From Concept to Process:
E. coli Metabolic Engineering for Production of Glucosamine and *N*-Acetylglucosamine

Bio-Technical Resources (BTR)

www.biotechresources.com

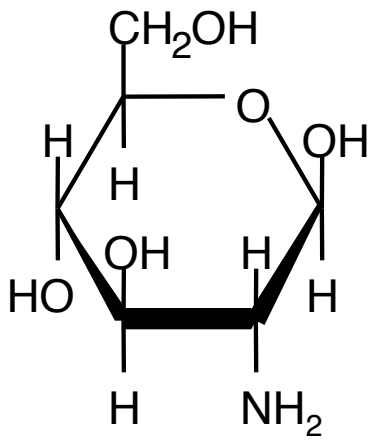
1035 S 7th Street, Manitowoc, WI 54220

Phone: (920) 684-5518

Fax: (920) 684-5519

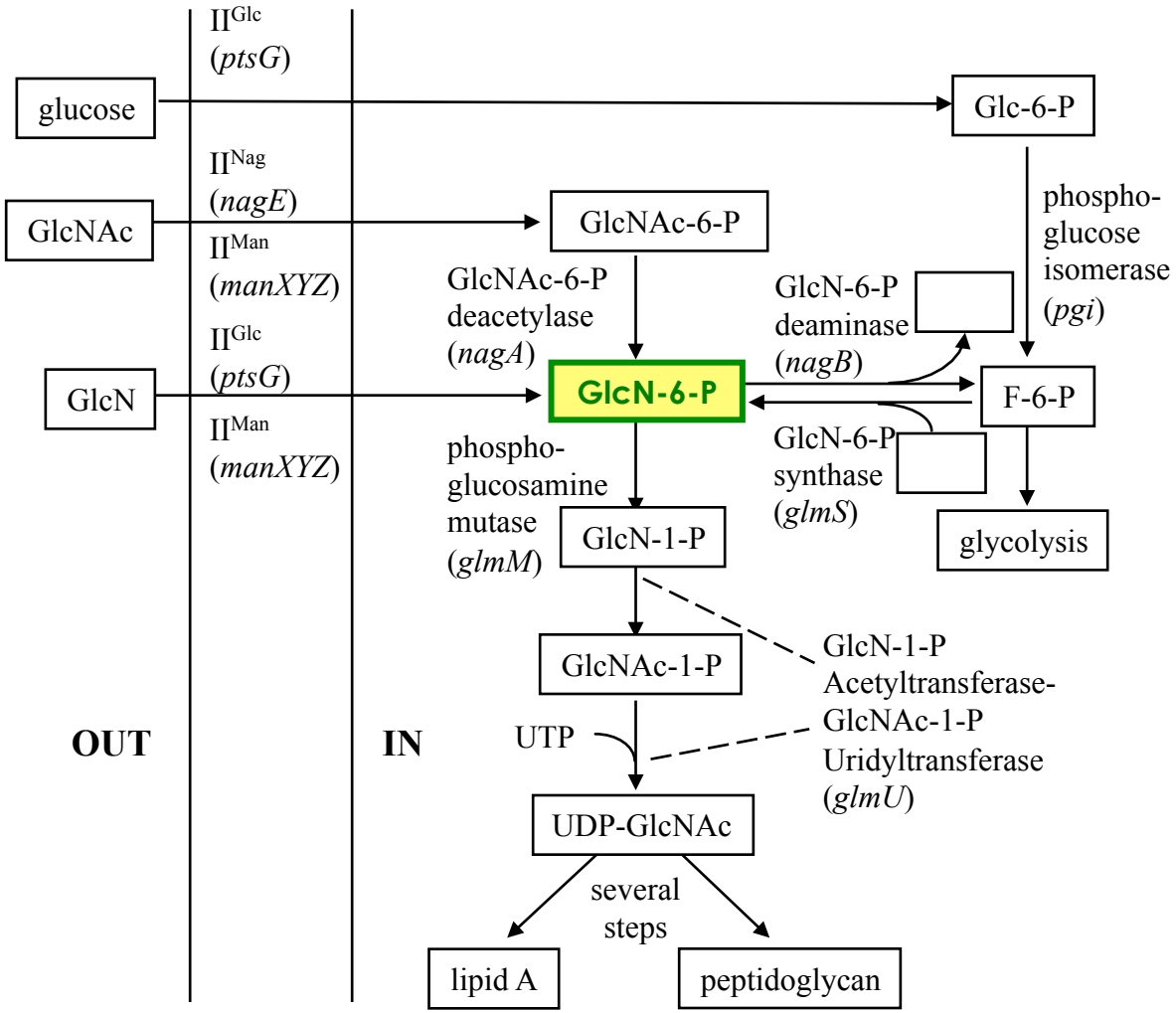
Email: info@biotechresources.com

Glucosamine

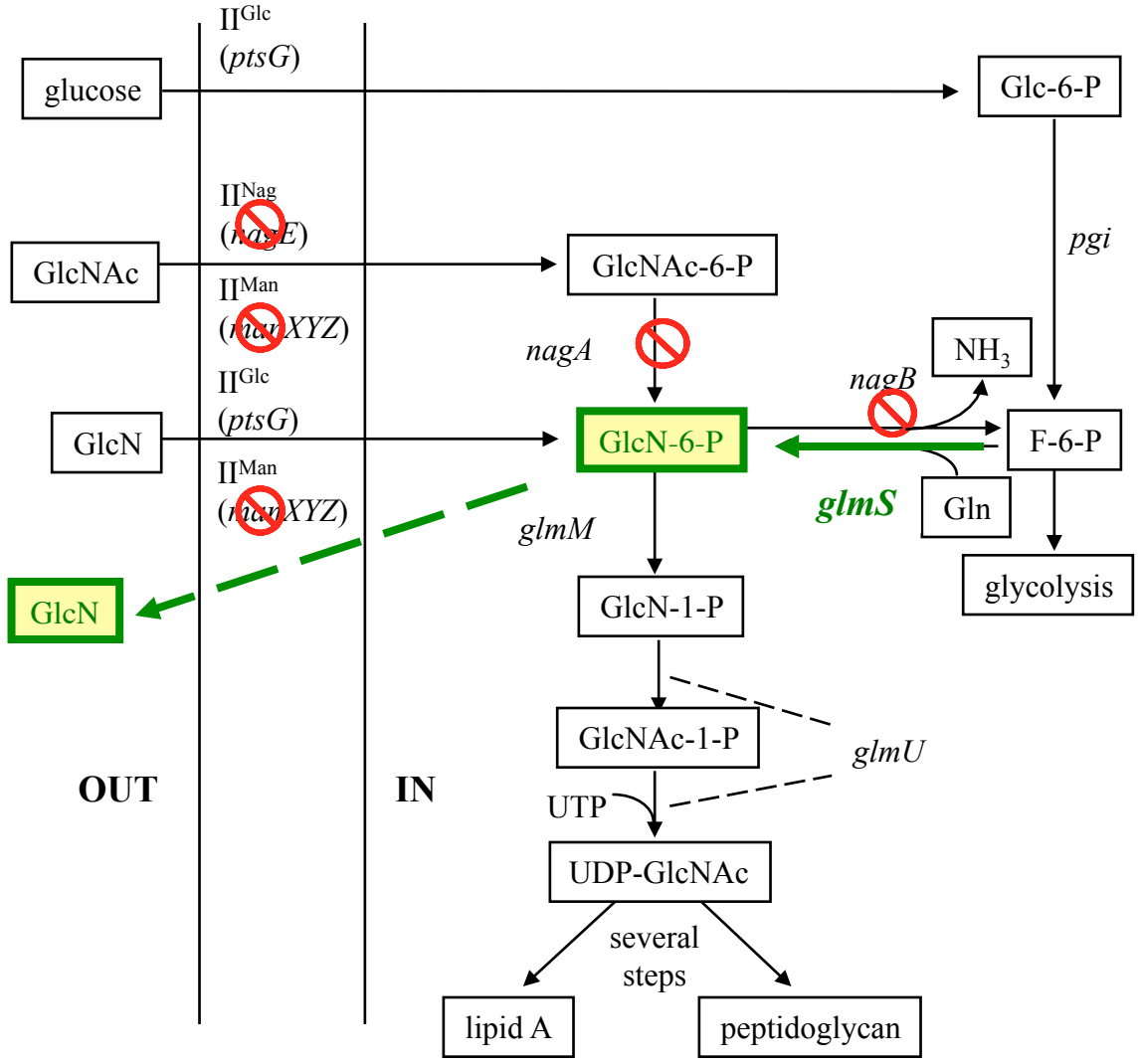


- Dietary supplement for the treatment of arthritis
- Increasing market: >$350 MM/year in the US
- Current manufacture by hydrolysis of chitin from shellfish
- Raw material supply limitation and shellfish allergy problem
- Fermentation process developed by Bio-Technical Resources

Glucosamine Pathway in *E. coli*



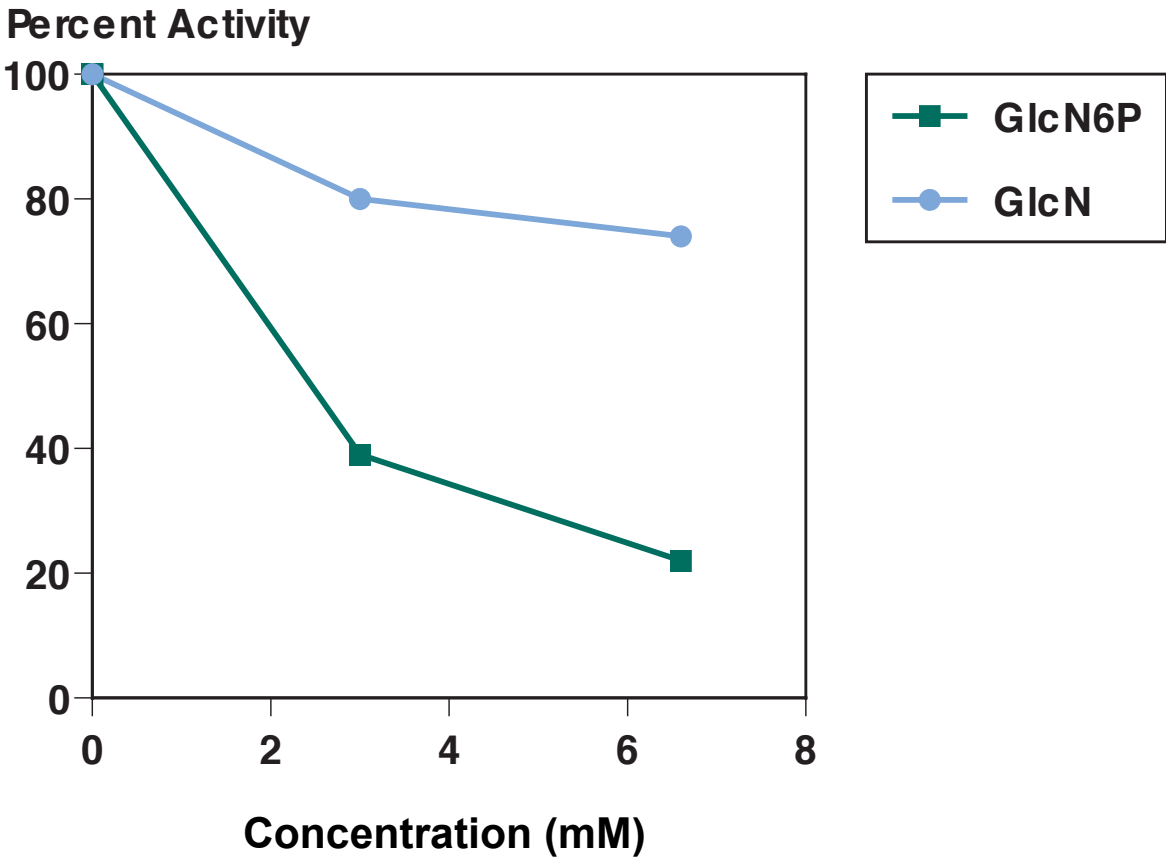
Metabolic Engineering Strategy to Produce Glucosamine



Production of Glucosamine in *E. coli*

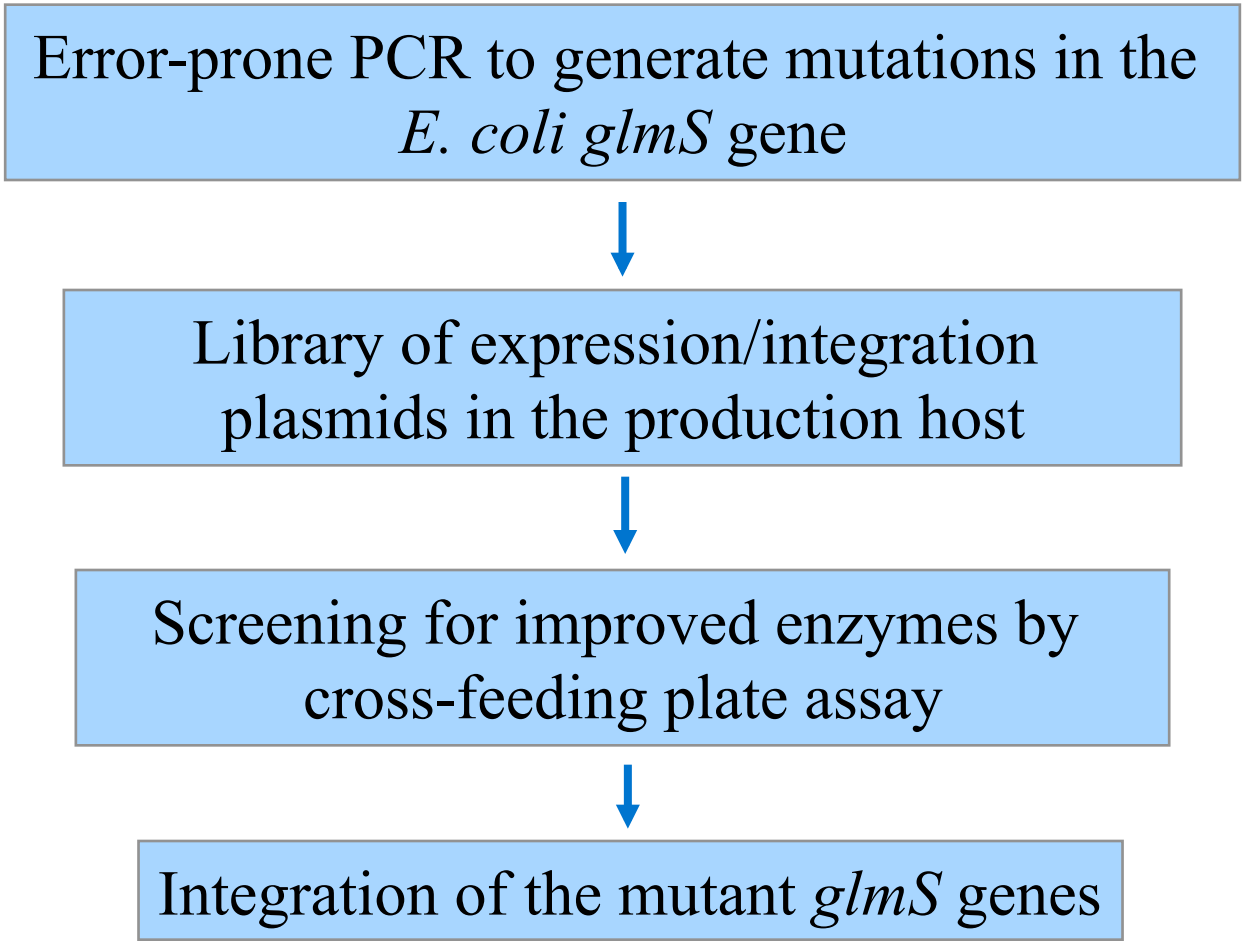
Genotype	Glucosamine, mg/L
Shake Flask:	
Host strain (Δ*nag manXYZ* DE3)	4
T7-glmS plasmid	37
T7-glmS::*ΔlacZ* (integrant)	75
Fermentor:	
T7- glmS::*ΔlacZ* (integrant)	400

Inhibition of GlcN6P Synthase

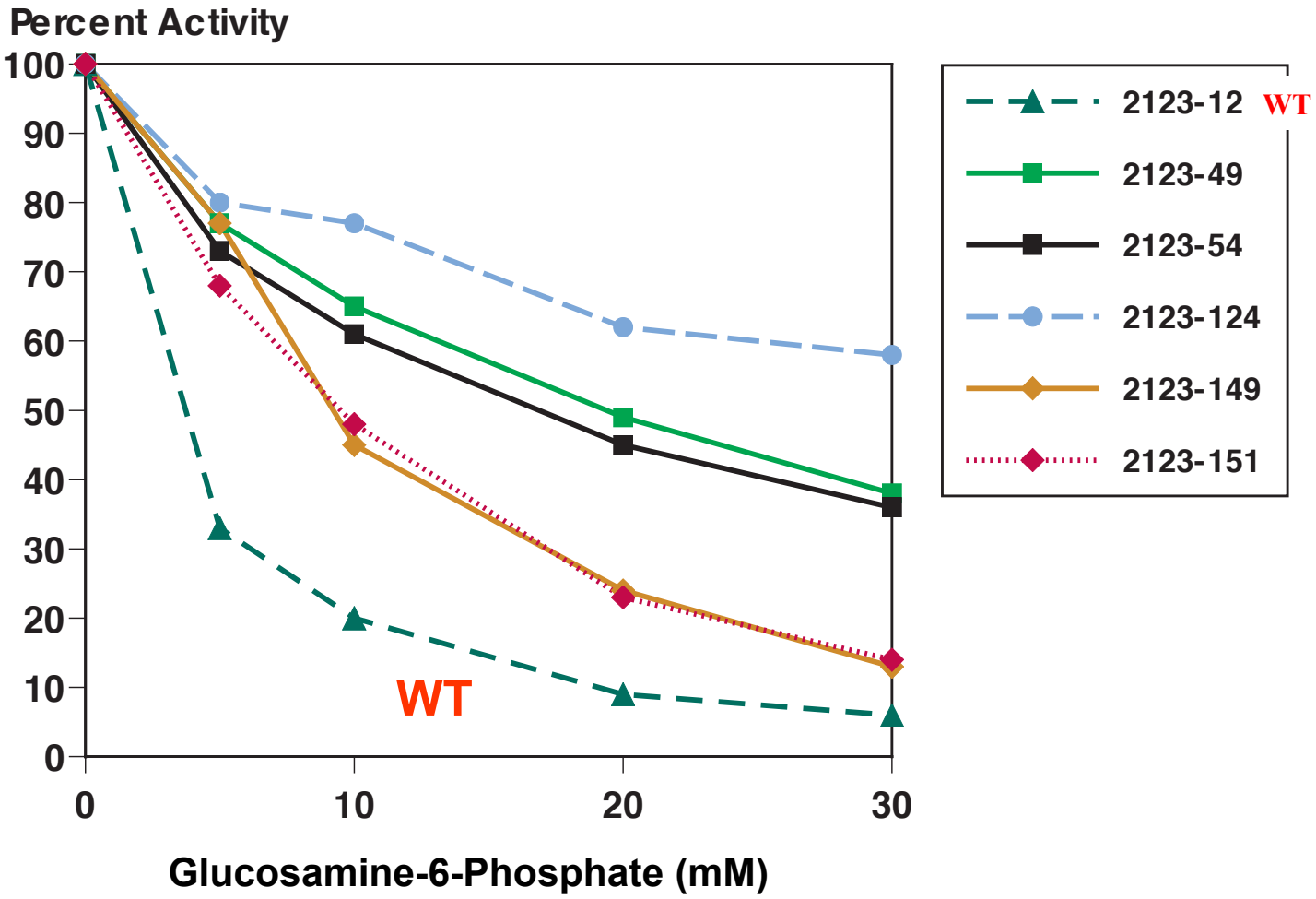


- The biosynthesis enzyme GlcN6P Synthase (GlmS) is product-inhibited
- Activity is lowered to ~20% at 6 mM GlcN6P
- Directed Evolution was undertaken to generate and select for variants resistant to product inhibition

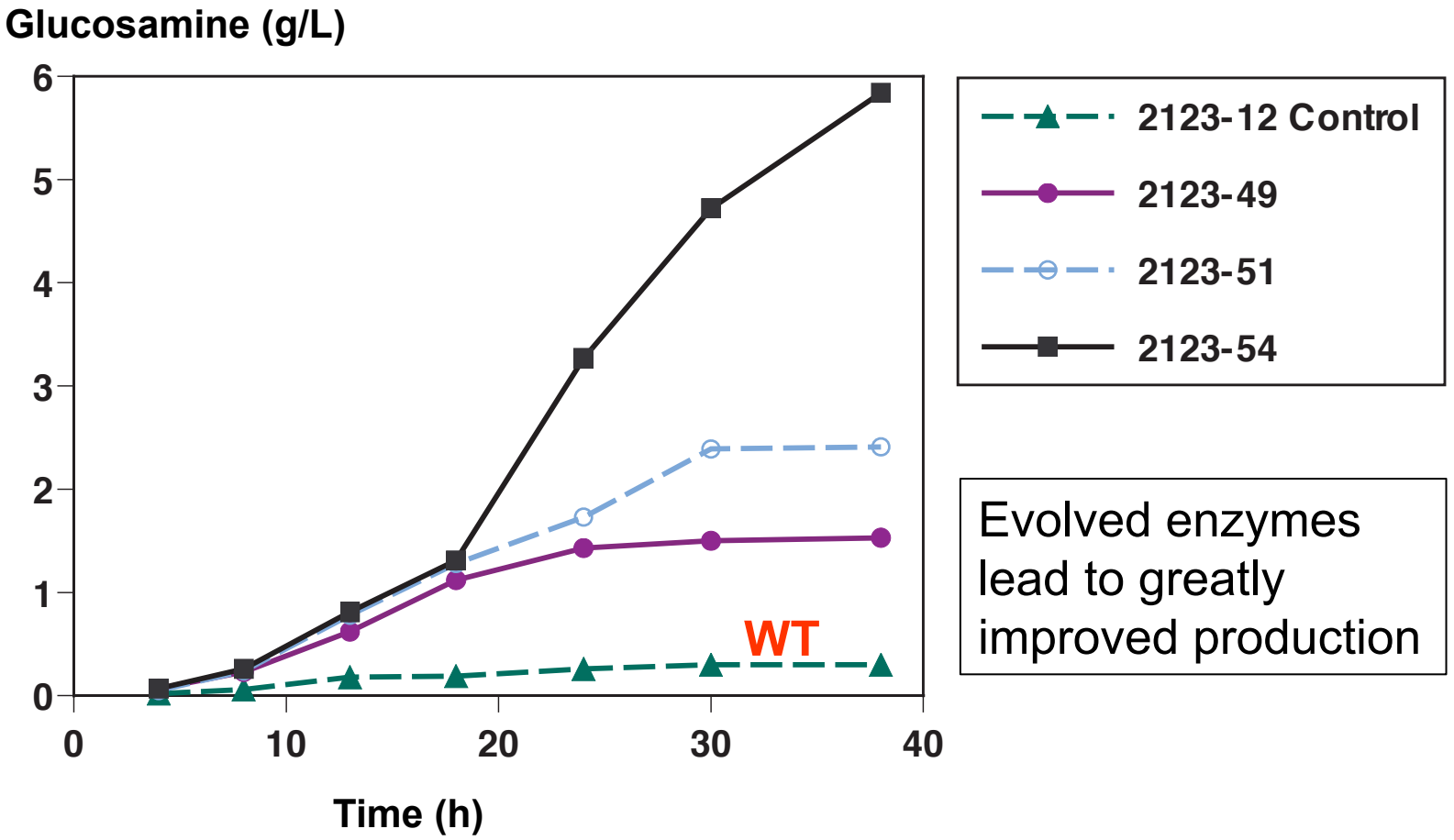
Generation of Mutant *glmS* Alleles



Product Inhibition of the WT GlmS and Evolved Variants



Glucosamine Production Using End Product-Resistant Mutants



Evolved enzymes lead to greatly improved production

Properties of GlcN6P Synthase in Mutant Strains

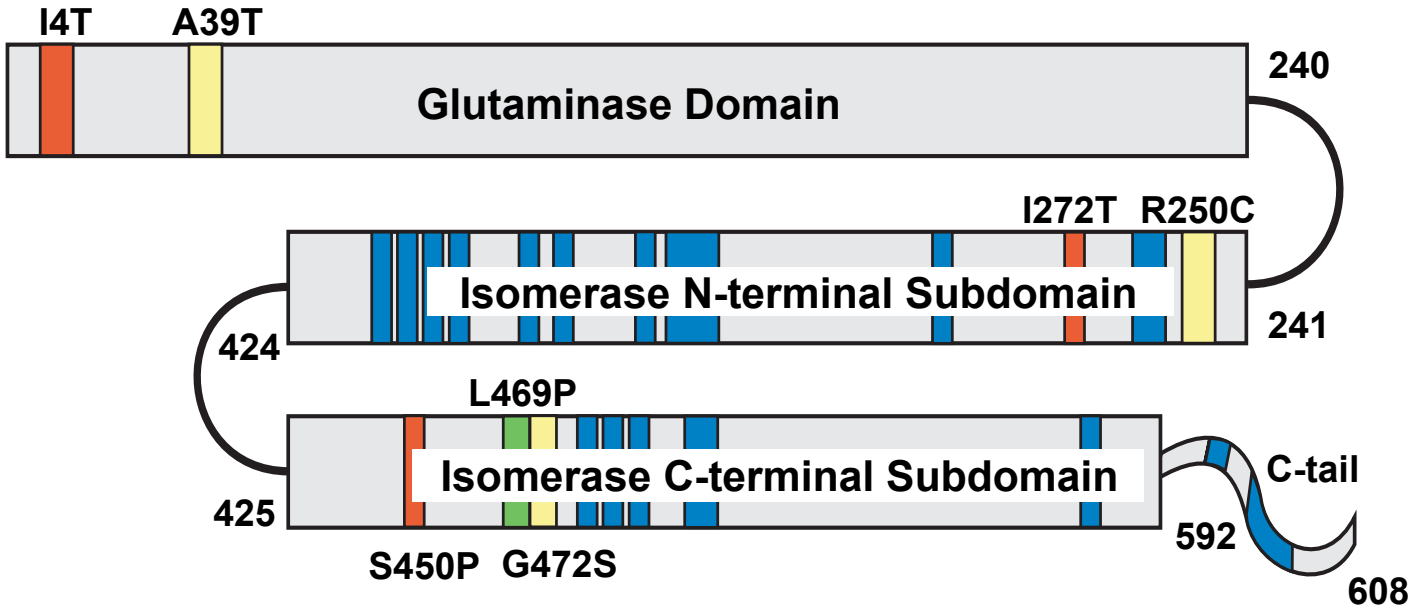
Strain	Mutations	Glucosamine Production[1]	Specific Activity	Product Resistance[2]	Thermal Stability[3]
2123-12	none	0.3	0.39	20	95
2123-49	I4T I272T S450P	4.6	0.38	65	8
2123-54	A39T R250C G472S	7.2	0.42	61	4
2123-124	L469P	5.3	0.008	77	101
2123-149	G472S	0.6	0.49	45	78

[1]Glucosamine production: g/L

[2]Percent activity with 10 mM glucosamine-6-phosphate

[3]Percent activity after 30 minutes at 50˚C

Locations of *glmS* Mutations and Invariant Residues



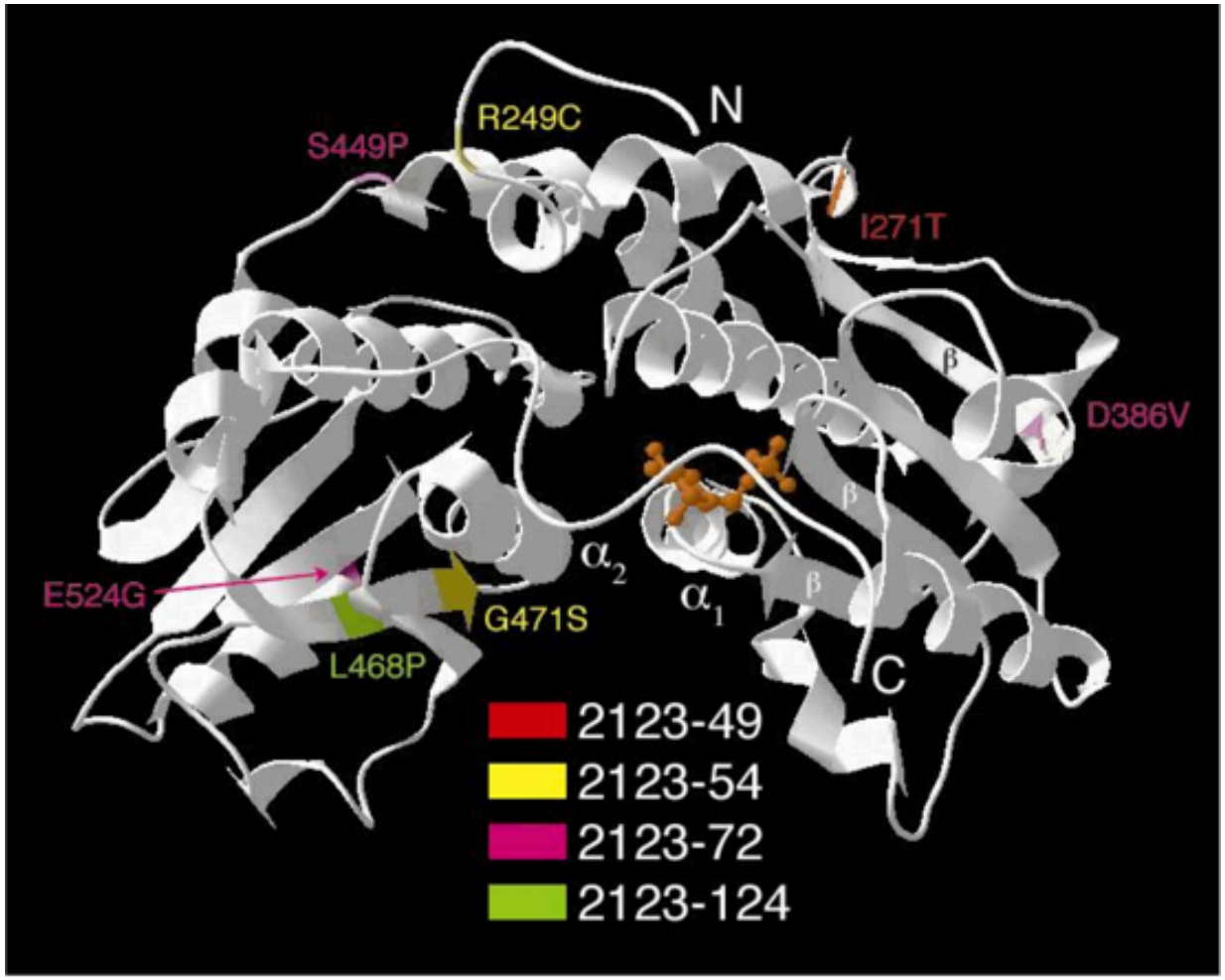
2123-49

2123-54

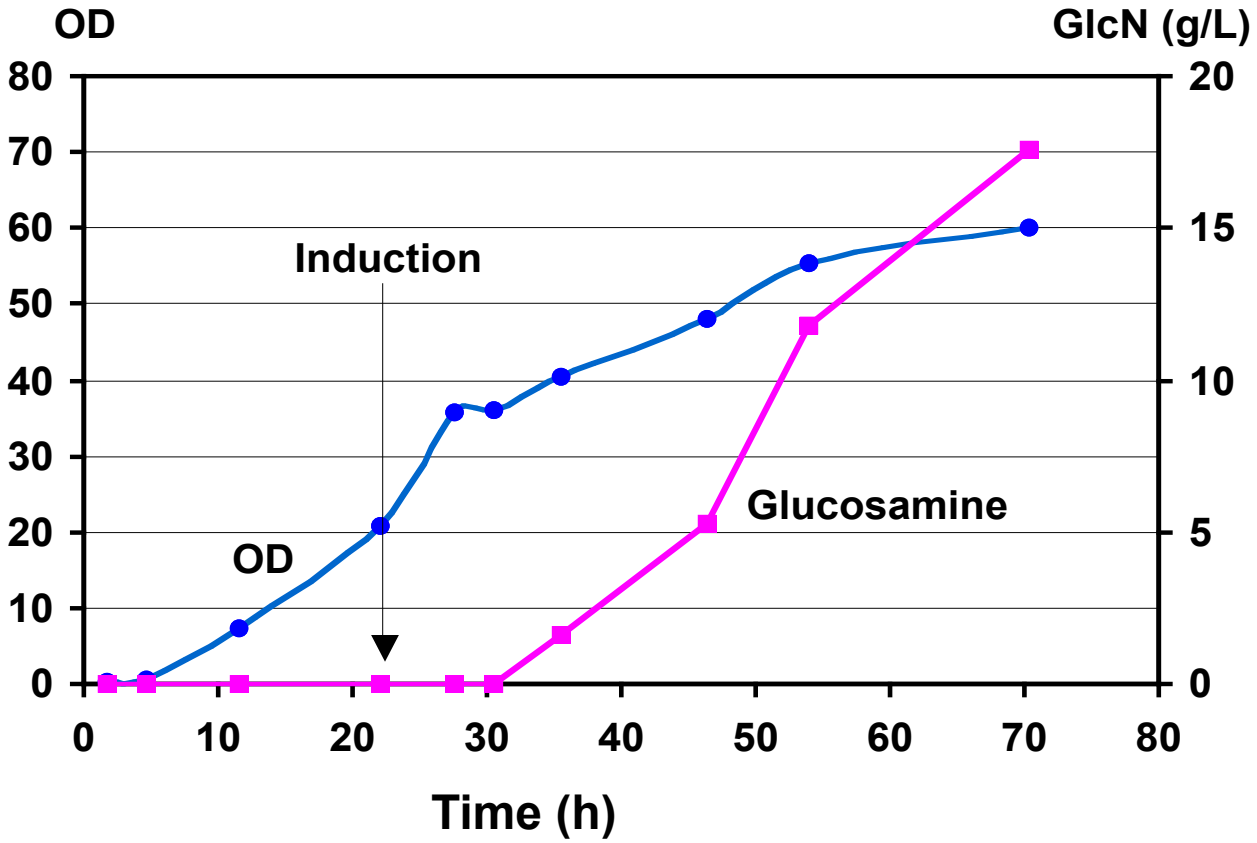
2123-124

Invariant Residues

Mutations in the GlmS Isomerase Domian

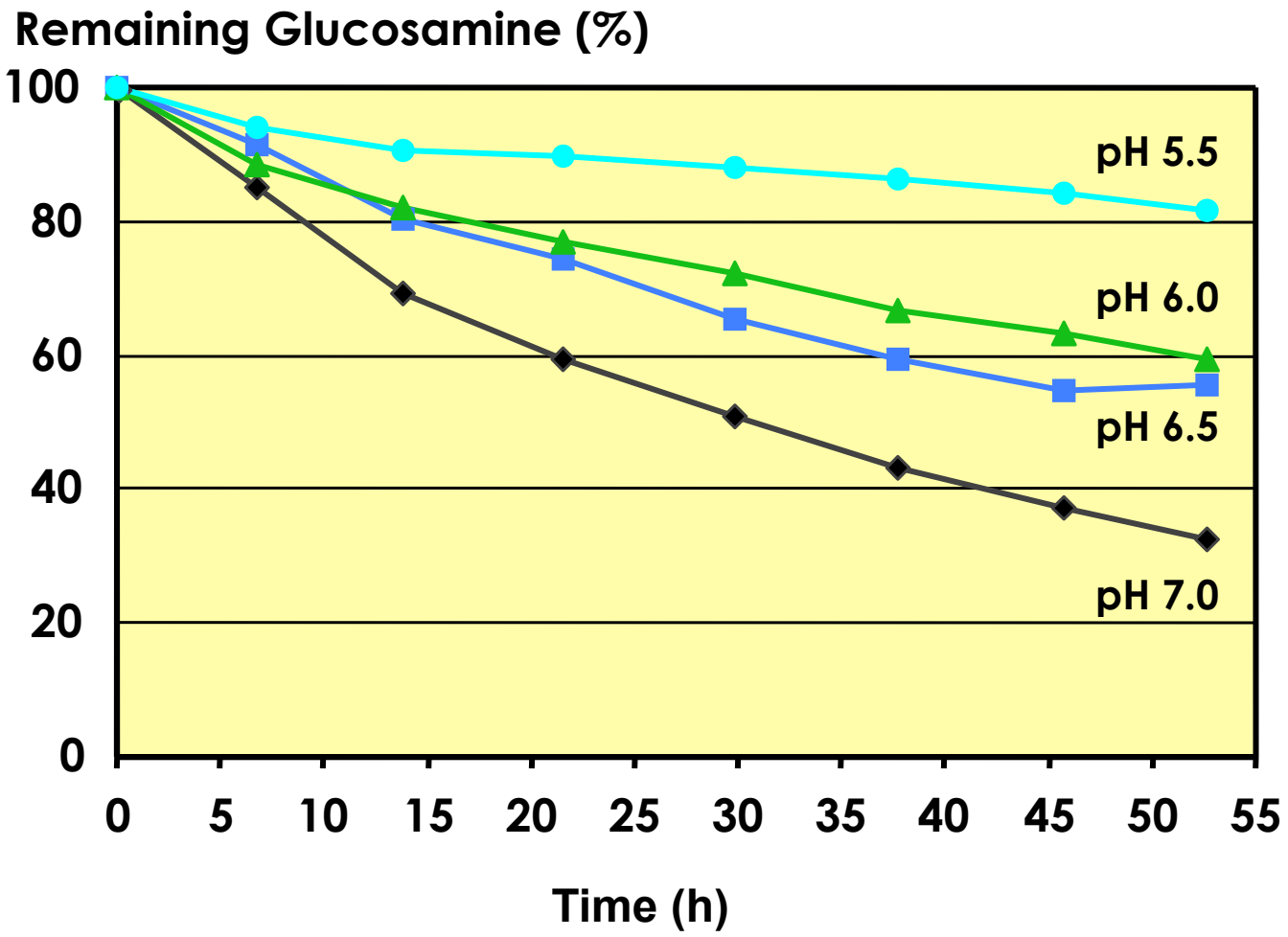


Lactose-Induced Glucosamine Production

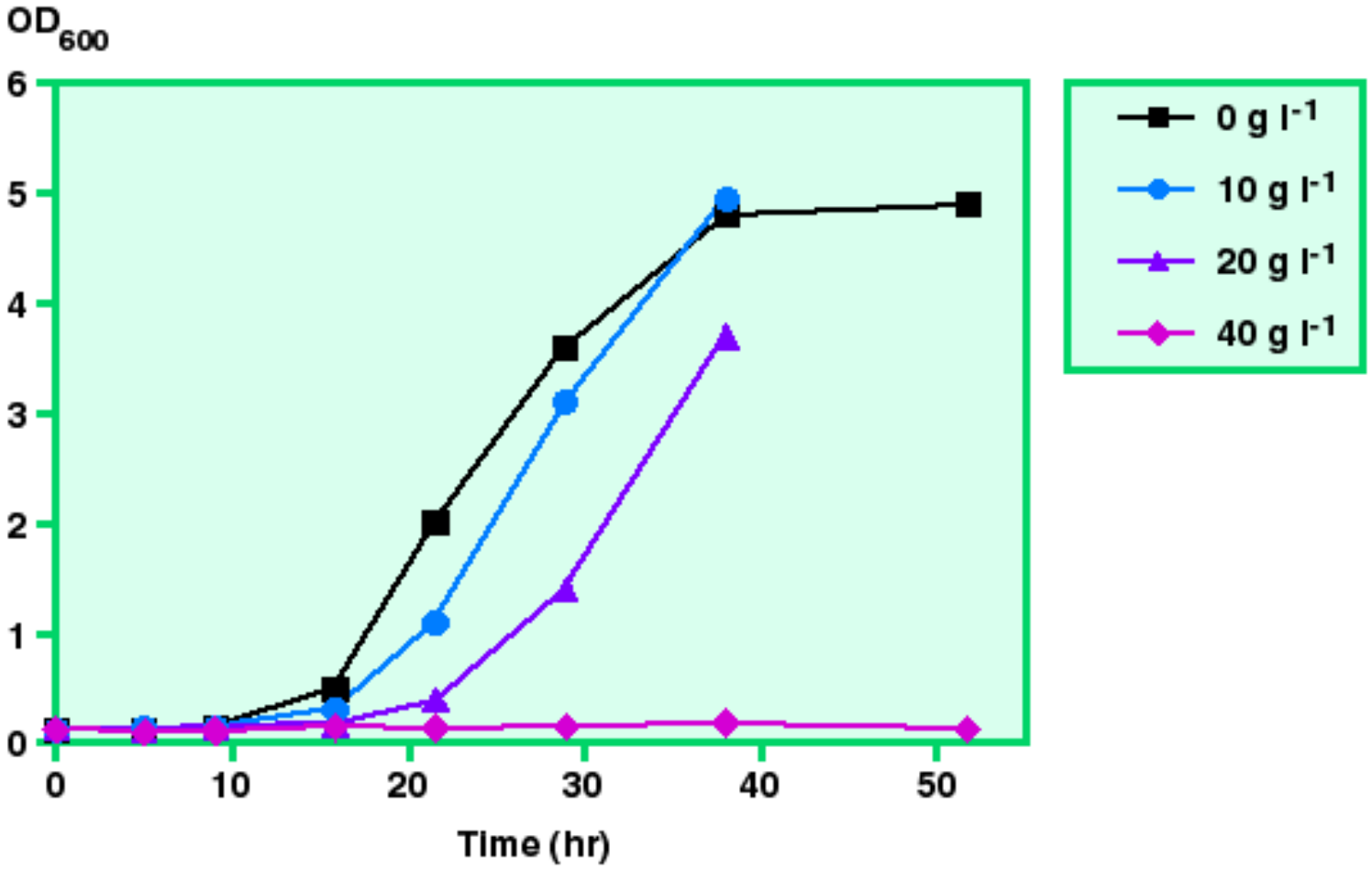


- Protocols for lactose induction of GlcN production were established in one-liter fermentors
- GlcN production reached 17 g/L, ~4,000-fold improvement over the wild type *E. coli* strain
- Titer was still far below the target for commercialization of 50 g/L

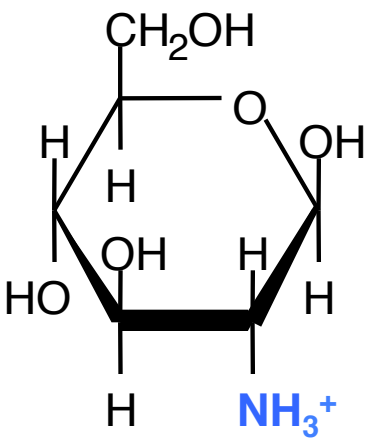
Glucosamine Degradation at Different pH



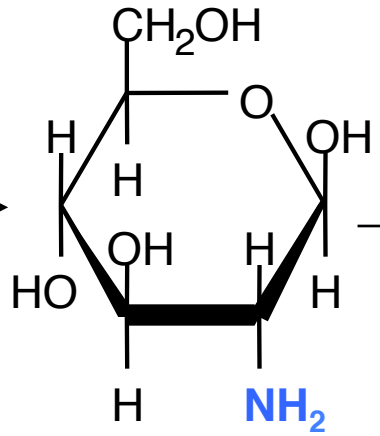
Growth Inhibition by Glucosamine at Different Concentrations



How to Overcome the Challenges?
Glucosamine Degradation and Toxicity

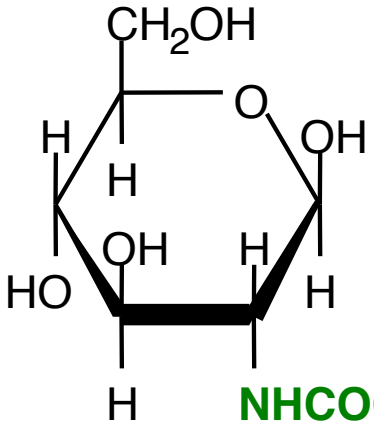


Low pH



High pH

Glucosamine Degradation

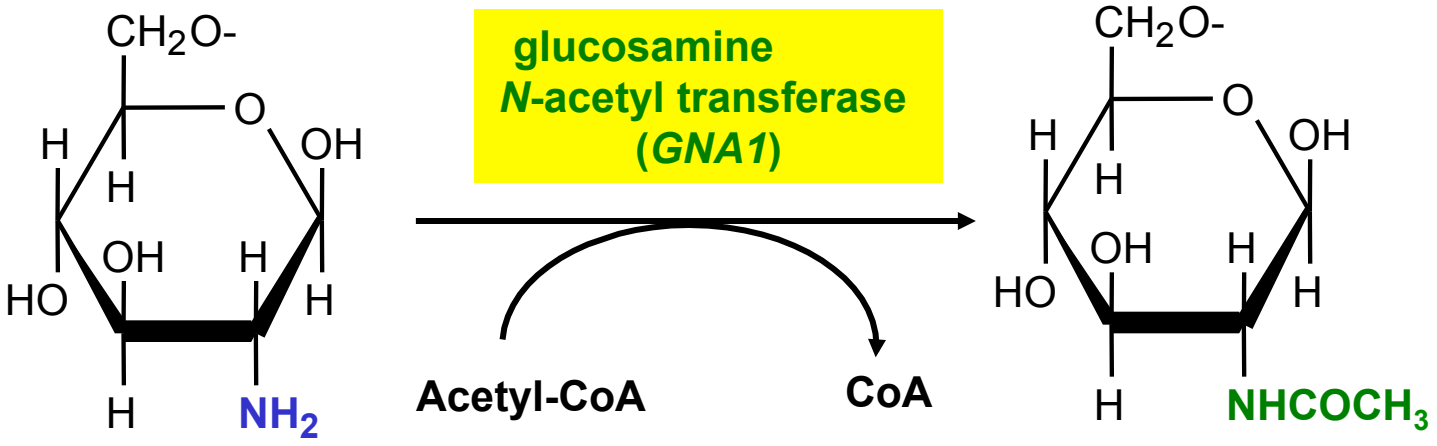


***N*-Acetylglucosamine (NAG)**
Very stable

N-Acetylglucosamine as a Fermentation Product

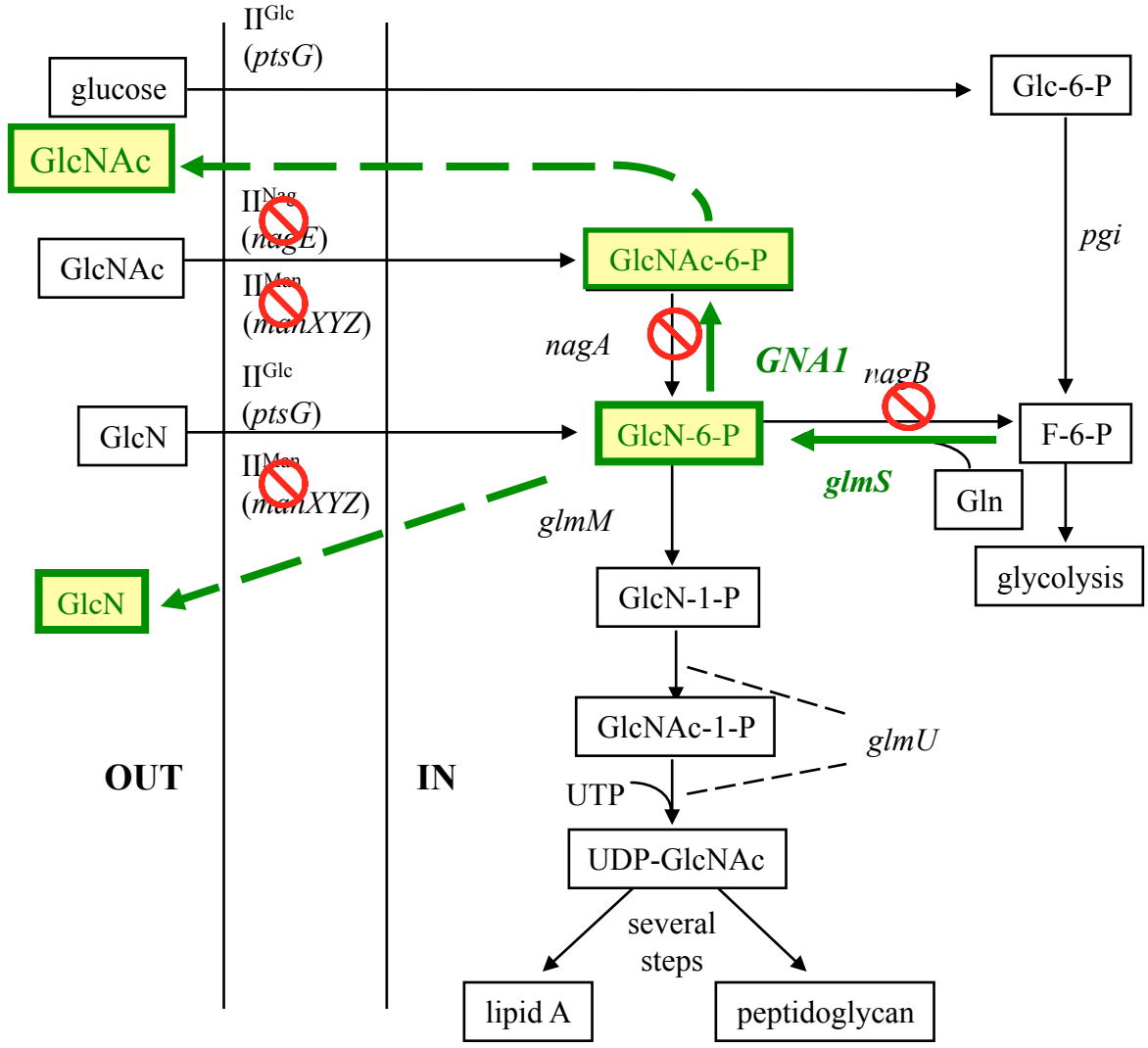
- **Favorable fermentation product characteristics**
  - Stable over a range of pH values
  - Non-inhibitory to growth
  - Readily converted to glucosamine by hydrolysis
- **Straightforward to modify the pathway**

Over-Expression of a Yeast *GNA1* Gene

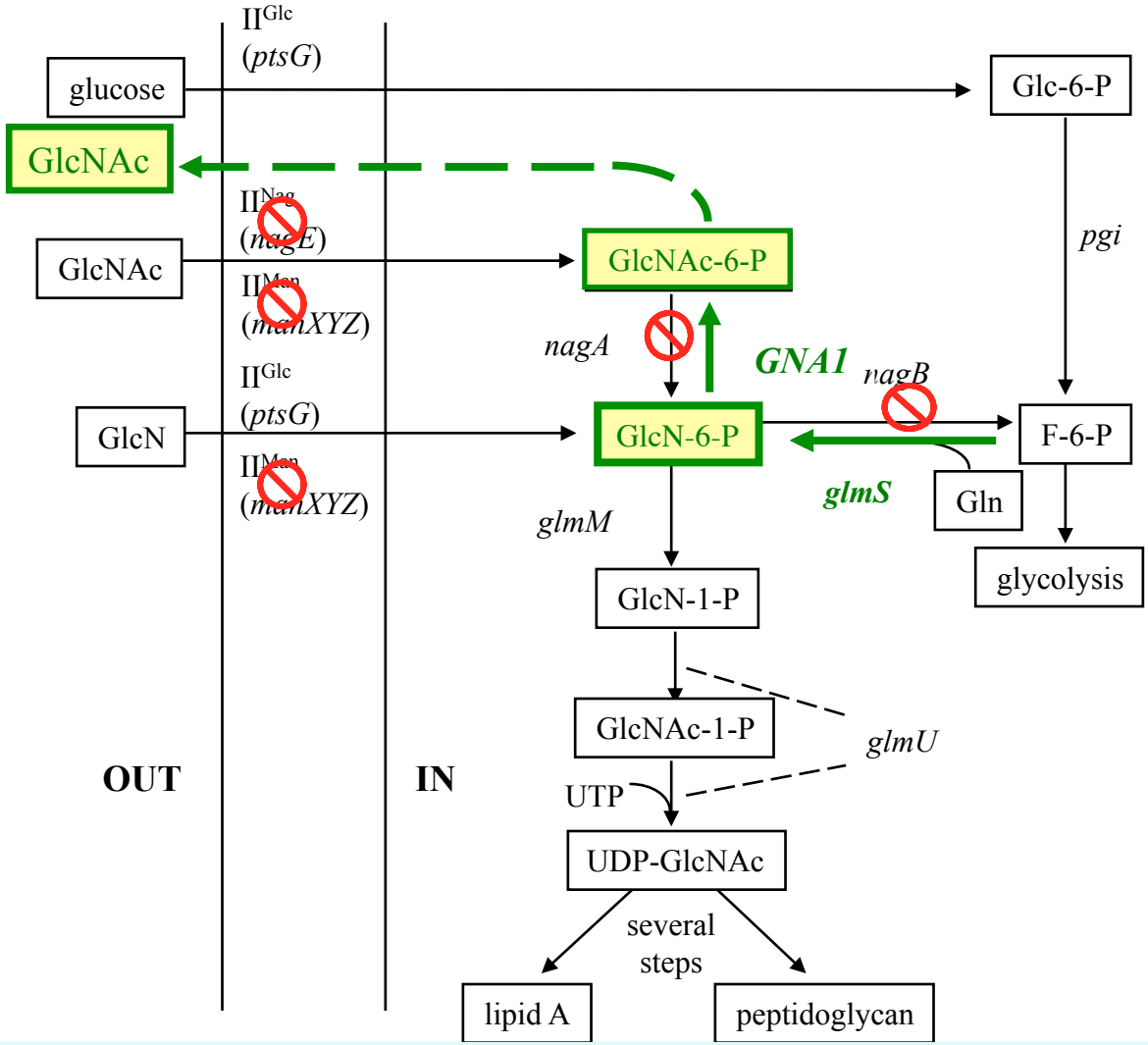


GlcN-6-P **GlcNAc-6-P**

Metabolic Engineering to Produce *N*-Acetylglucosamine

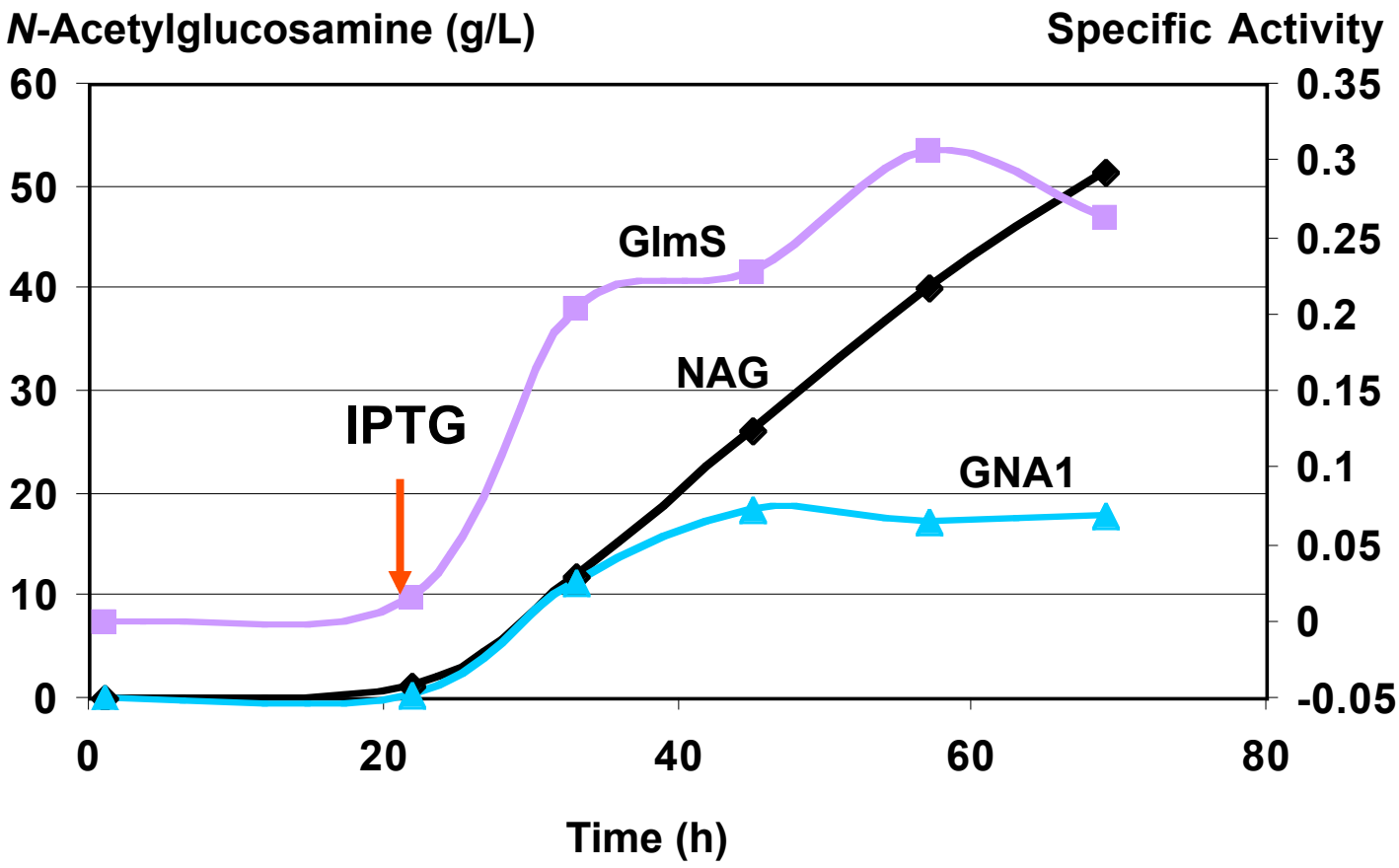


Metabolic Engineering to Produce *N*-Acetylglucosamine



- GlcNAC: accumulated in the medium as the only amino sugar product
- GlcN: not detectable

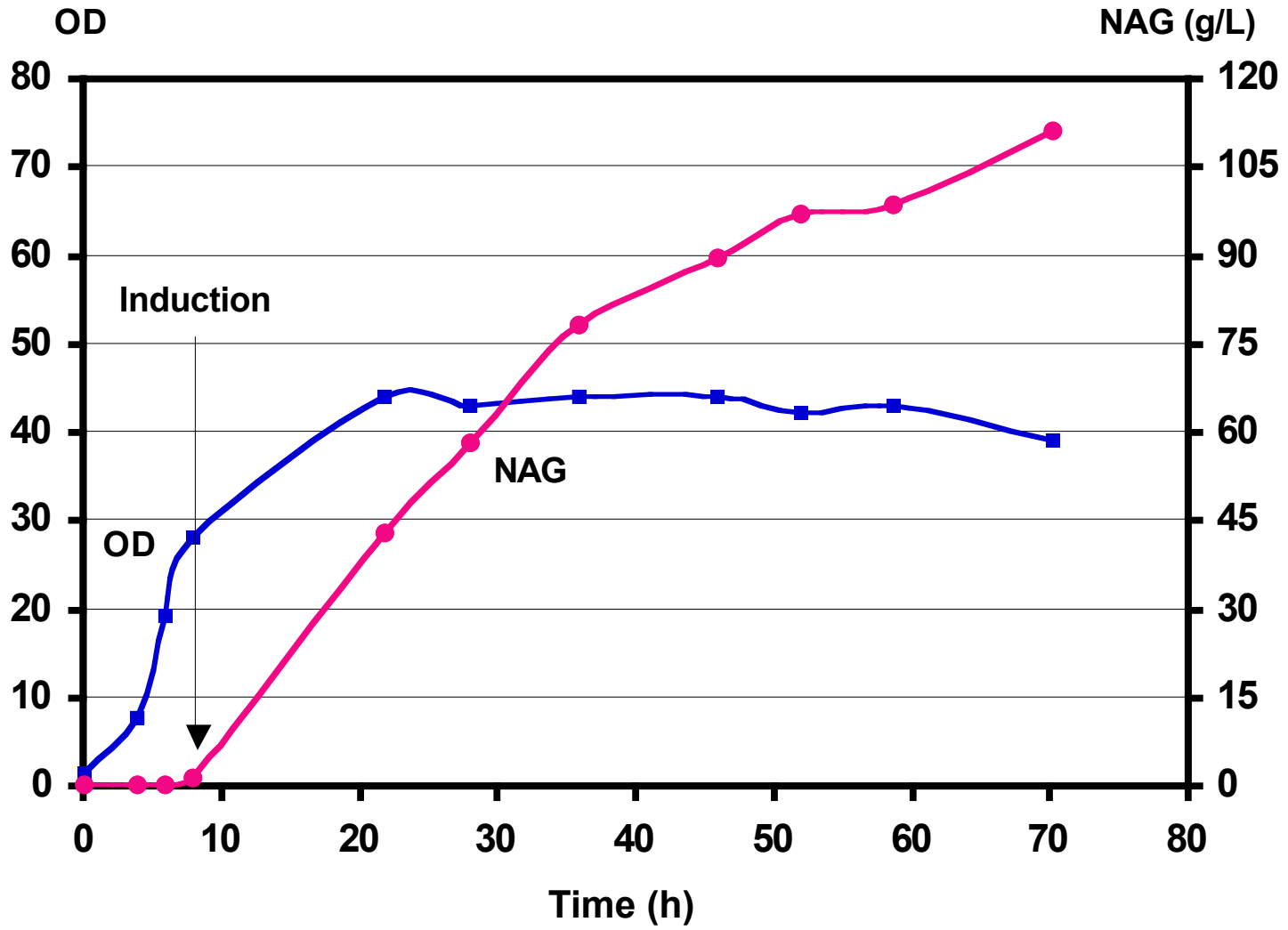
NAG Fermentation with a GNA1 Plasmid



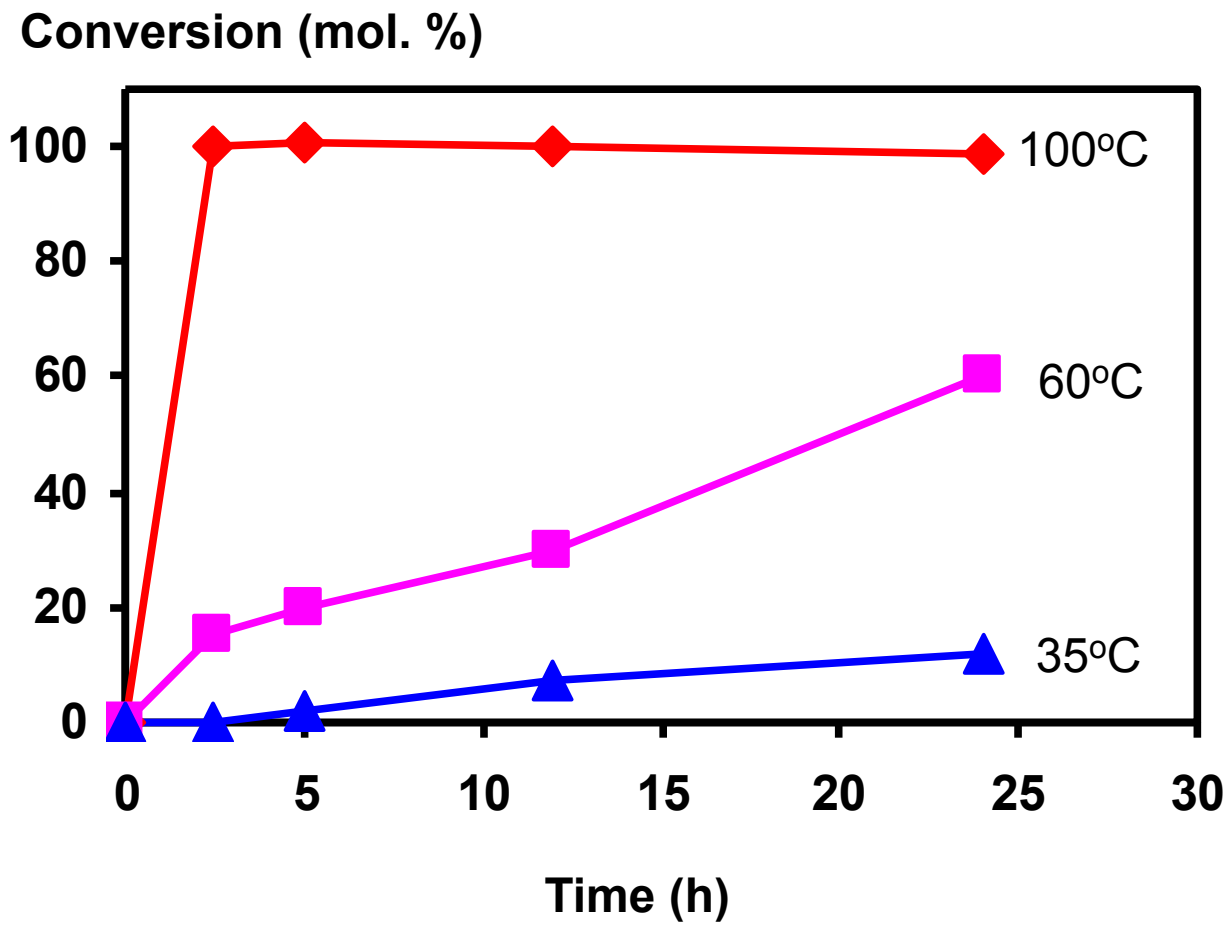
Process Parameters for *N*-Acetylglucosamine Fermentation

- **Physical**
  - Temperature: 30ºC
  - pH: 6.9 (controlled with ammonia)
  - Oxygen: 20% or higher
- **Process**
  - Medium: Defined, minimal salts + glucose and NH_3
  - Protocol: Bi-phasic (growth and production phases)
  - Induction: Lactose
  - Cell Density: 25 g/L

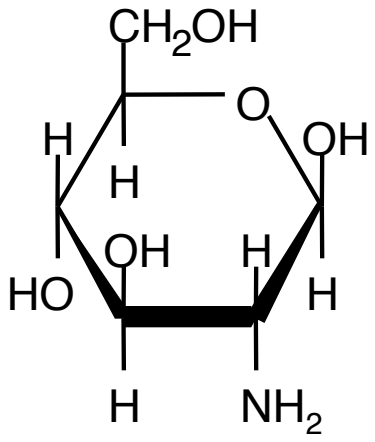
Lactose-Induced *N*-Acetylglucosamine Production



N-Acetylglucosamine Hydrolysis to Glucosamine

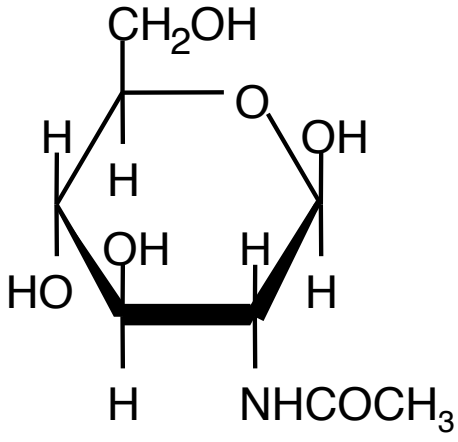


Glucosamine



- Increasingly used nutraceutical to treat arthritis
- Current manufacture by hydrolysis of chitin/chitosan from shellfish

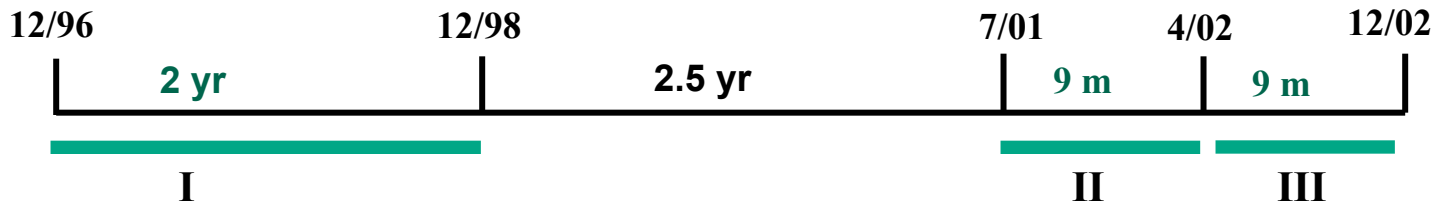
N-Acetylglucosamine



- Many nutraceutical, pharmaceutical and cosmetic applications
- Current manufacture by chemical *N*-acetylation of glucosamine

- Raw material supply limitation and shellfish allergy problem
- Fermentation processes developed by Bio-Technical Resources

Technology Development Time Line



- **Phase I**
  - Metabolic engineering feasibility
  - Generation of improved GlmS enzyme
- **Phase II:**
  - Glucosamine process improvement
  - Identification of the key problem: glucosamine degradation
  - Concept of NAG fermentation
- **Phase III:**
  - Modification of the pathway for NAG production
  - Development of a high-performance NAG process

Acknowledgments

Phase I:

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Phases II and III:

Ming-De Deng, Sarah Wassink, Candice Leanna, Kathy Nielsen, Al Grund, Jeff Running, Linsheng Song, Dave Severson, Brian Huckins, Bonnie Walsh, Troy Lutze, Reinhardt Rosson

References:

- Deng MD, Severson DK, Grund AD, Wassink SL, Burlingame RP, Berry A, Running JA, Kunesh CA, Song L, Jerrell TA and Rosson RA. Metabolic Engineering of *Escherichia coli* for industrial production of glucosamine and *N*-acetylglucosamine. Metabolic Engineering **7**:201-214 (2007)

- Deng MD, Grund AD, Wassink SL, Peng SS, Nielsen KL, Huckins BD, Walsh BL and Burlingame RP. Directed evolution and characterization of *Escherichia coli* glucosamine synthase. Biochimie, 2006, 88:419-429