inoculation (batch cultivation) or one hour after feeding start (fed-batch cultivation). Two hours after induction, the temperature was shifted to 24°C to initiate reactivation of inclusion bodies (indicated by dashed line). (**A**) Specific concentration *c* of α-glucosidase, determined from Coomassie-stained gels by densitometry (circles) and specific production formation rate q_p (squares) calculated as $q_p = d(c\,X)/X\,dt$, (**B**) specific α-glucosidase activity, (**C**) specific growth rate calculated from change of cell densities (batch: red; fed-batch: blue line). Time is given relative to the time of induction.

At this time point of identical product concentrations in both cultivations, the temperature was shifted from 37°C to 24°C to initiate the reactivation of α-glucosidase. The accumulation of active α-glucosidase was efficient in fed-batch cultivation. The specific activity increased linearly directly after temperature downshift, reached 1.8 U mg^{-1} two hours after the temperature downshift, giving volumetric activity of 4 U mL^{-1}. The slope of specific activity

in fed-batch cultivation was about 0.9 U mg^{-1} h^{-1}, which was lower than in shake flask experiment on complex medium under the same temperature profile (1.1 U mg^{-1} h^{-1}) and was twofold lower than the highest value in shake flask expreriments. In contrast, the reactivation was not efficient in the batch cultivation, which had higher production formation rate on the first two hours after induction. The specific activity reached only 0.3 U mg^{-1} after the same time of reactivation and did not increase until the end of cultivation (four hours after temperature downshift) (Fig. 14 B). The slope of specific α-glucosidase activity reached only 0.1 U mg^{-1} h^{-1}, which was nine times lower than in fed-batch cultivation. The amount of *inclusion bodies* was similar in both cultivations at time of initiation of reactivation (Fig. 14 A) and thus cannot be the reason for different accumulation of active α-glucosidase in batch and fed-batch cultivations. The stop of net accumulation in batch cultivation two hours after temperature downshift but not in fed-batch indicated that the new synthesised α-glucosidase was less in the batch than fed-batch cultivation. The slow reactivation in the batch culture was confirmed in shake flasks experiments on defined medium. The specific activity did not increase within 4 h reactivation without arrest of protein synthesis (not shown) and even after arrest of protein synthesis (Fig. 13 A). Thus, the slow reactivation is not due to special condition in the reactor (such as maintenance of pH value and high oxygen saturation). Since the reactivation took place in batch culture on complex medium, the slow reactivation in batch culture might connected to specific properties of defined medium.

3.1.2.2.2 Optimum feeding rate and temperature for production of soluble α-glucosidase

To test how the growth rate influences the *in vivo* reactivation, cultivations with fast growth rate of 0.18 h^{-1} and slow growth rate of 0.12 h^{-1} were performed (Fig. 15). The temperature was shifted to 30°C at four hours after induction.

In the first hour after induction, the actual growth rate of the cultures at high set growth rate followed the set point μ_{set} (Fig. 15 C). The growth rate, however, progressively slowed down one hour after induction and the slope of cell densities became similar to that at slow growth rate (Fig. 15 C). At slow growth rate of 0.12 h^{-1}, however, the actual growth rate followed the set point growth rate at least to the end of cultivation (7 h after induction) (Fig. 15 C). As the reactivation temperature was the same in both cultivations, the growth inhibition in culture with high μ_{set} was a consequence of fast feeding rate. The growth rate hence was affected at high μ_{set} but not at low μ_{set}. The stress caused by overexpression α-glucosidase in the fed-

batch culture with constant feeding rate, enabling a specific growth rate of 0.24 h^{-1} at the time of induction is characterised by induction of stress proteins, growth inhibition and decrease in the glucose uptake rate (Lin, 2000; Schweder et al., 2000). The data indicated that low reactivation temperature of 24°C might cause the slow growth after temperature downshift in the cultivations shown in Fig. 14, but stress contributed to this growth inhibition.

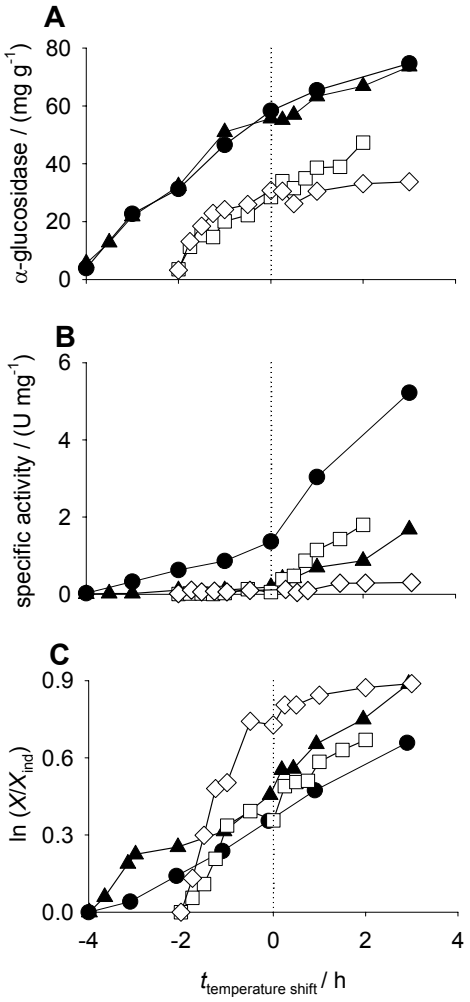

Fig. 15: Effect of feeding rate and temperature on in vivo reactivation of α-glucosidase. Inclusion bodies of α-glucosidase were produced at 37°C in fed-batch cultivations with feeding rates of μ_{set} = 0.18 h^{-1} (circle) or 0.12 h^{-1} (triangle). Reactivation was initiated 4 h after induction by temperature shift to 30°C. For comparision, data from Fig. 14 are shown with reactivation at 24°C (open symbols) for fed-batch with μ_{set} = 0.22 h^{-1} (square) and batch (diamond). (**A**) Specific total α-glucosidase concentration, (**B**) specific α-glucosidase activity, (**C**) relative cell density from cell dry mass determination. Time is given in hours relative to the time of temperature shift.

The product accumulation in production with temperature shift at four hours after induction was similar at μ_{set}= 0.18 h^{-1} and μ_{set}= 0.12 h^{-1} and followed the same slope as product accumulation in fed-batch cultivation at μ_{set}= 0.22 h^{-1} with temperature downshift at two

hours after induction (Fig. 15 A); no such saturation of product accumulation as in batch cultivation was observed. Feeding rate and temperature in the range studied had only little effect on the product accumulation during reactivation, as during direct production (Fig. 7 and 8).

With fast feeding rates, similar slopes of specific α-glucosidase activity versus time were obtained independent of reactivation temperature (Fig. 7 B). Thus, the temperature of 30°C or 24°C has similar effect on α-glucosidase folding after temperature downshift.

A pronounced effect on specific α-glucosidase activity was observed at low μ_{set} = 0.12 h^{-1}. The specific α-glucosidase increased after the temperature downshift with a slope of 1.3 U mg^{-1} h^{-1}, which higher than the 0.9 U mg^{-1} h^{-1} obtained in the cultivation with high μ_{set} = 0.18 h^{-1} and also resulted in 50% higher final yield. The lower growth rate correlated with faster accumulation of active α-glucosidase after temperature shift, which was similar to direct production. Thus, the slow growth rate favours the α-glucosidase folding both in direct production and production with temperature downshift.

3.1.2.2.3 Optimum time of temperature shift

α-Glucosidase IBs were produced in fed-batch cultivations with μ_{set}= 0.12 h^{-1} for optimisation of the time point of temperature downshift. At 0 h, 2 h, 4 and 7 h after induction, temperature was shifted from 37°C to 30°C.

The growth was not affected by prolonged production before temperature downshift; similar profiles of cell densities were obtained (Fig. 16 A).

Specific activities increased directly after temperature downshift in all cases and reached stationary level at about 10 hours after induction (Fig. 16 B). The slopes of specific activity were similar independent of time of temperature shift (Fig. 16 B). As the stationary levels were reached at the same time in all cultivations, the stationary level of specific activity was hence lower with prolonged production (Fig. 16 B). Production times prolonged to four or seven hours resulted in 25% and 60% lower final specific α-glucosidase activity, respectively, compared to the cultivation with direct temperature dowshift at 0 h.

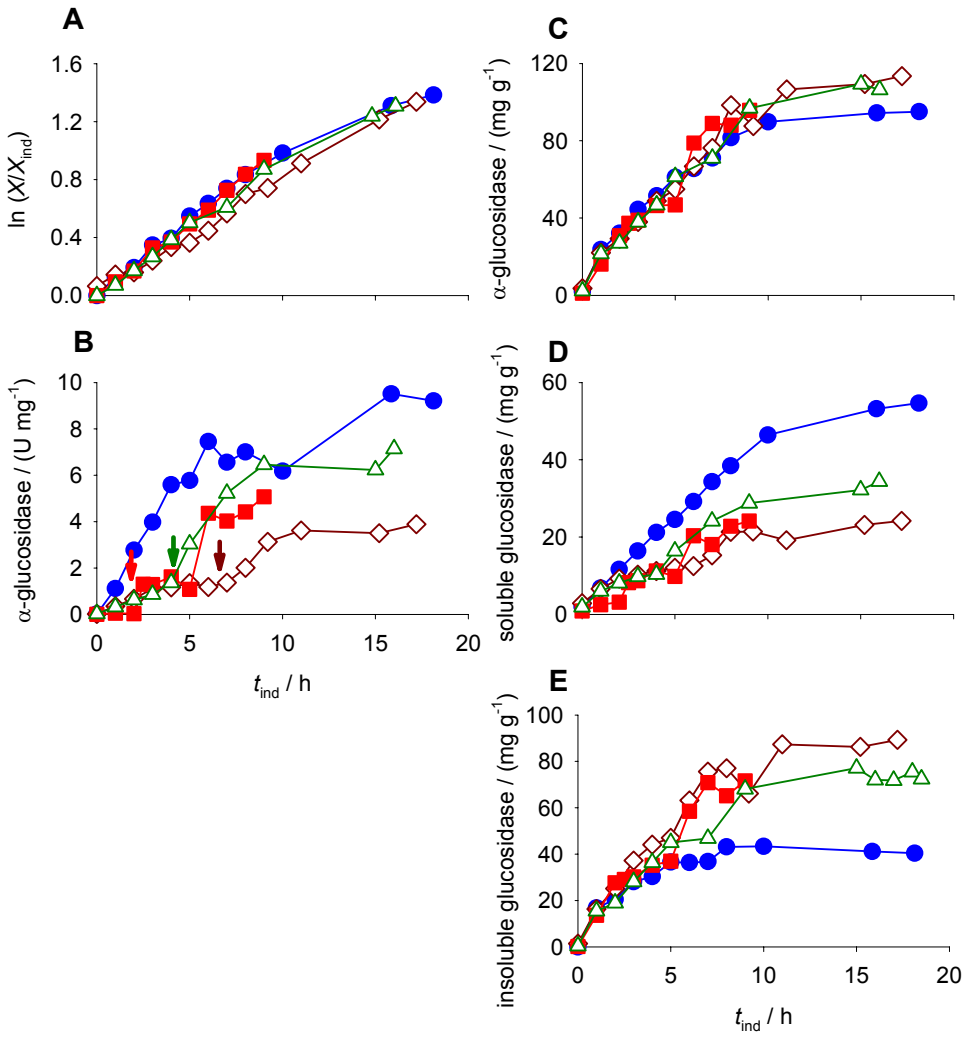

Fig. 16: Influence of production time before temperature downshift on production of active α-glucosidase. The cultures were grown at 37°C with a feeding rate $F(\mu_{set})$ = 0.12 h^{-1}. The temperature shift to 30°C was performed after 0 h (circle), 2 h (square), 4 h (triangle) or 7 h induction (diamond). (**A**) Cell density from cell dry mass determination, (**B**) specific α-glucosidase activity; arrows indicate time of temperature downshift; the colors of the arrows correspond to the colours of the graph; specific concentrations of α-glucosidase in (**C**) total, (**D**) soluble and (**E**) insoluble cellular fractions. Time is given relative to the time of induction.

The prolonged production time had only little effect on accumulation of total α-glucosidase; the profiles of the specific concentrations of α-glucosidase were very similar, althought the final specific concentration of α-glucosidase was slightly lower with direct temperature shift at 0 h (Fig. 16 C). The accumulation of total α-glucosidase stopped ten hours after induction in all cultivations regardless of the time of temperature shift.

The concentration of soluble product was lower with longer production time, as was seen for specific α-glucosidase activity (Fig. 16 D). More insoluble α-glucosidase was produced by prolonged production time (Fig. 16 E), which principally corresponded to more material for IBs resolution. The similar slope of soluble concentration of α-glucosidase or of specific α-glucosidase activity indicated that the reactivation in fed-batch cultivation under these conditions was not limited by the amount of insoluble α-glucosidase. Furthermore, the arrest of net accumulation of α-glucosidase 10 h after induction in all cultivations (Fig. 16 C) led to lower stationary level of soluble product and specific activity in all cultivation with longer production time. Althought the temperature downshift did not result on higher final yield of specific α-glucosidase activity, the concentration of insoluble α-glucosidase showed a lag phase of two hours after the temperature downshift in all cases, before it increased again (Fig. 16 E), whereas the concentration of soluble α-glucosidase increased immediately. It seems that the temperature reduction can prevent further aggregation within two hours.

3.2 Impact of IbpA/IbpB on inclusion bodies metabolism

3.2.1 Construction of the plasmid pKK177-3/GLUCP1_ibpAB

To study the role of IbpA and IbpB in the chaperone network, the effect of IbpA/IbpB availability on α-glucosidase accumulation as well as on metabolism of α-glucosidase IBs were investigated. For this purpose, a plasmid for coexpression of α-glucosidase and *ibpAB* was constructed.

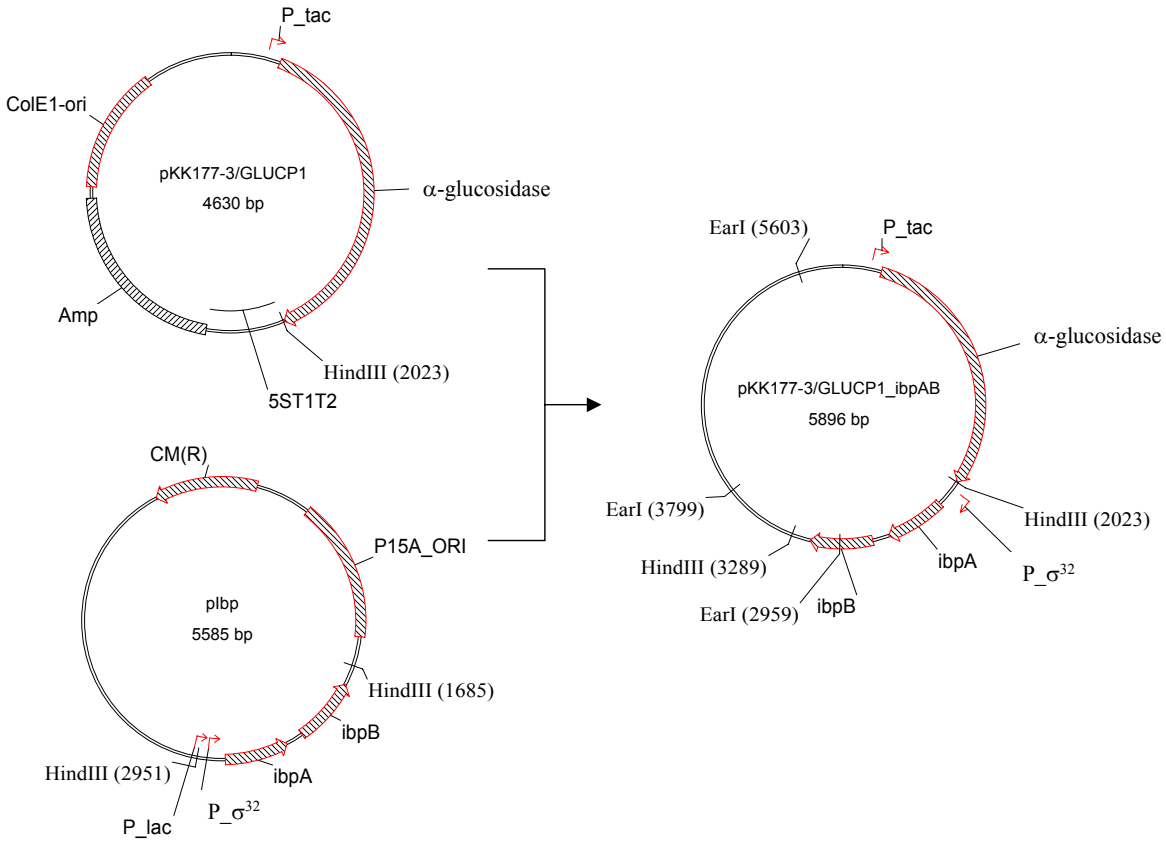

Fig. 17: Construction of the plasmid pKK177-3/glucP1 _ibpAB. The plasmids pKK177-3/GLUCP1 and pIbp were digested with *Hin*dIII. The *ibpAB* fragment of pIbp was then ligated with pKK177-3/GLUCP1. The proper orientation of *ibpAB* fragment was tested with *Ear*I digestion.

The plasmid pIbp has been constructed by insertion of the *ibpAB* operon including its own promoter as a *Hin*dIII fragment in pTG10 (Thomas and Baneyx, 1998). The plasmid pKK177-3/GLUCP1 has a single *Hin*dIII digestion site, which is situated immediately downstream of the α-glucosidase gene and upstream of the terminator $5ST_1T_2$ (Kopetzki et

al., 1989). In order to get a plasmid for overexpression of *ibpAB* from its heat-shock promoter and as a tricistronic operon with α-glucosidase from the IPTG regulated *tac* promoter, the *ibpAB* fragment was cut from the plasmid pIbp and inserted into pKK177-3/GLUCP1 (Fig. 17). The new plasmid was named pKK177-3/GLUCP1_ibpAB.

From 16 clones obtained after transformation of the wildtype strain MC4100 with the ligated plasmid, there were 5 clones with *ibpAB* insert, which were differentiated from clones without *ibpAB* insert by the presence of the second band with a size of about 1266 bp after digestion of the extracted plasmids with *Hin*dIII. Plasmids from the five positives clones were digested with the restriction enzyme *Ear*I to check the orientation of the insert (Fig. 18). There are three restriction sides for *Ear*I in pKK177-3/GLUCP1_ibpAB, one of which situated inside of the *ibpAB* fragment (Fig. 17). If the fragment is placed in the right direction, there must be three fragments after digestion with sizes of 840 bp, 1804 bp and 3252 bp; otherwise there must be fragments with sizes of 1446 bp, 1804 bp and 2646 bp.

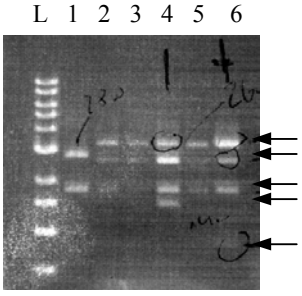

Fig. 18: Test for proper orientation of the *ibpAB* insert. Agarose gel of five plasmids after digestion with *Ear*I. L: 1 kb ladder with size of 10, 8, 6, 5, 4, 3, 2, 1.5, 1.0 and 0.5 kb (from the top); Lane 1: digestion of plasmid pKK177-3/GLUCP1; Lane 2 to lane 6: digestion of plasmids from 5 positives clones. Arrows indicate the position of fragments with (from top) sizes of about 3250 bp, 2650 bp, 1804 bp, 1446 bp and 840 bp.

The clone 6 was the clone with proper orientation of the insert, since there was a weak band with size of 800-900 bp and there was no band with size of 1400 bp, which was seen in clone 4 (Fig. 18). The appearance of a fourth band in these two clones was a result of uncompleted digestion, which was 1446 bp + 1804 bp = 3250 bp (the first arrow from top) in case with wrong direction (clone 4) and 1804 bp + 840 bp = 2644 bp (the second one) in case of the right one (clone 6). The plasmid from this clone 6 was transformed in strain MC4100 to get strain coproduced IbpAB and α-glucosidase upon induction with IPTG.

3.2.2 Cellular response to coproduction of IbpA/IbpB with α-glucosidase

3.2.2.1 Levels of IbpA/IbpB at various temperatures

The strain transformed with plasmid pKK177-3/GLUCP1_ibpAB (Fig. 17) was served as a strain with high level of IbpA/IbpB. The deletion strain JGT17 MC4100 *ΔibpAB* (Thomas and Baneyx, 1998) and wildtype strain MC4100 were transformed with plasmid pKK177-3/GLUCP1, which overproduces solely α-glucosidase and served as strains with low and wildtype level of IbpA/IbpB, respectively. The three strains were grown in complex medium at various temperatures. The levels of IbpA or IbpB two hours after induction with 1 mM IPTG were estimated from Coomassie-stained two-dimensional gels (Table 8).

Table 8. Level of IbpA/IbpB at various temperatures two hours after induction

	Temperature	*ibpAB*$^{++}$	wt	Ratio *ibpAB*$^{++}$ to wt[a]
IbpA	42°C	84[b]	3.3	25
	37°C	50	5.0	10
	30°C	19	1.6	12
IbpB	42°C	12	1	12
	37°C	7	1.4	5
	30°C	3	0.7	4
Ratio IbpA to IbpB	42°C	7	3	
	37°C	7	4	
	30°C	6	2	

[a] *ibpAB*$^{++}$: wildtype strain MC4100: pKK177-3/GLUCP1_ibpAB: pUBS520 with overexpression of *ibpAB* from the plasmid, wt: wildtype strain MC4100: pKK177-3/GLUCP1: pUBS520 without overexpression of *ibpAB*

[b] The levels of IbpA/IbpB were quantified by densitometry of Coomassie-stained two-dimensional gels, using reference spots, marked by cirle (cf. Fig. 25). Values were normalised to the value obtained for IbpB at 42°C without *ibpAB* overexpression.

The chromosomal *ibpAB* was already induced during α-glucosidase production at 30°C in the wildtype strain without *ibpAB* overexpression, whereas no IbpA/IbpB could be detected at this temperature in wildtype strain without α-glucosidase production, indicating the induction of IbpA/IbpB by unfolded α-glucosidase. Unfolded proteins are known to enhance the transcription driven by the heat-shock transcription factor σ^{32} (Bukau, 1993), and the *ibpAB*

operon is under control of σ^{32} (Allen et al., 1992). In fed-batch cultures of *E. coli* strain RB791 producing α-glucosidase at 35°C on glucose mineral salt medium, IbpA and IbpB were also detected in the insoluble cell fraction by two-dimensional gel electrophoresis (Jürgen et al., 2000).

Production of α-glucosidase in the wildtype strain at higher temperatures resulted in a moderate two to three fold increases of the IbpA and IbpB levels (Table 8). With overexpression of *ibpAB* from the plasmid, the levels of IbpA or IbpB at 30°C and 37°C were only 12 or 4 times higher than without overexpression of *ibpAB* respectively, and became 25 or 12 times higher at 42°C (Table 8). Whereas with *ibpAB* overexpression the levels of IbpA or IbpB were about 1.7-fold higher at 42°C than at 37°C, the levels of IbpA or IbpB were similar at both temperatures without *ibpAB* overexpression (Table 8).

The similarity of the levels of IbpA or IbpB at 37°C and 42°C without *ibpAB* overexpression indicated that the expression of *ibpAB* operon by unfolded α-glucosidase at 37°C was already completed and was not induced further by heat-shock. This observation collaborated with the analog observation, in which the expression of *ibpAB* and other heat-shock proteins are completely induced by heat-shock and not induced further by unfolded proteins. The further increase of IbpA/IbpB levels with *ibpAB* overexpression might be due to IPTG *tac*-regulated as well as heat-shock promoters on the plasmid (Fig. 17).

At all temperatures, the IbpA concentrations were 2-3 times or 6-8 times, respectively, higher than the IbpB concentrations with or without *ibpAB* overexpression. In contrast, in natural system, higher mRNAs level of *ibpA* than *ibpB* upon heat-shock was found (Chuang et al., 1993). In previously reported data, a higher synthese rate of IbpB than IbpA was found in cells producing human basic fibroblast growth factor under the control of a temperature-regulated promoter (Hoffmann and Rinas, 2000) or similar levels of IbpA and IbpB were obtained (Allen et al., 1992). The ratio of IbpA to IbpB might depend on the property of the recombinant proteins. Higher ratios of IbpA to IbpB may result from more frequent translation of IbpA-part of *ibpAB*- mRNA under these conditions. Furthermore, the higher ratio of IbpA to IbpB with *ibpAB* overexpression than without *ibpAB* overexpression suggests, that the translation inside the tricistronic operon of the third gene (*ibpB*) was worse than from the second gene (*ibpA*).

3.2.2.2 Physical association of IbpA/IbpB with α-glucosidase

The physical association of IbpA/IbpB with recombinant α-glucosidase in IBs was studied. The cells without or with *ibpAB* overexpression were used to produce α-glucosidase at 42°C for two hours. Purified IBs were prepared from cell pellets as described in Materials and Methods.

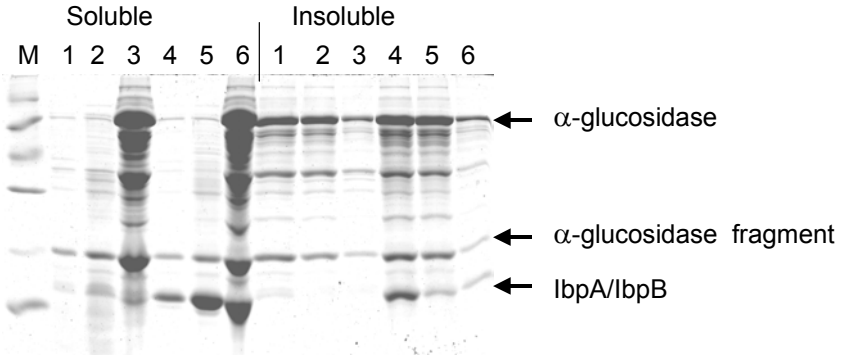

Fig. 19: Association of IbpA/IbpB with α-glucosidase IBs. Coomassie-stained SDS-gel of soluble and insoluble cellular fractions after extracting purified IBs with urea at concentrations of 2 M (lane 1, 4), 4 M (lane 2, 5) and 8 M (lane 3, 6) for 1 h at room temperature. Lanes 1, 2, 3: wildtype strain, lane 4, 5, 6: with *ibpAB* overexpression; lane M: molecular weight markers of (from top) 97.4, 66.2, 45, 31, 21.5 and 14.4 kDa respectively. The positions of α-glucosidase, α-glucosidase fragment due to second translation start site at position of 409 and IbpA/IbpB are indicated. The position of the α-glucosidase fragment was identified by comparison of its molecular mass with data from two-dimensional gels, cf. Fig. 25.

Extraction of insoluble proteins from purified IBs with 1% Triton X-100 three successive times (every time 10 min) resulted in solubilisation of only very small amount of α-glucosidase. No IbpA or IbpB could be detected on the Coomassie-stained SDS gel of the soluble cell fraction (not shown). After incubation for one hour at room temperature, IbpA and IbpB were partially dissolved with 2 M urea and almost completely extracted at a urea concentration of 4 M. α-Glucosidase, however, dissolved not up to at a urea concentration of 8 M (Fig. 19). This suggests that there is no tight contact between α-glucosidase and IbpA/IbpB. The full length α-glucosidase was even not dissolved at urea concentration of 6.7 M (data not shown). In contrast, protein human renin dissolves at the same urea concentration of 4 M as IbpA/IbpB, from which a tight association of recombinant protein with IbpA/IbpB was inferred (Allen et al., 1992). It is not clear, whether the association of IbpA/IbpB with human renin differed from association of IbpA/IbpB with α-glucosidase, or human renin dissolved only by chance also at 4 M. The dissolved amounts of α-glucosidase in wildtyp

strain with and without *ibpAB* overexpression were the same at different urea concentrations, indicating that IbpA/IbpB do not influence the dissolution of insoluble α-glucosidase.

3.2.2.3 Effect of IbpA/IbpB on growth and α-glucosidase accumulation

Without production of a recombinant protein, *ibpAB* deletion or overexpression affects cell growth only after challenge with conditions that are characterised by extensive aggregation of cellular proteins, such as extreme temperatures (Thomas and Baneyx, 1998; Kitawaga et al., 2000; Kuczyńska-Wiśnik et al., 2002).

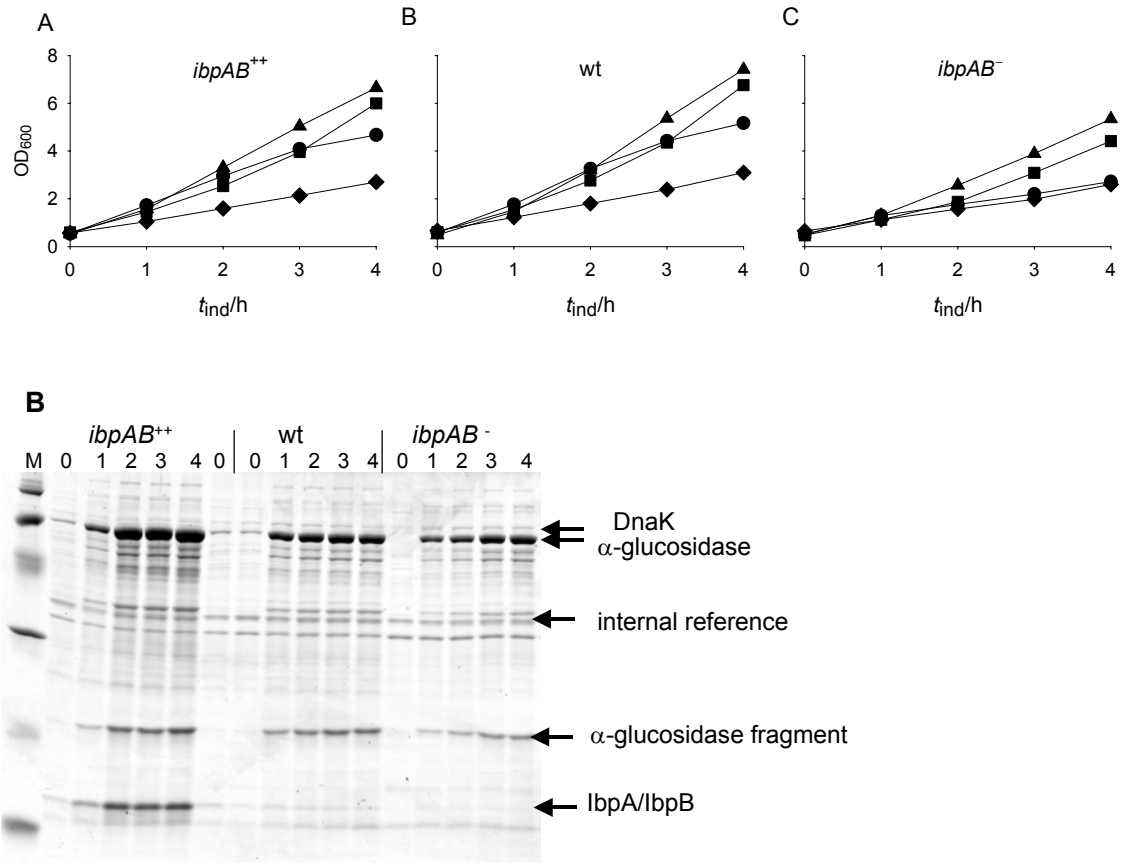

Fig. 20: Influence of IbpA/IbpB levels on growth and α-glucosidase accumulation. Cultures were grown at 37°C on complex medium to an OD600 of 0.5, induced with 1 mM IPTG and transferred to 24°C (diamant), 30°C (square), 37°C (triangle) and 42°C (circle), growth during the production phase was monitored by optical density OD600 in the strains (A) with overexpression of ibpAB, (B) without overexpression of ibpAB and (C) the ibpAB deletion strain. (B) Coomassie-stained SDS-PAGE of the insoluble cell fractions, samples were taken from the cultivation at 42°C. Genotypes and times relative to the time of induction are indicated above the lanes. The positions of DnaK, α-glucosidase, and reference proteins (cf. text), the α-glucosidase fragment and IbpA/IbpB are indicated; lane M: molecular weight markers.

Overproduction of aggregation-prone α-glucosidase mimics the permanently impaired protein folding, leading to marked reduction in culture growth of the *ibpAB* deletion strain already at 30°C (Fig. 20), whereas there was no significant difference in the specific growth rate at low temperatures, e. g. 24°C (Fig. 20) or within the investigated temperature range from 24°C to 42°C without induction of α-glucosidase synthesis (not shown). The wildtype levels of IbpA/IbpB are sufficient to prevent harmful effects to the cells, as the growth rates were not higher with overexpression of *ibpAB*. The slightly slower growth with *ibpAB* overexpression is probably due to the metabolic burden of additional recombinant protein synthesis.

Table 9. Effect of temperature and IbpA/IbpB levels on α-glucosidase concentrations and activities obtained four hours after induction

	genotyp	24°C	30°C	37°C	42°C
Total a-glucosidase/ (mg g^{-1}) [a]	*ibpAB*$^{++}$	38±5	52±1	66±11	47±11
	wt	39±2	47±5	56±16	38±7
	ibpAB^{-}	36±4	43±2	49±10	28±4
Change [b]		- 8%	± 10%	± 15%	± 25%
Vol. activity/ (U mL^{-1}) [a]	*ibpAB*$^{++}$	0.56 ±0.17	0.80 ±0.29	0.13 ±0.04	0.15 ±0.01
	wt	0.61 ±0.14	0.76 ±0.26	0.10 ±0.02	0.10 ±0.01
	ibpAB^{-}	0.55 ±0.02	0.74 ±0.20	0.11 ±0.04	0.10 ±0.02

[a] Mean values and 95% confidence intervals from at least three independent experiments. The variance between experiments is mainly caused by different times needed depending on the volume of the cultures to reach the set temperature after induction.

[b] percentage difference of α-glucosidase concentration in modified strain relative to wildtype strain; positive for strain with *ibpAB* overexpression, negative for *ibpAB* deletion strain. (No significant changes for strain with *ibpAB* overexpression at 24°C.)

The effect of IbpA/IbpB levels on α-glucosidase accumulation was evaluated. Some leaky α-glucosidase production was observed before induction in wildtype strain with and without *ibpAB* overexpression, but not in the *ibpAB* deletion strain (Fig. 20 D). An effect on the α-glucosidase levels was found at 37°C and 42°C (Table 9). Here, the amount of α-glucosidase obtained correlated with IbpA/IbpB availability, giving 15-25% more product with overexpression and about the same margin less product in the deletion strain (Table 9). The

plasmid contents of three strains during production at 42°C were checked (Fig. 21). Comparable plasmid levels were found in the three strains. During the production, the plasmid contents remained constant; in the deletion strain, the plasmid contents might even increase. Thus, lower α-glucosidase level in the deletion strain cannot be attributed to gene doses effects.

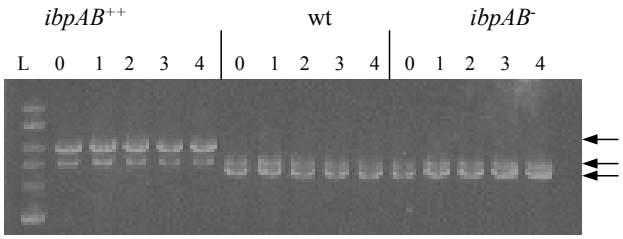

Fig. 21: Plasmid stability in three strains producing α-glucosidase at 42°C. Plasmids were extracted from the same amount of biomass (8/OD mL) and were digested with the restriction enzyme *Bam*HI at 37°C for two hours and separated on 0.8% agarose gel. Genotypes and times after induction in hours are given above the lanes. Arrows indicate the positions of pKK177-3/GLUCP1_ibpAB, pUBS520 and pKK177-3/GLUCP1 (from top to the bottom). Lane L: 1kb ladder.

At temperatures of 24°C, *ibpAB* overexpression had no effect on α-glucosidase levels, whereas a modest reduction was observed in the deletion strain (Table 9).

The α-glucosidase activities and levels of soluble α-glucosidase varied not significantly between the strains (Table 9 and not shown). The insoluble product accumulated to higher level with higher IbpA/IbpB levels at 37°C (not shown) and at 42°C (Fig. 20 D). Thus, the amount of α-glucosidase increased by IbpA/IbpB seems to be migrated in the insoluble cell fraction. Overepression of *ibpAB* was also found to stabilise aggregates in insoluble cellular fraction at 30°C and resulted on higher amount of aggregated at 45°C than without *ibpAB* overexpression (Kuczyńska-Wiśnik et al., 2002).

3.2.3 Impact of IbpA/IbpB levels on the heat-shock response

The accumulation of misfolded proteins induces the heat-shock response already at low temperatures (Parsell and Sauer, 1989). This response can be easily monitored by the increase of the intensity of the DnaK band in SDS-PAGE. The induction of the heat-shock response, as reflected by the level of DnaK in the soluble cell fraction, was unaffected by deletion of *ibpAB* (Fig. 22 A), despite the negative effect of the deletion on cell growth. In the strain with

ibpAB overexpression, however, a marked attenuation of the intensification of the DnaK band in the soluble cell fraction was observed at 42°C (Fig. 22 A) and 37°C (not shown).

In the insoluble fraction, DnaK/DnaJ are found after heat aggregation (Kucharczyk et al., 1991) and in IBs (Carrió and Villaverde, 2002). They are supposed to be trapped there when trying to prevent the aggregation of IBs proteins. With α-glucosidase production, almost two times more DnaK was detected in the *ibpAB* deletion mutant than in the wildtype strain, and with *ibpAB* overexpression, the DnaK concentration was two times lower (Fig. 20 D and Fig. 22 B). α-Glucosidase production causes the additional stress to the cell with *ibpAB* deletion as the growth inhibition was observed at 30°C in cell overproduced α-glucosidase, which otherwise can be only observed at 50°C in cells without α –glucosidase production. Also the twofold lower level of DnaK in *ibpAB* deletion strain was observed in cells overproduced α -glucosidase already after two hours production at 37°C but only can be observed in insoluble cellular fraction of cells without α -glucosidase after four hours incubation at 50°C (Kuczyńska-Wiśnik et al., 2002).

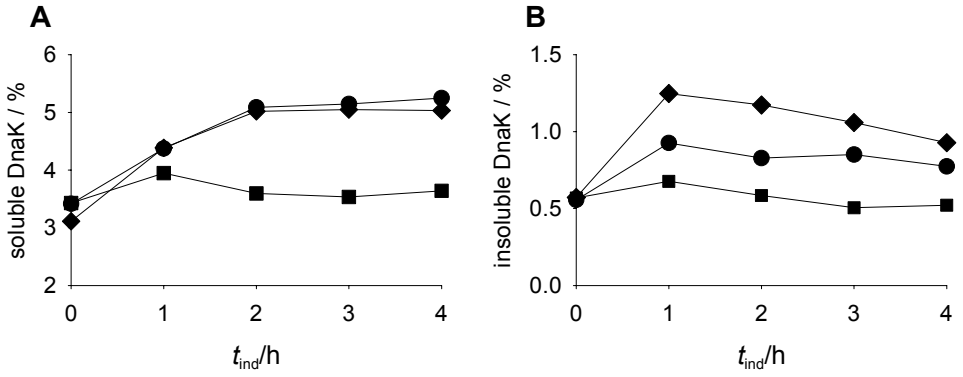

Fig. 22: Effect of IbpA/IbpB availability on induction of the heat-shock response during α-glucosidase production at 42°C. Concentration of DnaK (in % total cellular protein) in the (A) soluble and (B) insoluble cell fraction of the wildtype strains with *ibpAB* overexpression (square) or without *ibpAB* overexpression (circle) or the *ibpAB* deletion strain (diamant), was estimated by densitometry of Coomassie-stained SDS-PAGE gels. Time is given relative to the time of induction.

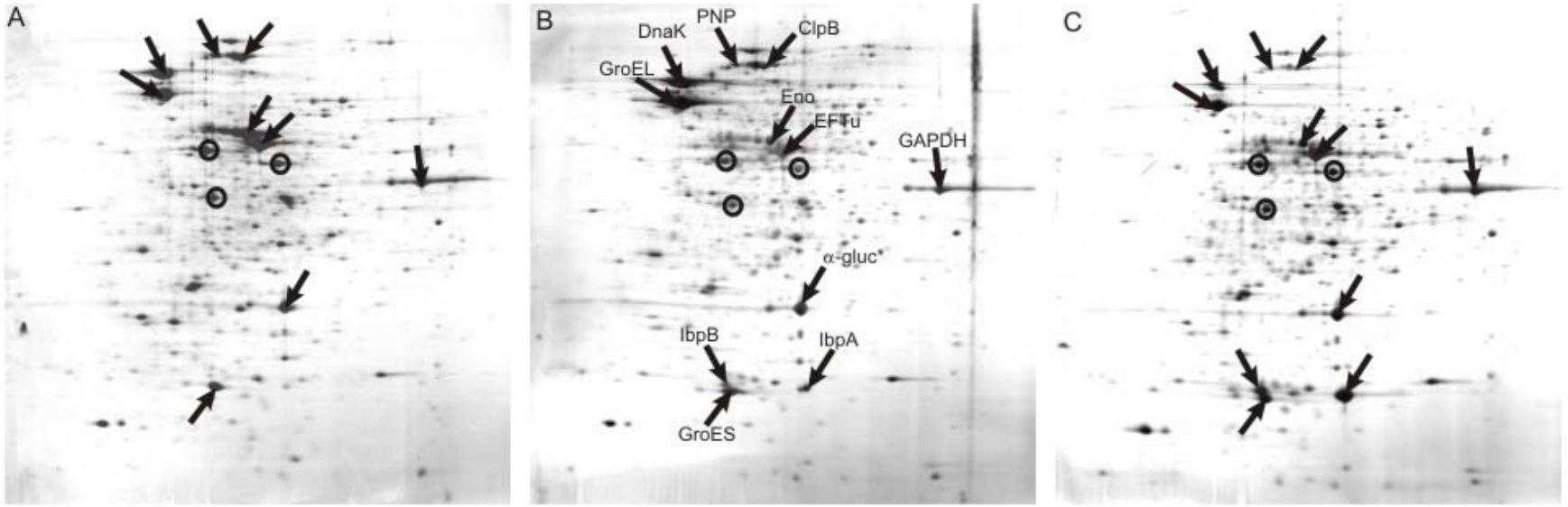

Fig. 23 a: Effect of IbpA/IbpB availability on the proteome composition after two hours of α-glucosidase production at 42°C. After growth at 37°C to OD_{600} of 0.5, cultures were induced with 1 mM IPTG, and transferred to 42°C. Two hours later, cells were harvested, solubilised in 8 M urea and the proteins were separated on two-dimensional gels (the concentration of urea is asumedly diluted by 8/OD amount of cells pellets and is therefore lower than 8 M). Silver-stained two-dimensional gels of (A) *ibpAB* deletion mutant, (B) wildtype strain without or (C) with *ibpAB* overexpression, . References spots are indicated by circles. Positions of selected proteins are indicated. Major heat shock proteins DnaK, GroES, GroEL ; PNP: protein polynucleotide phosphorylase, GAPDH: glyceraldehyde-3-phosphate dehydrogenase, and EF-Tu: thermo-unstable elongation factor were identified by comparison of the spot position with E. coli 2D-database (Wilkins et al., 1998). ClpB: ClpB identified by N-terminal sequencing (RLDRLTN), Eno: enolase identified by N-terminal sequencing (SKIVKII), α-glucosidase fragment due to a second translation start site at position 409, identified by N-terminal sequencing (MKNNYEIIKK). α-gluc* indicates a fragment of α-glucosidase; full length α-glucosidase is not resolved from IBs under conditions used (cf. Materials and Methods).

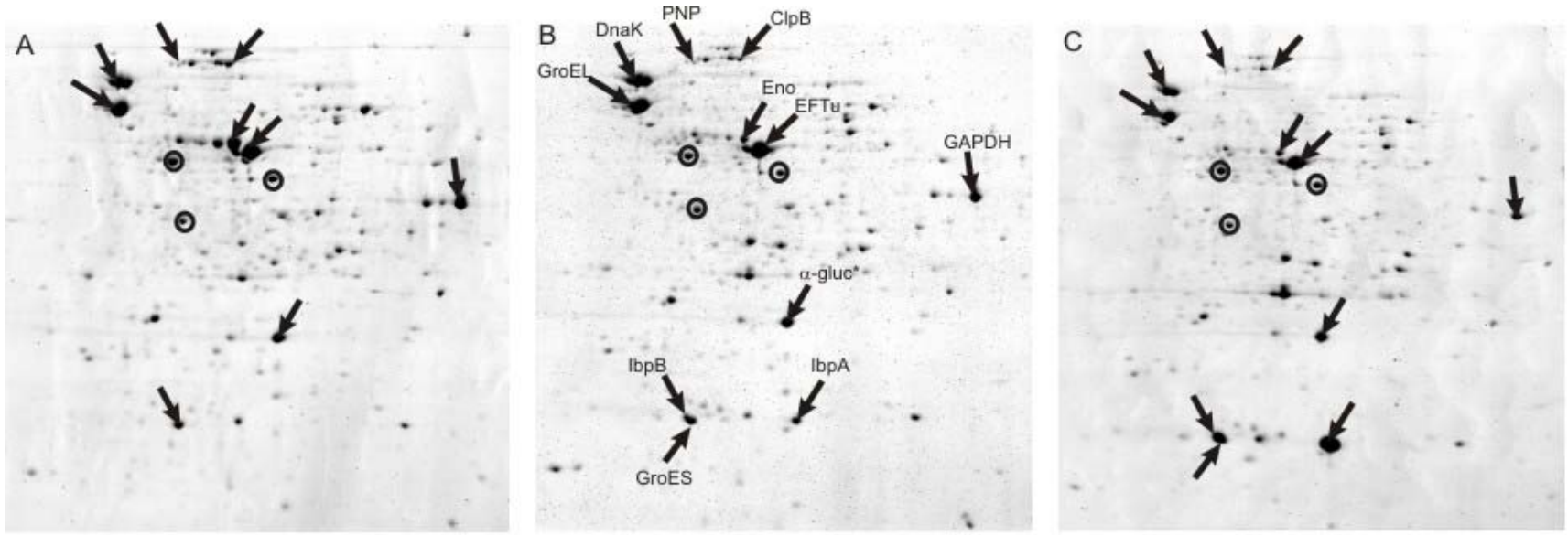

Fig. 23 b (continued): Effect of IbpA/IbpB availability on the proteome composition after two hours of α-glucosidase production at 37°C. Coomassie-stained two-dimensional gels.

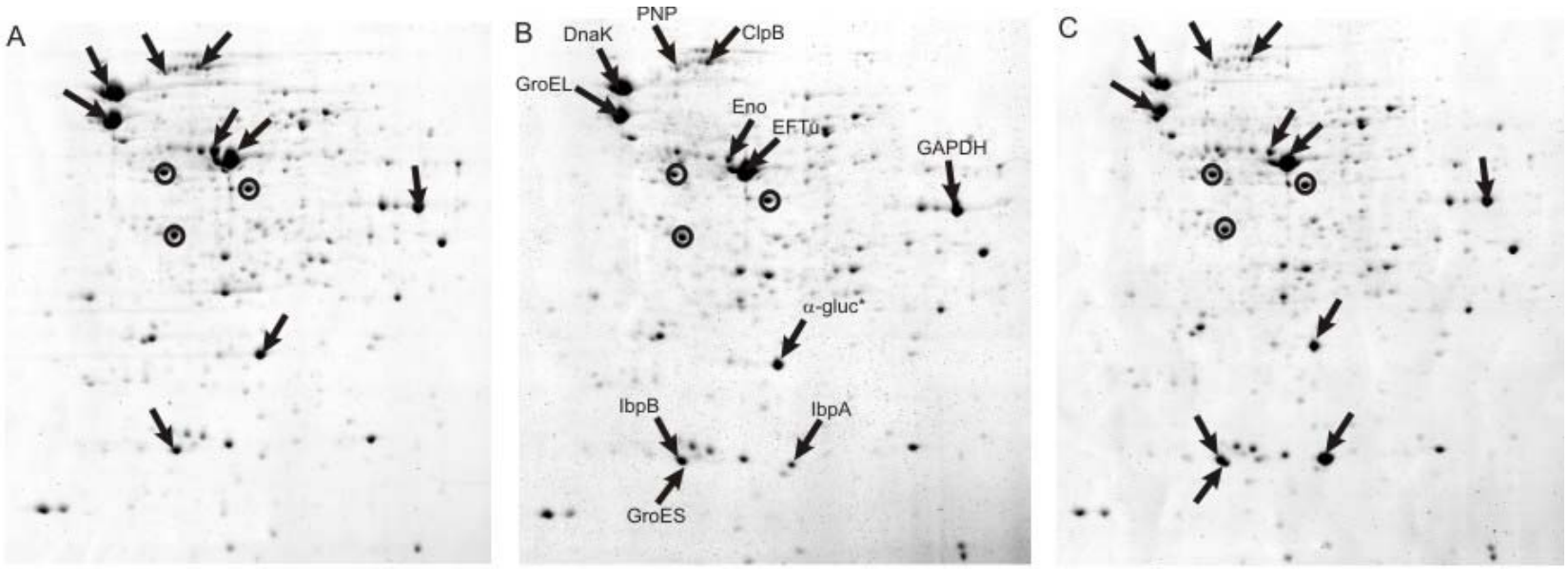

Fig. 23 c (continued): Effect of IbpA/IbpB availability on the proteome composition after two hours of α-glucosidase production at 30°C. Coomassie-stained two-dimentional gels.

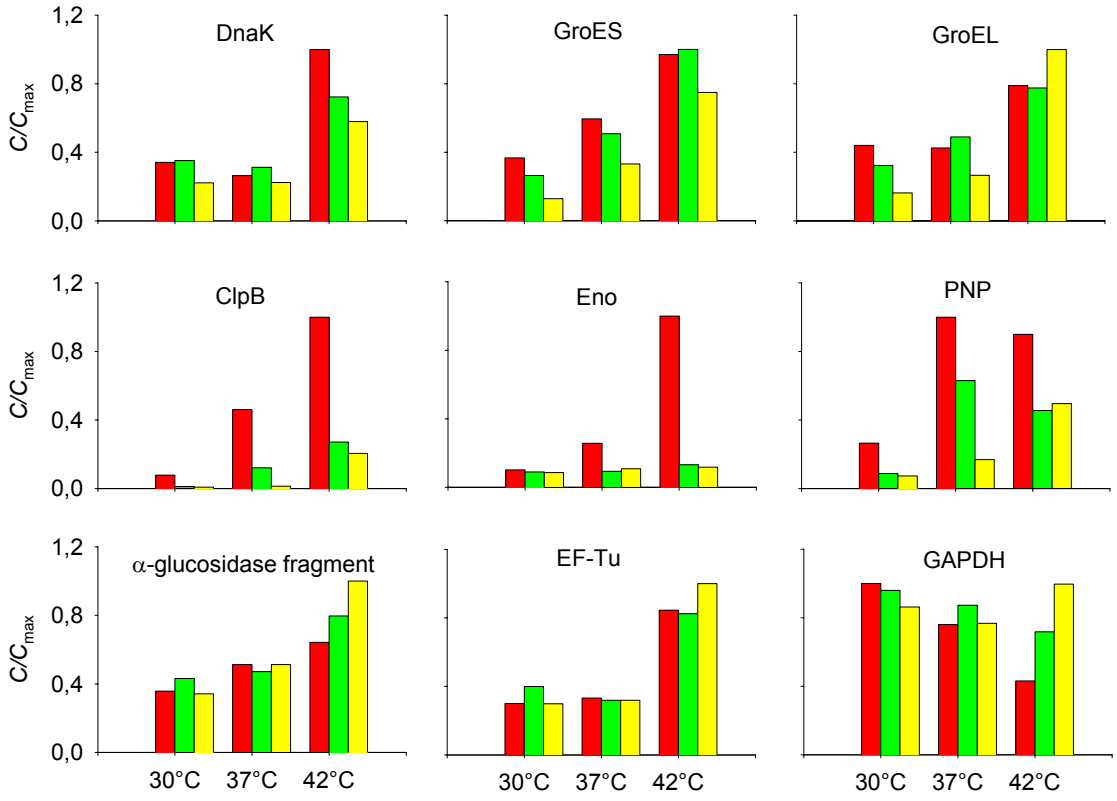

Fig. 24: Level of proteins after two hours of α-glucosidase production at different temperatures. Cultures were grown to an OD_{600} of 0.5 at 37°C, induced with 1 mM IPTG and transferred to the temperature of 30°C, 37°C or 42°C. After two hours, cells were harvested, solubilised in 8 M urea and the proteins were separated on two-dimensional gels (the concentration of urea is asumedly diluted by 8/OD amount of cells pellets and is therefore lower than 8 M). Spot volumes were determined from Coomassie-stained two-dimensional gels, and were normalised with the summed volumes of three internal references spots marked in Fig. 24 by circles. Concentrations are given relative to the maximum concentration determined for the respective protein to facilitate comparison. Levels of proteins (from left to right in the group of three bars) in: *ibpAB* deletion strain (red), in the wildtype strain without (green) and with (yellow) *ibpAB* overexpression. Protein designations are given above and production temperature below the bars. Major heat shock proteins DnaK, GroES, GroEL; PNP: protein polynucleotide phosphorylase, GAPDH: glyceraldehyde-3-phosphate dehydrogenase, and EF-Tu: thermo-unstable elongation factor were identified by comparison of the spot position with *E. coli* 2D-database (Wilkins et al., 1998). ClpB: ClpB identified by N-terminal sequencing (RLDRLTN), Eno: enolase identified by N-terminal sequencing (SKIVKII), α-glucosidase fragment due to a second translation start site at position 409, identified by N-terminal sequencing (MKNNYEIIKK).

To differentiate the impact of IbpA/IbpB on the heat-shock response further, the proteome of cultures producing α-glucosidase for two hours at various temperatures were separated by two-dimensional gel electrophoresis (Fig. 23). The heat-shock proteins DnaK, ClpB, GroEL and GroES were induced during α-glucosidase production at 30°C, and their concentrations

were higher at higher temperatures. Generally, their concentrations were inversely correlated with IbpA/IbpB levels (Fig. 24). Overexpression of *ibpAB* attenuated the heat-shock response; the concentrations of major heat shock proteins with *ibpAB* overexpression were lower at all temperatures studied. With ClpB, a chaperone involved in disaggregation, there was a marked higher level in the deletion strain, while its induction was completely repressed in the wildtype strain at 30°C, and even at 37°C with *ibpAB* overexpression. The level of DnaK at 42°C was clearly negatively correlated with IbpA/IbpB availability (Fig. 22 and Fig. 24).

A similar pattern as with ClpB was found with enolase. Its concentration was much higher in the *ibpAB* deletion strain (Fig. 24). As enolase is a glycolytic enzyme as well as a putative structural component of the RNA degradosome (Kuhnel and Luisi, 2001), an other structural component of the RNA degradosome, protein polynucleotide phosphorylase (PNPase) and another metabolic enzyme, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were identified by comparison of their positions with the *E. coli* 2D-database, and their concentrations were determined. While PNP was also induced at higher temperature and accumulated to higher level in the *ibpAB* deletion strain, the GAPDH concentration was hardly affected by temperature and clearly lower in the deletion strain at 42°C (Fig. 24). Thus, enolase seems to be induced as a part of stress response rather than as a metabolic enzyme. Enolase homologues have been reported to be induced by heat shock in various species, including *Bacillus subtilis* (Miller et al., 1991), but not yet in *E. coli*.

Elongation factor Tu (EF-Tu), the most abundant protein in *E. coli*, which functions as carrier of amino acyl-tRNA to the ribosome, showed a similar level in three strains. At 42°C, nearly 3-fold higher EF-Tu concentrations were found than at the low temperature (Fig. 24). The increased accumulation at higher temperature might be related to thermal instability of EF-Tu.

No intensive spot at the position of α-glucosidase can be detected, since full length α-glucosidase should be completely solubilised only at 8 M urea concentration. However, the fragment of α-glucosidase was clearly seen on the gel, as this fragment is already dissolved at a urea concentration of 4 M (Fig. 19). The concentration of the fragment of α-glucosidase was similar in the three strains at low temperature. At 42°C, twofold higher level of the fragment of α-glucosidase in all strains was observed. Furthermore the positive correlation of fragment with IbpA/IbpB levels was seen, which was similar to full length α-glucosidase (Fig. 24).

Thus, an inverse correlation of IbpA/IbpB availability and the induction of individual heat-shock proteins were found. The enhanced synthesis of other heat-shock proteins, however, cannot compensate the lack of IbpA/IbpB in terms of reduced growth or product accumulation of the deletion strain during α-glucosidase production at higher temperatures.

3.2.4 Impact of IbpA/IbpB on recovery of α-glucosidase from *inclusion bodies*

3.2.4.1 *In vivo* reactivation after arrest of protein synthesis

3.2.4.1.1 Impairment of inclusion bodies disaggregation and reactivation in *clpB* and *dnaK* deletion strains

α-Glucosidase was produced at 37°C in wildtype strain with and without *ibpAB* overexpression and in isogenic strains carrying deletions of *ibpAB*, *dnaK* or *clpB*. Two hours after induction, the cultures were treated with tetracycline to arrest the protein synthesis and subsequently transferred to 30°C.

Two hours after arrest of proteins synthesis, with all levels of IbpA/IbpB, an increase of the α-glucosidase concentration in the soluble cellular fraction (not shown) concomintant to fivefold increase of α-glucosidase activity (Table 10), a decrease of the α-glucosidase concentration in the insoluble cellular fraction (Table 10) was observed.

The yield of reactivated α-glucosidase was highest in the wildtype strain without overexpression of *ibpAB*. Deletion of IbpA/IbpB did prevent neither the disaggregation nor reactivation, but reduced the reactivation yield by nearly 30% compared to the wildtype. Overexpression of *ibpAB*, however, did not improve the reactivation yield; rather resulted on about 16% lower yield (Table 10).

Not all α-glucosidase removed from the insoluble cellular fraction was recovered in the soluble cellular fraction, since the total amount of α-glucosidase decreased with time. The loss during IBs disaggregation due to degradation was most pronounced in the *ibpAB* deletion strain. Wildtype level of IbpA/IbpB slightly reduced the degradational loss, while overexpression of *ibpAB* diminished the degradation considerably (Table 10).

Table 10. Disaggregation and reactivation of α-glucosidase from the insoluble cellular fraction after arrest of protein synthesis and reduction of temperature in different genetic backgrounds

Genotype	before arrest of protein synthesis[a]		two hours after arrest		Loss[b]
	activity /(U mg^{-1})	α-glucosidase / %[c]	activity /(U mg^{-1})	α-glucosidase / %[c]	/%
ibpAB^{++}	0.30±0.05	12.6±1.4	1.44±0.09	11.5±1.2	9
wildtype	0.28±0.06	11±1.4	1.7±0.03	8.4±0.8	24
ibpAB^{-}	0.26±0.03	9.4±0.6	1.2±0.2	6.0±0.03	36
clpB^{-}	0.08±0.01	12.2±1.4	0.056±0.002	12.8±1.0	0
dnaK^{-}	0.04±0.02	14±0.6	0.002±0.005	13.6±0.4	3

[a] Cultures of wildtype *E. coli* MC4100 and MC4100 mutants of *ibpAB* (JGT17), *dnaK* (BB1553), and *clpB* (BB4561) carrying the plasmids pKK177-3/GLUCP1 (or pKK177-3/GLUCP1_ibpAB in case *ibpAB* overexpression *ibpAB*$^{++}$) and pUBS520 were grown in complex medium at 37°C to an OD of 0.5; α-glucosidase synthesis was induced by 1 mM IPTG; after two hours, the protein synthesis was stopped by addition of 25 µg mL^{-1} tetracycline and the cultures were transferred to 30°C.

[b] Difference in total α-glucosidase before and two hours after arresting protein synthesis

[c] Amount of total α-glucosidase in percentage of total cellular proteins, determined from Coomassie-stained SDS-PAGE.

In *dnaK* or *clpB* deletion strains with impaired ability to dissolve protein aggregates (Mogk et al., 1999), none of these observation could be made (Table 10): the α-glucosidase activity did not increase after the temperature downshift and the arrest of protein synthesis, α-glucosidase was not removed from the IBs within two hours after the temperature downshift and the intensity of the α-glucosidase band even fainted in SDS-PAGE gels of the soluble cellular fraction of the *dnaK* strain (not shown).

3.2.4.1.2 Impact of IbpA/IbpB on disaggregation, reactivation and degradation

The disaggregation kinetics of α-glucosidase IBs were investigated at modified levels of IbpA/IbpB, i.e. with deletion or overexpression of *ibpAB*. After production of α-glucosidase for two hours at 37°C, protein synthesis was arrested by addition of tetracycline, and the

cultures were transferred to temperatures of 15°C, 24°C, 30°C or 37°C. The amount of soluble and insoluble α-glucosidase during reactivation at various temperatures was quantified from Coomassie-stained SDS-PAGE by densitometry.

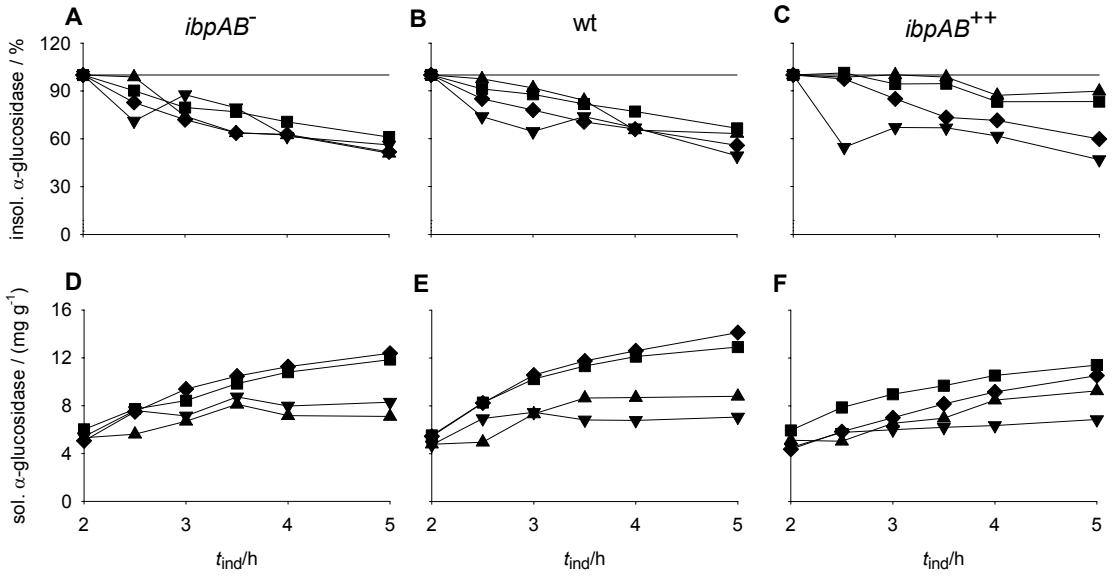

Fig. 25: Disaggregation kinetics of α-glucosidase IBs. The cultures of the *ibpAB* deletion strain (A, D) and wild-type strain without overexpression of *ibpAB* (C, F) or with overexpression of *ibpAB* (B, E) were grown at 37°C to an OD_{600} of 0.5, and then were induced with 1 mM IPTG. Two hours after induction, tetracycline was added and the cultures were transferred to 15°C (down triangle), 24°C (diamant), 30°C (square) or 37°C (triangle), respectively. (A, B, C) The amount of insoluble α-glucosidase was estimated by densitometry of Coomassie-stained SDS-PAGE and was referred to the value at the time of reactivation start (49 mg g^{-1}, 54 mg g^{-1} and 58 mg g^{-1} for *ibpAB* deletion, wildtype strain without and with *ibpAB* overexpression, respectively). (D, E, F) The amount of soluble α-glucosidase determined from SDS-PAGE analysis. Time is given relative to the time of induction.

The amount of α-glucosidase in the insoluble cellular fraction decreased with time. In the *ibpAB* deletion strain, the temperature had no influence on the disaggregation kinetics; the α-glucosidase was removed from IBs with similar kinetics (Fig. 25 A). In the wildtype strain, the removal of α-glucosidase from the IBs was fastest at 15°C proceeding with similar rates as in the *ibpAB* deletion strain. The disaggregation was, however, progressively retarded at higher temperature (Fig. 25 B). This delay of IBs dissolution became more pronounced with overexpression of *ibpAB*; at 37°C, α-glucosidase was largely preserved in the insoluble cellular fraction with *ibpAB* overexpression, whereas in the deletion strain as much of α-glucosidase as at 15°C was removed from IBs (Fig. 25 C).

Concomitant with reduction of the α-glucosidase concentration in the insoluble cellular fraction, the concentration of α-glucosidase increased in the soluble cellular fraction (Fig. 25 D, E, F). The resolubilisation, monitored as the increase of soluble α-glucosidase concentration, was faster at 24°C or 30°C and slower at 15°C or 37°C in all three strains, althought the difference was more pronounced in the *ibpAB* deletion strain and without *ibpAB* overexpression. The highest soluble α-glucosidase concentration was obtained at 30°C and 24°C without *ibpAB* overexpression

A large part was subjected to proteolysis: within three hours after arrest of protein synthesis, about 25-40% of α-glucosidase was lost due to degradation at 15°C in all three strain (Fig. 26), and in the *ibpAB* deletion strain at all temperatures studied (Fig. 26 A). Similar to the effect on disaggregation, higher *ibpAB* expression levels or elevated temperatures synergistically retarded degradation, reducing the loss to 5-10% in *ibpAB* overexpression strain (Fig. 26 C).

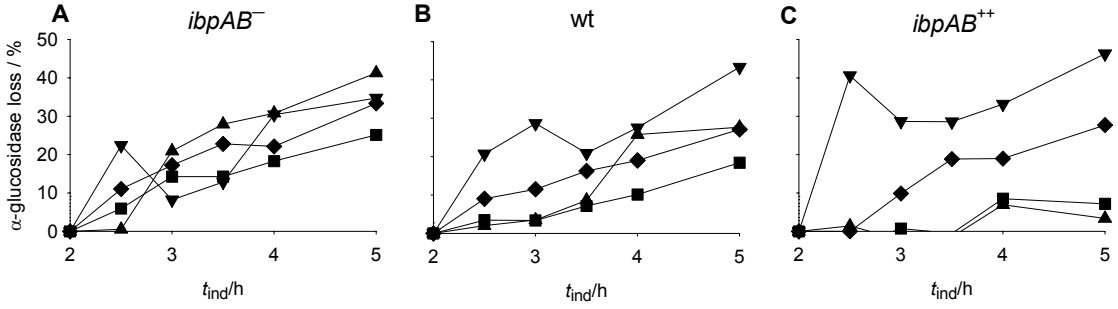

Fig. 26: Loss of α-glucosidase after arrest of protein synthesis. The cultures of the *ibpAB* deletion strain (A), the wildtype strain without (B) or with overexpression of *ibpAB* (C) were grown at 37°C to an OD_{600} of 0.5, and then were induced with 1 mM IPTG. Two hours after induction, tetracycline was added and the cultures were transferred to 15°C (down triangle), 24°C (diamant), 30°C (square) or 37°C (up triangle), respectively. The α-glucosidase loss was calculated as difference of the sum of soluble and insoluble α-glucosidase to the value obtained before addition of tetracycline. Time is given relative to the time of induction.

The reactivation of three strains as determined by specific α-glucosidase activities was more efficient at 30°C and 24°C (Fig. 27); the specific α-glucosidase activities increased within three hours reactivation sevenfold up to 2 U mg^{-1}, whereas they reached only maximum 0.75 U mg^{-1} at 37°C or 15°C (Fig. 27). The level of IbpA/IbpB had an effect on the rate and yield of α-glucosidase reactivation, whose significance depended on the temperatures. At

24°C and 30°C, both *ibpAB* deletion and overexpression impaired reactivation slightly compared to the wildtype (Fig. 27). At the more extreme temperatures, the effect of IbpA/IbpB became more pronounced. At 37°C, reactivation was very inefficient without IbpA/IbpB (Fig. 27 A), while IbpA/IbpB coproduction enhanced the final yield of active α-glucosidase (Fig. 27 C). At 15°C, on the contrary, appreciable activity was found only in the *ibpAB* deletion strain, mainly due to a higher initial reactivation rate compared to wildtype strains with and without *ibpAB* overexpression.

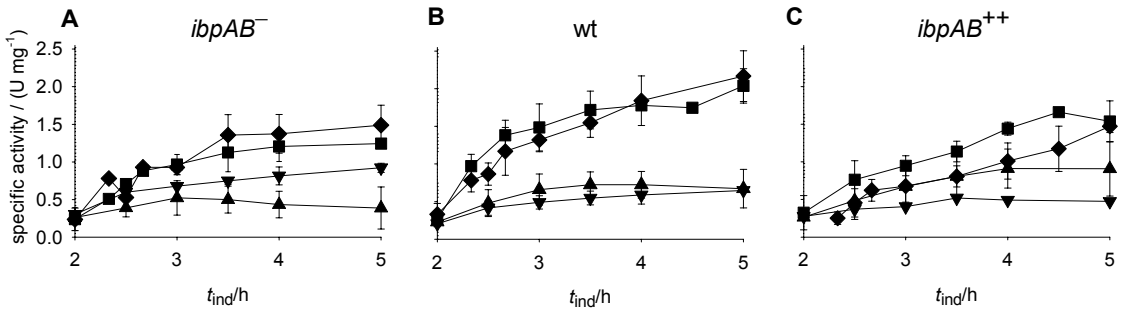

Fig. 27: Reactivation of α-glucosidase after arrest of protein synthesis. Cultures of the *ibpAB* deletion strain (A), wild type strain without *ibpAB* overexpression (B), or with *ibpAB* overexpression (C) were incubated at 37°C to an OD_{600} of 0.5 and then were induced with 1 mM IPTG. Two hours after induction, tetracycline was added to a final concentration of 25 µg mL^{-1}, and the cultures were transferred to 15°C (down triangle), 24°C (diamant), 30°C (square) or 37°C (trianagle), respectively. Time is given relative to the time of induction.

Thus, IbpA/IbpB influenced disaggregation, resolubilisation, reactivation and degradation in a temperature-dependent manner. Specifically, the small heat-shock proteins interfere with degradational removal of disaggregated proteins.

3.2.4.1.3 Effect of temperature during production on subsequent disaggregation and reactivation of α-glucosidase

As IbpA/IbpB influence the IBs dissolution as well as degradation at high temperatures, the effect of high temperature during the production phase was investigated. α-Glucosidase was produced for two hours at 42°C, followed by reactivation at 30°C.

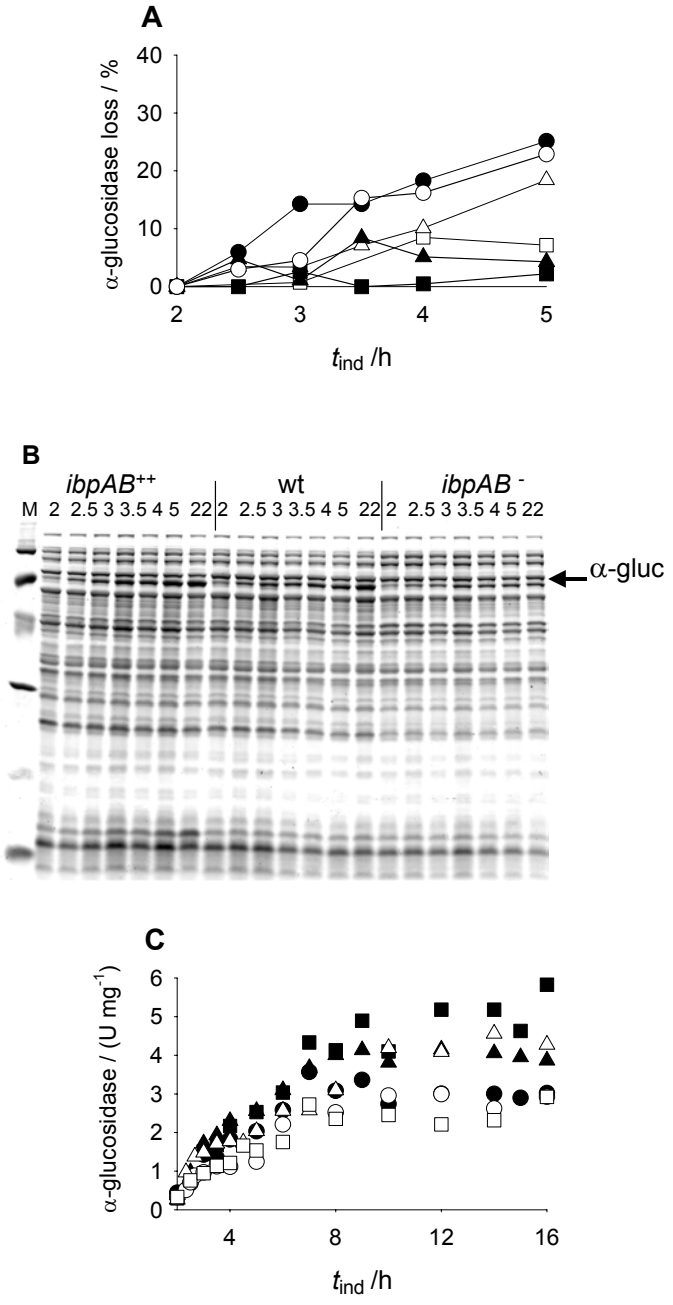

Fig. 28: Reactivation of α-glucosidase and disaggregation of IBs after production at 42°C. IBs of α-glucosidase were produced for two hours at 37°C (open symbols) or at 42°C (closed symbols) with *ibpAB* overexpression (square) or without *ibpAB* overexpression (triangle), the *ibpAB* deletion strain (circles); reactivation was monitored after addition of tetracycline and transfer of the cultures to 30°C. Time is given relative to the time of induction. (A) Degradational loss of α-glucosidase, (B) Coomassie-stained SDS-PAGE gel of soluble cell fraction extracted from the same amount of biomass; sample times (in hours after induction) and genotypes are given above the lanes; the position of α-glucosidase is indicated; lane M: molecular weight markers. (C) specific α-glucosidase activity.

While production at 37°C resulted in 15 % degradational loss of α-glucosidase within 3 hours incubation at 30°C in the wildtype strain without *ibpAB* overexpression, the loss was much smaller after production at 42°C and became similar to that in the *ibpAB* overexpression strain (Fig. 28 A). In contrast, the degradational loss of α-glucosidase remained at high level in the *ibpAB* deletion strain (Fig. 28 A).

Disaggregated α-glucosidase was quantitatively transferred to the soluble cell fraction after production at 42°C with *ibpAB* overexpression and became the most prominent band in the gel. The band intensity increased from 2 to 7 % of the total cellular protein (Fig. 28 B). Significantly less α-glucosidase accumulated in the soluble cell fraction of the *ibpAB* deletion strain even after prolonged incubation, the band intensity reached only 4 % of the cellular protein (Fig. 28 B).

Also at the position of IbpA/IbpB, the band intensity in the soluble cell fractions increased with *ibpAB* overexpression. The small heat-shock proteins that was located almost exclusively in the IBs fraction during production seemed to be transferred into the soluble cell fraction when the IBs were dissolved upon arrest of protein synthesis with subsequent reduction of the temperature (Fig. 28 B). IbpA/IbpB was observed to migrate in the soluble cell fraction after dissociation from protein aggregates by heat-shock (Mogk et al., 2003).

The reactivation after production at 42°C was not impaired anymore in *ibpAB* overexpression strain; the α-glucosidase activity initially increased with similar slopes as without *ibpAB* overexpression (Fig. 28 C). The activities in the *ibpAB* deletion strain were 25 % lower than in the wildtype strain (Fig. 28 C). Moreover, the α-glucosidase activity reached stationary level after 6 hours and 8 hours of reactivation in deletion strain and the wildtype strain without *ibpAB* overexpression respectively, while it continued to increase for about 12 hours after production at 42°C with *ibpAB* overexpression, giving highest yield of reactivated α-glucosidase in *ibpAB* overexpression strain after prolonged incubation at 30°C (Fig. 28 C).

Compared to the production at 37°C, the reactivation yield after production at 42°C with *ibpAB* overexpression was much improved; the specific α-glucosidase was about twofold higher, while the yield of α-glucosidase activities after 12 hours of reactivation without *ibpAB* overexpression or with *ibpAB* deletion remained at the same values regardless to the production temperature (Fig. 28 C).

While the high production temperature reduced the degradational loss during disaggregation in strains with IbpA/IbpB, it did not affect the disaggregation kinetics (Fig. 29 B, C), indicating that the impact on degradation and disaggregation are separate. Nor did the production temperature change the residual α-glucosidase concentration after overnight incubation (Fig. 29 B, C). With *ibpAB* overexpression, about one third of α-glucosidase remained in the insoluble cell fraction after prolonged incubation independent of the production temperature (Fig. 29 B, C), which was almost three times as much as without *ibpAB* overexpression or with *ibpAB* deletion.

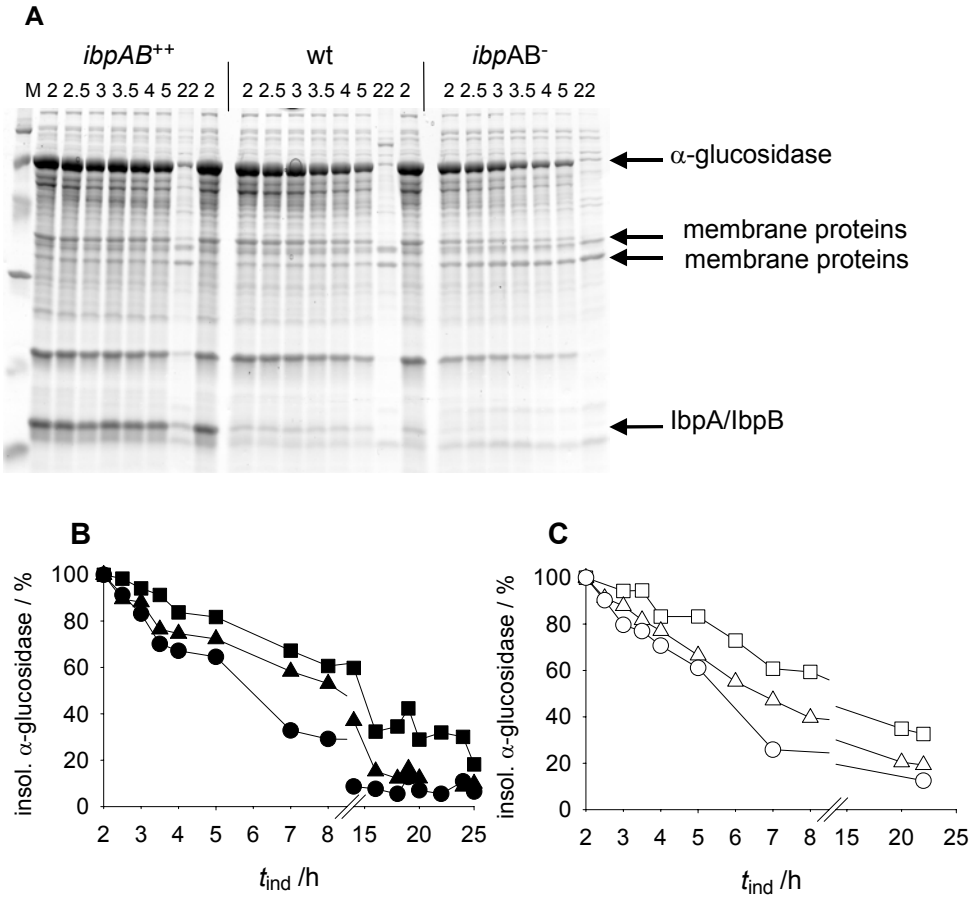

Fig. 29: Disaggregation of IBs after production at 42°C. (A) Coomassie-stained SDS-PAGE gel of the insoluble cell fraction extracted from the same amounts of biomass; sample times (in hours after induction) and genotypes are given above the lanes; positions of α-glucosidase, IbpA/IbpB and membrane proteins (Mogk et al., 1999) are indicated. Disaggregation kinetics of α-glucosidase IBs produced at 42°C (B) or 37°C (C) with subsequent transfer to 30°C.

Also the removal of other proteins in the IBs was retarded with *ibpAB* overexpression (Fig. 29 A), indicating that the delay of disaggregation by IbpA/IbpB is not specific to α-glucosidase. Only the membrane proteins were retained in the insoluble cell fractions after prolonged

incubation (Fig. 29 B). They are supposed to coprecipitate unspecifically with IBs proteins during IBs purification (Rinas et al., 1993).

4. DISCUSSION

4.1 Improving the folding of recombinant proteins *in vivo*

4.1.1 Optimum growth rate for maximising the yield of active protein

Impact of growth rate on the accumulation of α-glucosidase

Low specific growth rate can limit the accumulation kinetics of α-glucosidase. With μ_{set} = 0.06 h^{-1}, the specific product concentration of α-glucosidase seven hours after induction was 50 % lower than at μ_{set} = 0.12 h^{-1} (Fig. 8). However, other factors seem to limit product accumulations too, as similar product levels were obtained with specific growth rates in a range between 0.12 h^{-1} and 0.18 h^{-1} (Fig. 7). At high growth rates, sustainability of production becomes of major importance. In batch cultures with μ_{max} = 0.56 h^{-1}, production was initially fast, but stops early. The product formation declined very fast one hour after induction that the specific product formation rate was by almost 60% slower than at time of induction. Therefore two hours after induction, the specific α-glucosidase concentration was similar to the one obtained at the fed-batch cultivation with much slower growth rate (0. 22 h^{-1}) (Fig. 14). The higher specific growth rate results on higher concentration of ribosomes (Herbert, 1961). The lower specific concentration at specific growth rate of 0.06 h^{-1} is probably due to lower capacity of protein synthesis system. The specific growth rate correlated with specific concentration of human glucagon and human growth hormone in the range of low specific growth rate from 0.02 h^{-1} to 0.1 h^{-1} (Shin et al., 1998). A similar level of specific product concentration in the specific growth rate in range between 0.12 h^{-1} to 0.18 h^{-1} suggest that the change of ribosome concentration in this range might not influence the product accumulation. Hellmuth et al. (1994) also did not find an influence of the growth rate on the specific concentration of their model protein Aprotinin::ß-galactosidase in the fed-batch cultivation with variation of growth rate from 0.11 h^{-1} to 0.16 h^{-1}.

The stop of net accumulation of α-glucosidase after two hours intensive protein synthesis in batch cultivation is a consequence of stress caused by high rate of recombinant protein synthesis. The stress caused by high rate of protein synthesis affecting the cells and preventing their normal function, causes not only earlier growth inhibition but also earlier

stop of recombinant product accumulation. The competition between house-keeping and recombinant proteins at transcription and translation level was reported (Vind et al., 1993) and consequently was enhanced at higher recombinant protein synthesis rate. High product formation rate at high feeding rates results in degradation of ribosomal RNA for supply of precursors and energy (Sandén et al., 2002). Moreover, degradation of α-glucosidase at higher growth rate is not excluded since stress induced protease activity during recombinant production was observed (Jordan and Harcum, 2002). mRNA levels of the heat-shock protease Lon in production of recombinant α-glucosidase is increased (Schweder et al., 2002). The faster arrest of protein accumulation at higher rate of protein synthesis is common evidence (Seo and Bailey, 1986; Turner et al., 1994; Kim et al., 1996; Hoffmann et al., 2001).

Thus, the specific product concentration seems to be limited by the rate of protein synthesis at low growth rate due, but limited by duration of net product accumulation at high growth rate due to stress caused by high synthesis rate of recombinant product .

The strong stress caused by production of α-glucosidase was observed in fed-batch cultivation with constant feeding of 53.2 g L^{-1} glucose, which corresponds to specific growth rate of μ_{set} = 0.24 h^{-1} at the time of induction (Lin, 2000). The stress led to inhibition of cell growth and stop of net accumulation of product three hours after induction (Lin, 2000) and to reduction by 50 % of the capacity for glucose uptake (Lin et al., 2001; Neubauer et al., 2003), which resulted in glucose accumulation up to 1.5 g L^{-1} already two hours after induction (Lin, 2000). In this thesis, the stress caused by production of α-glucosidase was successfully avoided using the low specific growth rate of 0.12 h^{-1}. The actual growth rate was equal to the set point of specific growth rate for at least seven hours after induction (Fig. 15). This was in contrast to the cultures with set growth rates $\mu_{set} \geq 0.18$ h^{-1}, in which the growth was inhibited already one hour after induction, resulting in actual growth rates lower than the set specific growth rates (Fig. 15). At μ_{set}= 0.12 h^{-1}, no glucose accumulated until ten hours after induction. Furthermore, the product accumulation is not affected at this low μ_{set}, yielding a similar value as at high $\mu_{set} \geq 0.18$ h^{-1}.

Impact of growth rate on the activity of α-glucosidase

Even more pronounced than the impact of specific growth rate on the accumulation of α-glucosidase is the effect on the fraction of native α-glucosidase and its activity. During production at 37°C, the specific α-glucosidase activity obtained with a feeding rate $F(\mu_{set} = 0.12\ h^{-1})$ was six times higher than those obtained with $F(\mu_{set} = 0.18\ h^{-1})$, despite similar level of total α-glucosidase (Fig. 7). The fraction of sol α-glucosidase, that was only 4 mg g^{-1} with the higher growth rate, reached 11 mg g^{-1} with $\mu_{set} = 0.12\ h^{-1}$. Although high temperatures are known to interfere with proper protein folding (Schein and Noteborn, 1988; Schein, 1989) and are found also to affect accumulation of active α-glucosidase (cf. below), a substantial amount of active α-glucosidase could be produced under conditions that minimised the stress. Moreover, using the more favourable temperature of 30°C, the basal α-glucosidase activity with $\mu_{set} = 0.2\ h^{-1}$ was 12-times higher than at 37°C. Nevertheless, a further enhancement by a factor of two was achieved with $\mu_{set} = 0.12\ h^{-1}$, resulting in a specific activity of 8 U mg^{-1} seven hours after induction, corresponding to 40 mg g^{-1} of soluble α-glucosidase (Fig. 7).

In addition, as growth could be maintained at conditions that minimised the stress, the volumetric yield of α-glucosidase per OD present at the time of induction was raised from 0.04 U mL^{-1} to 0.18 U mL^{-1} seven hours after induction even at production temperature of 37°C (Fig. 7); at favourable temperature of 30°C it increased to 1.33 U mL^{-1}after the same time of induction and increased to 3.13 U mL^{-1} on the end of cultivation (18 h after induction (Fig. 5).

The specific growth rate hence plays very important role in improving the yield of recombinant product. The reduced stress allows prolonging the production time. In that way high cell densities can be achieved and high volumetric product activity can be reached.

The lower protein production rate at low growth rate might reduce the overload of the chaperone systems, which are responsible for proper folding, and thereby increase the folding yield concomitant to a decrease of aggregation. The measured enzymatic activity of protein Aprotinin::ß-galactosidase was strongly depended on the growth rate; it reached maximum at growth rate of 0.13 h^{-1} and decreased at higher or slower growth rate (Hellmuth et al., 1994).

The specific α-glucosidase activities were decreased at growth rate higher than 0.12 h^{-1}, however, were similar at 30°C at growth rate of 0.06 h^{-1} and 0.12 h^{-1} (Fig. 8). This similarity

is probably due to the limited solubility of α-glucosidase. According to limited solubility, there is a limitation for solubility of protein, over which all new synthesised proteins aggregated (Klein and Dhurjati, 1995). The limited solubility *in vivo* might connect to the stress, which is already minimal at the specific growth rate of 0.12 h^{-1} (Fig. 15).

Aggregation of α-glucosidase could be almost completely avoided at the slow growth rate of 0.06 h^{-1} but the specific product accumulation was 50 % lower than at growth rate of 0.12 h^{-1} (Fig. 8). Thus, higher concentration of product favours the aggregation. Fast protein synthesis has been supposed to encourage the precipitation of partially unfolded recombinant product in cytoplasm, leading to increasing the aggregation (Kiefhaber et al., 1991). Greygory et al. (1992) found higher amount of aggregated periplasmic protein ß-lactamase at higher synthesis rates. The aggregation of ß-lactamase is only observed when the expression rate exceeds 2.5 % of the total protein synthesis rate (Bowden and Georgiou, 1990). Since specific α-glucosidase activity despite reduced aggregation was not higher, but the specific product concentrations and also cell densities were lower, the volumetric activity showed its maximum bei growth rate of 0.12 h^{-1}. Thus high specific growth rates decrease the yield of specific activity, while low growth rates decrease the yield of biomass. The growth rate is hence an important parameter to optimise the yield of active proteins and to reduce the aggregation. Fed-batch cultivation with exponentially feeding rate for constant growth rate is an efficient tool not only for to achieve high cell densities but also for enabling a desired to control the growth rate of cultures at a desired value.

Other factors influencing α-glucosidase production

With partial induction, the product concentration 4 h after induction on complex medium at 0.05 mM IPTG was lower than full induction by 27% (Table 7). Low concentrations of IPTG result in low level of gene expression, leading to low rate of protein synthesis (Wood and Peretii, 1991). Accordingly, lower IPTG concentration reduced the stress from overexpression of recombinant proteins to the cells as low growth rates do. Low IPTG concentration reduced the aggregation considerably on complex medium, but did not influence growth or the concentration of soluble product (Table 7), indicating that stress play less roles under this condition. Furthermore, reducing synthesis rate by partial induction or low growth rate (μ_{set} = 0.06 h^{-1}) both result on aggregation, but not alter the solubility limit. The accumulation of activity is hence already optimum under those conditions. Full induction result in higher

synthesis rates of the recombinant protein. These have been postulated to favour aggregatin (Kiefhaber et al., 1991).

On defined medium, partial induction ameliorated growth considerably. The final optical density with partial induction was twofold higher than with full induction. In contrast to defined medium, the cultures grew similarly at both IPTG concentrations on complex medium. The inhibition factor, i.e. the ratio of growth rates with and without induction, is higher on defined medium than on complex medium. Thus, stress seems to play a more pronounced role on defined medium than on complex medium. The stronger inhibition of growth with induction than without induction on defined medium compared to complex medium also reveals about that.

The strong stress at full induction in defined medium leads to similar final specific product concentration compared to partial induction, although the growth was strongly inhibited at full induction (and possibly the synthesis rate was also higher). As consequence of strong stress, the specific and volumetric α-glucosidase activity was less improved on defined medium at low IPTG concentration compared to high IPTG concentration, in contrast to the observation with cultures on complex medium (Table 7).

There are numerous reports about an influence of medium compositions on recombinant production such as on accumulation of soluble (Kopetzki et al., 1989) or total product (Lee and Chang, 1995), on plasmid maintenance (Brownlie et al., 1990), and on the growth (Rothen et al., 1998). Although the author did not explicitely refer to the impact of medium composition on stress intensity, they observed reduction of degradation by addition of amino acids (Harcum et al., 1993) which normally are present in complex medium, reduction of IBs formation on complex medium (Hoffmann et al., 2004), higher yield of active protein (Winter et al., 2001). Furthermore, the lower plasmid contents in complex medium than defined medium (Seo and Bailey, 1985) might also contribute to reduction of stress.

During production at low pH value, the specific α-glucosidase activity was twofold higher. Concomitantly, the growth rate was twofold lower. As has been discussed above, low growth rate is able to promote proper folding; it is not clear whether the pH value has direct effect on specific α-glucosidase activity (Fig. 3). Low pH value of the medium has an effect on the patterns of protein synthesis in *E. coli* (Heyde and Portalier, 1990; Hickey and Hirshfield,

1990) and decrease the internal pH value from 7.73 to 6.25 concomitant to reducing the external pH value from 7 to 5 (Hickey and Hirshfield, 1990).

Diverse physical factors have been proposed to influence the yield of soluble α-glucosidase Low pH values and low temperatures both reduce the growth rate. These effects were separated in fed-batch cultivation with exponential increasing feeding rate to keep the growth rate constant. It was found that 1) growth rate has a strong impact on yield of active protein; 2) Temperature in range between 24°C and 30°C has little effect on yield of active protein. High temperature, however, affect the yield. It can be concluded that the physical factors such as pH values and the temperatur of 24°C influence the yield of soluble α-glucosidase mainly indirectly mediated by reduction of growth rate. Moreover, these parameters are of little use to enhance production, as the volumetric yield is not ameliorated. In contrast, in fed-batch process, the volumetric productivity can improve considerably by controlling the growth rate and temperature.

4.1.2 Impact of temperature on the folding of α-glucosidase

Temperature plays an important role on accumulation of soluble active α-glucosidase. At a constant specific growth rate of $\mu_{set} = 0.12\ h^{-1}$, specific α-glucosidase activity at 30°C was seven times higher compared with 37°C (Fig. 7). High temperatures affect the solubility of α-glucosidase by promoting aggregation, because the insoluble α-glucosidase concentration was considerably higher at higher temperatures (Fig. 7). Since high temperatures promote aggregation, α-glucosidase activity at 37°C or 42°C was not detected in shake flask experiments. Under this condition, the effect of high temperature dominates the effects of other factors. Furthermore, as both low temperature and low growth rate favour the correct folding, they both contribute to the higher specific activities obtained at lower temperature in shake flasks (Fig. 3).

High temperature decreases the solubility *in vivo* and increases the IBs formation (Chalmers et al., 1989; Schein, 1989; Donovan et al., 1996). Pulse-chase labelling experiments showed migration of soluble protein into insoluble fraction if temperature elevated (Klein and Dhurjati, 1995). *In vitro*, lower temperatures suppress the aggregation and promote chaperonin-dependent refolding of some proteins (Viitanen et al., 1990). It is supposed that high temperature reduces the productive folding pathways by destabilisation of productive folding intermediates (Haase-Pettingell and King, 1988). Furthermore, low temperature

decreases the proteolytic degradation and stabilises numerous recombinant proteins (Baneyx et al., 1991; Chesshyre and Hipkiss, 1989; Kosinski et al., 1991).

Stress caused by production of recombinant proteins at high temperature is possible. Recombinant proteins need molecular chaperones for correct folding. The need for chaperones of cellular proteins is enhanced at high temperatures, which is still not full covered through accelerated synthesis by heat-shock response. Accordingly the amount of unbound recombinant proteins at high temperatures is higher and thus aggregation increases. Slightly lower cell density at 37°C than at 30°C despite at the same specific growth rate of 0.12 h^{-1} might support this suggestion.

Specific α-glucosidase activity at 24°C is not higher than at 30°C at a growth rate of 0.06 h^{-1} (Fig. 8). In shake flasks, the specific α-glucosidase activity obtained at 24°C was higher than that obtained at 30°C. As temperature in this range is found not to influence the activity by itself, the increase is most likey due to slower growth rate at lower temperature.

4.2 Production by *in vivo* reactivation from inclusion bodies

The IBs can be disintegrated and proteins released from IBs can be reactivated (Carrió et al., 1999). To exploit the potential of *in vivo* reactivation for improvement the production processes, appropriate cultivation conditions for IBs dissolution or reactivation were set up and optimised in this thesis.

On complex medium, the α-glucosidase IBs readily disaggregated after arrest of protein synthesis (Fig. 25 B). The temperatures during production phase and reactivation phases play an important role for the yield of reactivated α-glucosidase. The reactivation was most efficient at intermediate temperatures of 24°C or 30°C, which were also optimum for *de novo* folding of α-glucosidase (Fig. 8). Both higher reactivation temperatures of 37°C or lower of 15°C, respectively, resulted in nearly twofold lower specific activity after three hours of reactivation (Fig. 9). High temperatures interfere with protein folding as discussed for direct production (see 4.1.2). The low reactivation yield of α-glucosidase at temperature of 15°C is somehow not expected from previously reported data: Is has been shown that temperature of 15°C inhibits the initiation of protein synthesis (Farewell and and Neidhardt, 1998), but there is no information about disturbance of folding at this temperature *in vivo* or *in vitro*.

Furthermore, the proteins can still fold correctly *in vitro* even at 0°C. There is, however, some evidence about improper function of chaperone systems at low temperatures. The function of DnaK relies on the chaperones cycle, in which DnaK changes between two states, the ATP-bound low affinity state with fast binding and release of the substrate and the ADP-bound high affinity state with slow kinetics. The cofactor DnaJ and the ATP/ADP exchange factor GrpE cooperate with DnaK to fulfil this cycle. Temperature controls the chaperone activity of DnaK system since the rate of chaperone cycles of DnaK/DnaJ/GrpE is increased at higher temperature; the rate at temperature of 37°C is about 60 times as much as at 15°C (Grimshaw et al., 2003). Furthermore, the chaperonin activity is also reduced at lower temperature. *In vitro*, the chaperonin-assisted refolding of rhodase is inhibited at low temperature; the activity of rhodase is not recovered after incubation of rhodanese-chaperonin complex at 10°C, in contrast to 50% recovered activity at 25°C (Mendoza et al., 1991). The low activity of chaperonin at low temperature might connect to lower hydrolysis of ATP by the GroEL at low temperatures (Mendoza, 2000).

The disaggregation of IBs was not affected at low temperature (Fig. 25); the low temperature hence does not interfere with disaggregation but specifically with reactivation. Disaggregated proteins, which cannot be refolded at 15°C, might be more susceptible to degradation.

Based on the optimum temperature profiles for reactivation, the production by *in vivo* reactivation from IBs was optimised in shake flask on complex medium and in fed-batch cultivation on defined glucose mineral salt medium.

On defined medium, reactivation was considerably slower than on complex medium (Fig. 13). After IBs production, the reactivation was initiated by temperature downshift. The specific growth rate has an effect on the reactivation of α-glucosidase also after temperature downshift. Similar to the direct production, the specific α-glucosidase activity was higher at $\mu_{set} = 0.12\ h^{-1}$ relative to $\mu_{set \geq}\ 0.18\ h^{-1}$ (Fig. 15). Prolonged production time prior to reactivation phase resulted on lower final specific and volumetric α-glucosidase activities (Fig. 16). Since the cultivations were both optimised in direct and in vivo reactivation, since the direct production in fed-batch cultivation (compared with shake flasks) very efficient and since the reactivation was slowly on defined medium, the best time for temperature downshift was direct by induction (Fig. 16)

On complex medium, in contrast, the yield of active product was improved considerably by *in vivo* reactivation protocol. Specifically, under optimised conditions, which were 42°C for productin of IBs and three hours for reactivation at 30°C, the activity yield on complex medium after temperature shift without arrest of protein synthesis was improved nearly twofold 5 h after induction relative to highest value in direct production (Fig. 12). Although the production at 42°C resulted in very little accumulation of active α-glucosidase, high production temperature of 42°C resulted on highest specific α-glucosidase activity three hours after temperature downshift without arrest of protein synthesis (Fig. 12). The higher reactivation yield might be contribute to this highest specific activity, since the reactivation yield with arrest of protein synthesis was higher after production at 42°C than at 37°C (Fig. 9). Furthermore higher yield of *de novo* folding due to higher concentration of chaperones at higher temperatures before shift to lower temperature (Table 9) might also contribute to. Higher yield of soluble protein obtained after temperature stress has been reported (Strandberg and Enfors, 1991; Chen et al., 2002). The application of disaggregating action of bi-chaperone network ClpB/DnaK in production of recombinant protein to improve the soluble active product was suggested (Schlieker et al., 2002; Mogk et al., 2002). A marked increase of recovery from IBs was reported when a set of chaperones genes *dnaK*, *dnaJ*, *grpE*, *clpB* and *groEL* was overexpressed after overexpression of target protein (de Marco and De Marco, 2004). The data indicate that further improvement of production of α-glucosidase in shake flask by *in vivo* reactivation by coexpression of chaperones genes could be expected.

4.3 Role of IbpA/IbpB in the production of recombinant α-glucosidase

4.3.1 IbpA/IbpB protect cells under DnaK depletion condition

Compared to other heat-shock proteins such as the DnaK system plus ClpB or the GroE system, IbpA/IbpB play only a minor role of in quality control system. Lack of *ibpAB* does not affect the growth even at high temperature as 42°C (Thomas and Baneyx, 1998). Also the removal of proteins that aggregated during incubation for 15 min at 50°C has similar kinetics in wildtype and *ibpAB* deletion strains (Kuczyńska-Wiśnik et al., 2002). *ibpAB* deletion has, however, a strong impact under conditions, which are characterised by depletion of free DnaK, such as prolonged incubation at high temperature or reduced DnaK synthesis (Thomas and Baneyx, 1998; Kuczyńska-Wiśnik et al., 2002; Mogk et al., 2003b). Prolonged incubation of an *ibpAB* deletion strain for 4 h at 50°C results in retardation of the removal of heat-

aggregated proteins and affects the cell viability (Kuczyńska-Wiśnik et al., 2002). Double mutant *ΔibpAB ΔdnaK756* causes a growth defect at 50°C (Thomas and Baneyx, 1998). In the *ibpAB* deletion strain, which carries the plasmid for expression of DnaK, whose amount could be regulated by IPTG concentration, higher level of DnaK is needed to compensate the growth defect caused by the lack of IbpA/IbpB (Mogk et al., 2003b).

Overproduction of aggregated-prone α-glucosidase mimics stress to the cell: the growth in the *ibpAB* deletion strain was already impaired at 30°C, whereas there was no significant difference to the isogenic wildtype strain without α-glucosidase synthesis. The lack of *ibpAB* also affected the growth after the temperature downshift. After two hours of α-glucosidase production at 37°C, a marked inhibition of the growth was observed even after three hours incubation at 24°C (not shown).

Recombinant protein production induces the heat-shock like response as a result of the accumulation of misfolded proteins (Goff and Goldberg, 1985). Also during α-glucosidase production at 35°C, the induction of heat shock genes such as *dnaK*, *groEL, ibpB*, *lon* and other major stress proteins was reported (Jürgen et al., 2000). In this thesis, the heat-shock response during α-glucosidase production at various temperatures was studied. The lack of free DnaK to bind to σ^{32} during α-glucosidase production even at low temperature became evident by synthesis of IbpA/IbpB (Table 9) and other heat-shock proteins (Fig. 24). The impact of IbpA/IbpB availability on heat-shock response was evaluated. Overexpression of *ibpAB* attenuated the heat-shock response. At all temperatures studied, the amounts of major heat-shock proteins generally were lower concomitant to overexpression of *ibpAB* (Fig. 24). Overexpression of *ibpAB* prevented the induction of the heat-shock response even at 42°C (Fig. 24); the concentrations of the disaggregating chaperones ClpB and DnaK were inversely correlated with IbpA/IbpB levels. As the levels of other cell proteins GAPDH or EF-TU were not affected, the lower level of major heat-shock proteins was unlikely the consequence of metabolic burden of *ibpAB* overexpression. Rather, IbpA/B are able to disburden the DnaK system. The heat-shock gene expression is feedback controlled by the availability of DnaK, and is induced if misfolded proteins are titrated by free DnaK (Bukau, 1993). DnaK and IbpA/IbpB seem to recognise similar traits of aggregationed-prone proteins as higher levels of DnaK are required to compensate for missing IbpA/IbpB (Kuczyńska-Wiśnik et al., 2002; Mogk et al., 2003b; Laskowska et al., 2004). In this context, unfolded α-glucosidase that normally is bound by IbpA/IbpB will be bound by DnaK in the *ibpAB* deletion mutant. As a

consequence of feedback regulation of DnaK, the negative correlation of other heat-shock proteins with IbpA/IbpB level was observed (Fig. 24). Vice versa, the overexpression of *ibpAB* can disburden the DnaK system, making resources available to repress the stress response to accumulation of α-glucosidase. The resistance to heat and oxidative stress conferred by *ibpAB* overexpression (Kitagawa et al., 2000) may rely on this mechanism.

Deletion of *ibpAB* did effect only little the levels of GroEL and GroES, the chaperonin for assisting protein folding at all temperatures, but strongly increased the need for the disaggregating chaperone ClpB and at 42°C also for DnaK. Thus, the lack of one partner in the disaggregation triad of DnaK, ClpB and IbpA/IbpB (Mogk et al., 2003) is compensated by enhanced synthesis of the others.

4.3.2 IbpA/IbpB inhibit *inclusion bodies* dissolution

The disaggregation kinetics of IBs depended on the levels of IbpA/IbpB. The IBs disaggregation was fastest in the *ibpAB* deletion strain and slowest with *ibpAB* overexpression (Fig. 25). The IbpA/IbpB activity is, however, temperature-dependent: IbpA/IbpB inhibited the IBs disaggregation only at high temperatures of reactivation or production (Fig. 27 and Fig. 29). At 15°C, the kinetics of disaggregation were similar independent of the IbpA/IbpB availability.

During a heating-cooling cycle *in vitro*, changes in the secondary structure of IbpB are fully reversible only at temperatures between 32°C and 55°C, but not between 10°C and 32°C (Shearstone and Baneyx, 1999. The irreversible structural changes at low temperatures might diminish the affinity of IbpA/IbpB to bind to disaggregated α-glucosidase and thus reduce the impact of IbpA/IbpB. As IBs dissolution was not inhibited in *ibpAB* deletion strain in the temperature range tested, the inhibition should be mediated by IbpA/IbpB function. Since the IbpA/IbpB were induced during production phase, they were similar in each strain before temperature shift to different temperatures; the effect is hence due to temperature but not due to IbpA/IbpB levels.

No disaggregation or reactivation of α-glucosidase IBs could be observed in *clpB* and *dnaK* deletion strains (Table 10). Since disaggregating chaperones ClpB/DnaK are essential for α-glucosidase IBs disaggregation, their low concentration with *ibpAB* overexpression (Fig. 24) might contribute to slow disaggregation. Furthermore, proteases can directly act on IBs before

IB solubilisation (Carrió et al., 2000). The concentrations of heat-shock proteases are probably lower at higher concentration of IbpA/IbpB, by the same mechanism as other heat-shock proteins. The reduced amount of heat-shock proteases hence might contribute to the delay of IBs dissolution.

The direct role of IbpA/IbpB seems also possible. Abundant IbpA/IbpB may block the access of the disaggregating/degrading machinery, as has been observed for other chaperones delivered in excess, such as ClpB or DnaJ (Weibezahl et al., 2003; Laskowska et al., 1996b). IbpA/IbpB may bind the solubilised α-glucosidase to prevent degradation and thus increase the pool for re-aggregation. In addition, as IbpA is the component that drives IbpB and other proteins in the insoluble fraction (Kuczyńska-Wiśnik et al., 2002); the higher ratio of IbpA relative to IbpB (Table 9) might contribute to the delay in disaggregation (Kuczyńska-Wiśnik et al., 2002).

4.3.3 IbpA/IbpB prevent proteolytic degradation

Not all proteins disaggregated from IBs were recovered in biological active form. The proteins released from IBs can be subjected to proteolytic degradation (Carrió and Villaverde, 2000). The degradation of α-glucosidase was decelerated at higher IbpA/IbpB levels (Fig. 26). The IbpA/IbpB activity again was dependent on temperature: the degradation was not prevented at low temperatures by IbpA/IbpB (Fig. 26), while the degradation was avoided with *ibpAB* overexpression during reactivation at 30°C (Fig. 26). High temperature during production diminished the degradation even without *ibpAB* overexpression, but not in *ibpAB* deletion strain (Fig. 29). Thus, the IbpA/IbpB decelerate the degradation. Han et al. (2004) has showed that the recombinant products were protected by IbpA/IbpB from cytoplasmic protease during production.

The function of IbpA/IbpB in preventing degradation could be a consequence of reduced concentration of protease (see 4.2.2). As degradation is less efficient with putative larger IBs, higher levels of the recombinant protein in direct correlation with IbpA/IbpB availability would accumulate, as was in fact observed with α-glucosidase accumulation (Table 10). Lower production levels in an *ibpAB* deletion strain have been reported (Carrió and Villaverde, 2002). The direct role of IbpA/IbpB in preventing degradation is not excluded. The misfolded proteins might bind to IbpA/IbpB and thereby block the access for proteases. IbpA/IbpB might drive proteins into insoluble fraction as speculated (Carrió and Villaverde,

2002; Kuczyńska-Wiśnik et al., 2002) and thus prevent the degradation. IbpA/IbpB do not affect the resolubilisation and reactivation

The yield of resolubilisation and reactivation is determined by the difference in the rates of disaggregation and degradation. As both processes were influenced in parallel by IbpA/IbpB levels, the yield of resolubilised, reactivated α-glucosidase was hardly affected by the IbpA/IbpB availability. This is in accordance with previous results, where little effect of *ibpAB* deletion on reactivation could be observed (Carrió and Villaverde, 2001; Kędzierska et al, 2001; Kuczyńska-Wiśniket al., 2002).

The reactivation yield in both strains with modified levels of IbpA/IbpB during reactivation at optimum temperature for reactivation of 24°C or 30°C was about 15-30% lower than in the strain with wildtype IbpA/IbpB levels (Fig. 27). The reactivation yield at 15°C was highest in the absence of *ibpAB*, whereas with higher temperatures for production or reactivation of α-glucosidase, overexpression of *ibpAB* slightly enhanced the reactivation yield (Fig. 27 and Fig. 28).

Since IbpA/IbpB are believed to hold the misfolded proteins in folding-competent intermediates in the soluble fraction, as sHsp from other organisms do (Haslbeck et al., 1999; Ehrnsperger et al., 1997; Lee et al., 1997), the previous reports are more or less concentrated in this direction. Therefore small effect of IbpA/IbpB could be seen. Recent data revealed that IbpA/IbpB accelerate the disaggregation of the heat-aggregated proteins *in vivo* and *in vitro* (Mogk et al., 2004a, b). We showed here that the disaggregation of α-glucosidase IBs *in vivo* is decelerated with *ibpAB* overexpression. Different properties of IBs and heat aggregates might explain this opposite behaviour. Whereas IBs are very dense and light-scattered particles (Marston, 1986), heat aggregates are rather diffuse and have smaller density (Kucharczyk et al., 1991). Whereas heat-aggregates quickly disaggregate after temperature reduction (Kucharczyk et al., 1991), IBs disaggregation and proteolyse are not possible or only partial possible (Corchero et al., 1997). Therefore, the ClpB might be more important for big compact IBs with smaller specific surface than small loose heat aggregates and thereby IBs dissolution is more sensitve to small concentration of ClpB. Furthermore, the IBs from different recombinant proteins showed different need for ClpB and thus might result in different effect of the IbpA/IbpB. The removal of IBs α-glucosidase is impossible without

clpB, whereas the removal of IBs consisting of a β-galactosidase fusion protein is unaffected by *clpB* (Carrió and Villaverde, 2003)

High concentration of IbpA/IbpB can increase the protection of recombinant protein from the degradation during production, resulting on higher yield of recombinant product (Table 10) or during disaggregation (Fig. 28). However since IbpA/IbpB is only one member from the cell network and apparently has overlapping substrate-spectrum with DnaK, too high concentrations of IbpA/IbpB supresses the heat-shock response and delays the disaggregation.

IbpA/IbpB seem to facilitate refolding after production at high temperature. A prolonged reactivation phase was observed after production of α-glucosidase for two hours at 42°C with *ibpAB* overexpression. The final reactivation yield under these conditions was slightly higher than the yield without *ibpAB* overexpression (Fig. 28). Thus, whereas high levels of IbpA/IbpB can increase the reactivation yield only after production at high temperature, their wildtype levels can increase the reactivation yield already after a mild shock.

4.3.4 Temperature-regulation of the IbpA/IbpB activity

Temperature plays an important role in regulating IbpA/IbpB activity. It does not only have an effect on the level, but also on the activity of IbpA/IbpB. α-Glucosidase accumulated to the higher levels with *ibpAB* overexpression and to the lower levelc in *ibpAB* deletion strain than wild type strain with wild type level of IbpA/IbpB. This effect is smaller at 24C and greater at higher temperatures (Table 10). Since the levels of IbpA/IbpB varied in parallel with the temperature (Table 9), the stronger effect at higher temperature can be interpreted as effect of IbpA/IbpB levels. However, similar IbpA/IbpB levels were obtained in the wildtype strain at 37°C and 42°C (Table 10); nevertheless, the difference in product accumulation between in the wildtype and the *ibpAB* deletion strains was bigger at higher temperature of 42°C than at 37°C (Table 10). This is attributed to the stimulating effect of higher temperature on IbpA/IbpB activity. The higher IbpA/IbpB activity can also explain the lower degradational loss of α-glucosidase (Fig. 29) and the higher reactivation yield (Fig. 9) after production at high temperature of 42°C.

The temperature-dependent activity of IbpA/IbpB might be a result of temperature-driven conformation changes of IbpA/IbpB. Stimulation of the transition to an active state by higher temperatures is known for other small heat-shock proteins (Haslbeck et al., 1999), and

IbpA/IbpB undergo similar structural changes from large complexes to smaller entities exposing hydrophobic putative client binding sites at higher temperatures (Kitagawa et al., 2002; Shearstone and Baneyx, 1999).

Thus, with proper reactivation by high temperatures, IbpA/IbpB is an efficient tool to manage protein reactivation and prevent protein degradation.

4.4 Application to production of recombinant proteins

Overexpression of *ibpAB* can increase the product accumulation, as shown for α-glucosidase. A prevention of degradation of recombinant proteins by *ibpAB* overexpression hence can be used for production of protease-sensitive protein. Han et al., (2004) has showed, that overexpression of *ibpAB* is necessary for production of numerous other recombinant proteins at temperature of 37°C. Furthermore, since (1) IbpA/IbpB could not promote protein folding but can deliver folding-competent intermediates to the DnaK system for folding and (2) overexpression of *ibpAB* repress the synthesis of other heat shock chaperones, further coexpression of *ibpAB* with chaperones *dnaKJ, grpE* might ameliorate the production of active α-glucosidase.

In *in vivo* reactivation protocol, specific α-glucosidase activities were twofold higher after 5 h incubation at 30°C in shake flasks experiments after production at 42°C (Fig. 12).
Coexpression of *ibpAB* with *dnaK*/*dnaJ*/*grpE* and *clpB* can be considered. The recovery of proteins from IBs was increased after coexpression with a set of chaperones *dnaK/dnaJ/grpE* and *clpB* and *groEL/groES* (de Marco and De Marco, 2004).

Also coexpression of *ibpB* alone can be benificial, since IbpB alone was found to drive the accumulation of the product in the soluble fraction (Kuczyńska-Wiśnik et al., 2002), in contrast to coexpression of *ibpA* alone or together with *ibpB*.

There was a report about use of *ibpAB* deletion strain as producing strain (Ochocka et al., 2002). The human Rho GTPase activating protein (ARHGAP6) was successful produced only in *ibpAB* deletion strain. The production in wildtype strain causes the cell lysis, which might be due to accumulation of denatured proteins. Thus, dependent on the properties of individual proteins, the overexpession or elimination of *ibpAB* could be beneficial for recombinant production.

5. CONCLUSION

The advances in genetic engineering permit the production of large amounts of valuable proteins, which are used for research, therapy and diagnostics. *Escherichia coli* is an often used organism for this purpose. The production of recombinant proteins in *E. coli*, however, results in most cases in accumulation of aggregated inactive proteins (inclusion bodies, IBs), thus remains a challenge for biological research. In this thesis, the cultivation parameters were optimised to maximise the yield of soluble α-glucosidase. Furthermore, a new approach of reactivating the IBs *in vivo* in order to improve the production of soluble α-glucosidase was examined. Cultivation processes were implemented and the influence of cultivation parameters on the productivity was analysed. The small heat shock proteins of *E. coli* IbpA and IbpB have been found tightly associated with IBs of numerous recombinant proteins and were implicated to change the kinetics of IBs disaggregation. Hence, their roles in the metabolism of IBs and in cell protection were investigated.

1. Important parameters that influence the α-glucosidase folding are temperature and growth rate. Temperatures that maximise the growth rate almost extensively promote aggregation of α-glucosidase, while temperatures of 30°C or below were compatible with production of active α-glucosidase. Moreover, as the growth rate is a function of temperature in shake flask experiments, maximum specific α-glucosidase activities were obtained at 24°C. But as growth was decelerated at lower temperature, the volumetric activities resulting from specific activity and cell density were equally high at 24 and 30°C.

   Fed-batch cultivations with exponentially increasing feeding rates enable to control cultures to grow with constant rates. The specific growth rate set by the feeding rate had a strong impact on the folding of α-glucosidase. At 37°C, the specific α-glucosidase activity obtained using specific growth rates of $\mu_{set} \leq 0.12\ h^{-1}$ was sixfold higher than with $\mu_{set} \geq 0.18\ h^{-1}$. Furthermore the actual growth μ was lower μ_{set} at higher feeding rates $F(\mu_{set} \geq 0.18\ h^{-1})$, whereas it was equal to μ_{set} at feeding rate $F(\mu_{set} \leq 0.12\ h^{-1})$. The data demonstated that a minimised stress at lower feeding rates can facilitate the correct folding and therefore improve the yield of active proteins. Additionally, due to lesser biomass formation during slow growth ($\mu_{set} < 0.12\ h^{-1}$), the highest volumetric product yields and productivities were achieved with a controlled growth rate of $\mu_{set} = 0.12\ h^{-1}$.

2. Disaggregation of *inclusion bodies* can be initiated by an arrest of protein synthesis and/or a reduction of the cultivation temperature. The temperatures during production and reactivation phases determine the reactivation yield. The reactivation yield is highest with production at 42°C and reactivation at 30°C. The α-glucosidase activities by this optimised protocol surpassed that values obtained with direct production under optimum conditions on complex medium: specific and volumetric α-glucosidase activities were nearly twofold higher. In fed-batch cultivations on defined medium, however, the α-glucosidase production was already very efficient: while the specific activity reached in shake flasks experiments on complex medium after five hours production 3 U mg^{-1} in direct production without reactivation and rose to 4.5 U mg^{-1} with reactivation under optimised temperature profiles, it reached 6 U mg^{-1} in fed-batch cultivation under optimised condition and rose to 9 U mg^{-1} on the end of cultivation. Since the optimised conditions for *in vivo* reactivation in fed-batch cultivations were the same as for direct production, the highest yield of α-glucosidase activity was achieved by temperature reduction at the same time of induction.

3. IbpA/IbpB protect the cells under harsh condition that overload the refolding chaperones network such as overexpression of aggregation-prone α-glucosidase; IbpA/IbpB can disburden the central chaperone DnaK, moderating the heat-shock response and enabling continual growth. Furthermore, IbpA/IbpB prevent the disaggregated proteins from degradation, with pronounced effects only at high temperatures of aggregation or disaggregation, as this is the condition of massive protein aggregation they are designed for. Therefore, cell growth during α-glucosidase production and recovery of α-glucosidase from *inclusion bodies are*, on the one hand, impaired by lack of IbpA/IbpB. Excess of IbpA/IbpB, on the other hand, suppresses the synthesis of chaperones and delay the disaggregation. Abundant IbpA/IbpB is degraded after dissociation from protein aggregates to ensure low sHsp level (Mogk et al., 2003). Moreover, induction of *ibpAB* by recombinant proteins take place only with completely aggregating products (Allen et al., 1992), while the synthesis of IbpA/IbpB is shut down with partially soluble proteins despite on-going synthesis of other heat-shock proteins (Hoffmann and Rinas, 2000). It seems that high IbpA/IbpB levels have to be restricted to requiring stress conditions. The interplay of all components of the quality control network thus needs to be finely balanced to optimise weathering of stressful situations.

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RESUME

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