E. coli Fermentation Process Development for Production of Glucosamine and *N*-Acetylglucosamine

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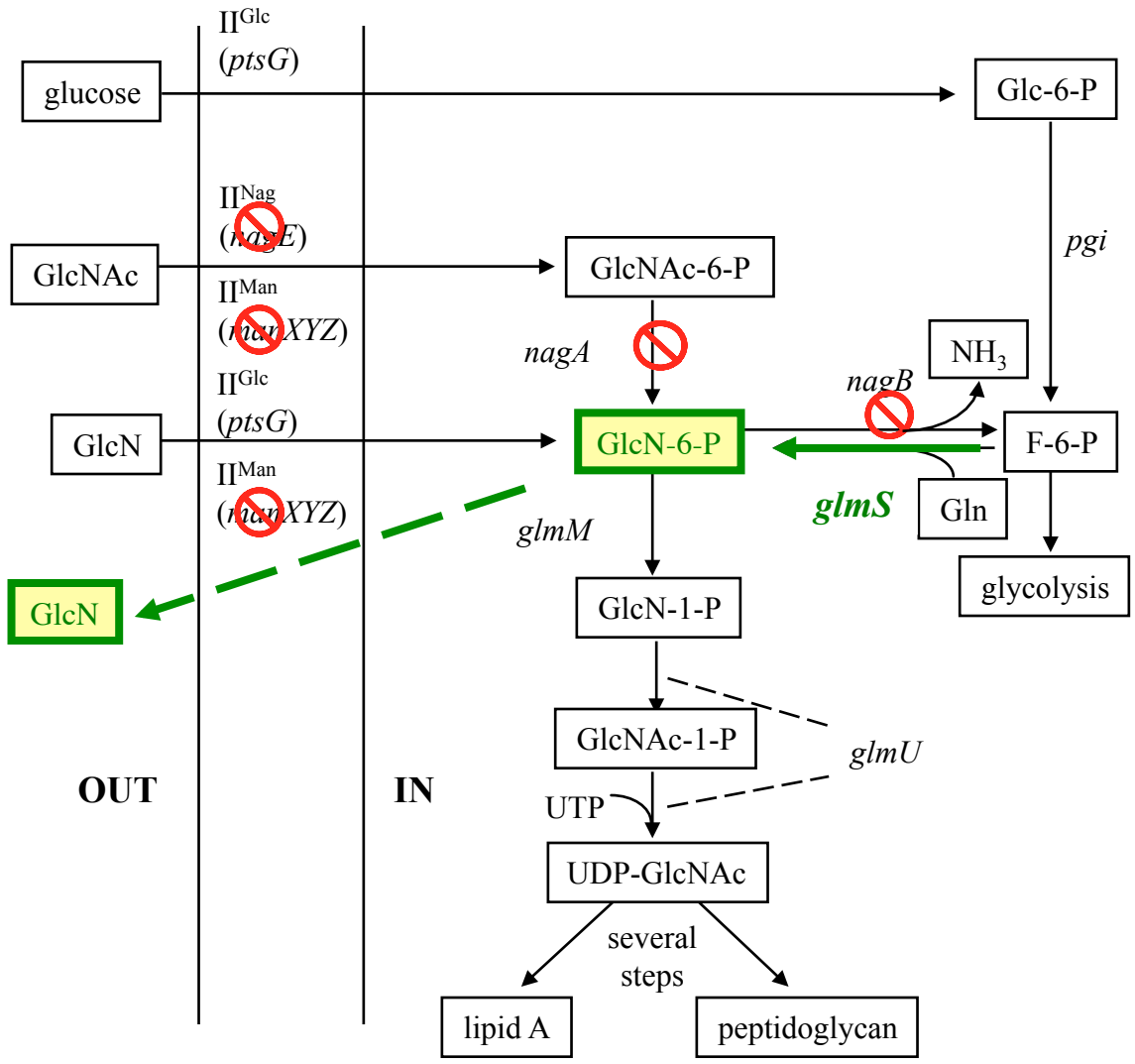
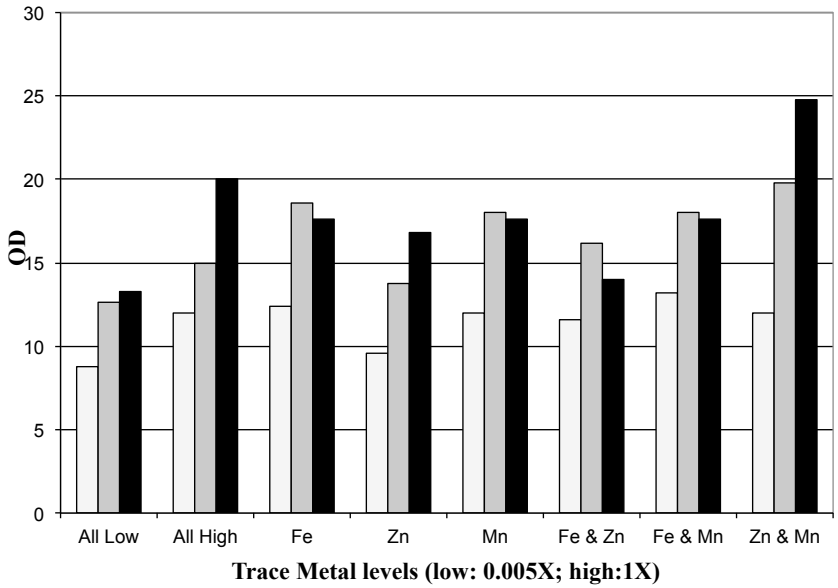
Introduction

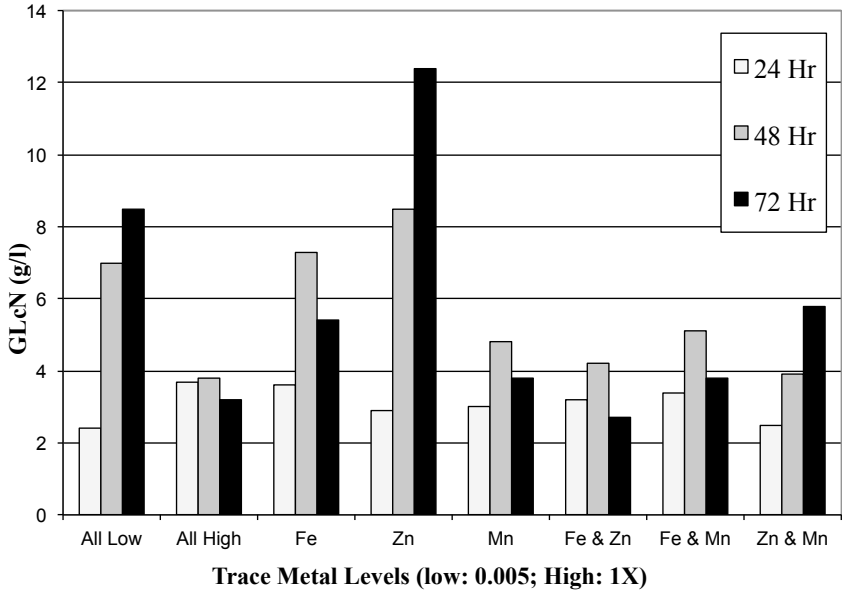
- Glucosamine (GlcN): nutraceutical for osteoarthritis.
- Current manufacture: acid hydrolysis of chitin from shellfish waste or fungal biomass.
- Direct microbial process offers many potential advantages: higher yields, lower cost and Kosher.
- *E. coli* strains engineered at BTR: over-expressing glucosamine synthase (*glmS*), and inactivating glucosamine transport and catabolism genes.
- Production: inducible by IPTG and lactose.

E. coli Metabolic Engineering to Produce Glucosamine



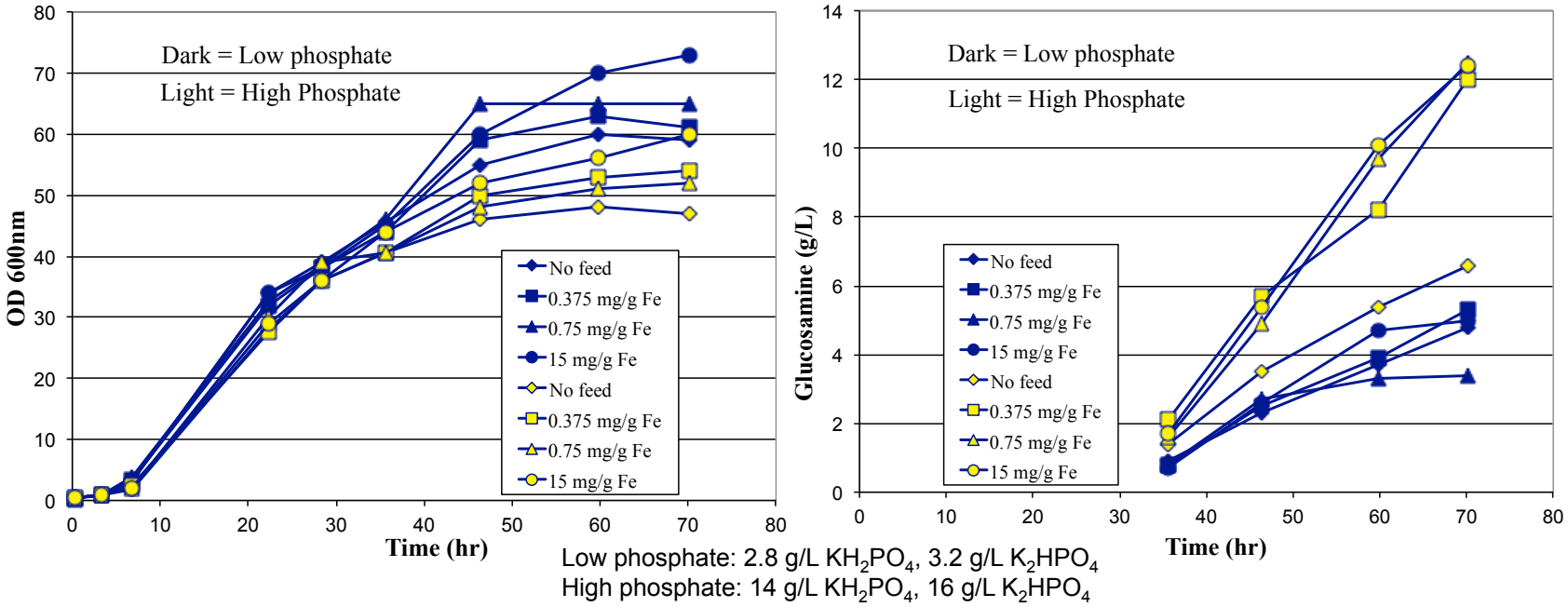
Effects of Trace Metals on Growth and GlcN Production in Shake Flasks





- Medium development initiated in shake flask cultures, allowing for rapidly exploring many different areas. Further optimization was conducted in 1-liter fermentors.
- A defined mineral salts medium (M9A): used as the base medium; trace elements added to support higher cell densities, but concentrations of some elements to be critical.
- Example shown: trace metals added alone or as different combinations

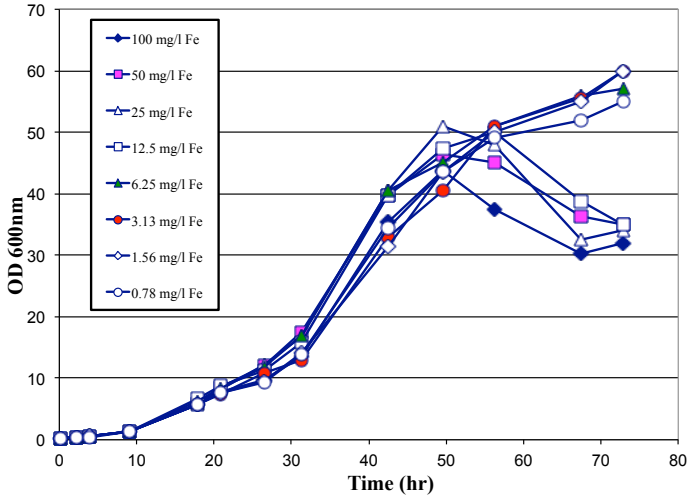
Optimization of Fed-Batch Fermentation for GlcN Production

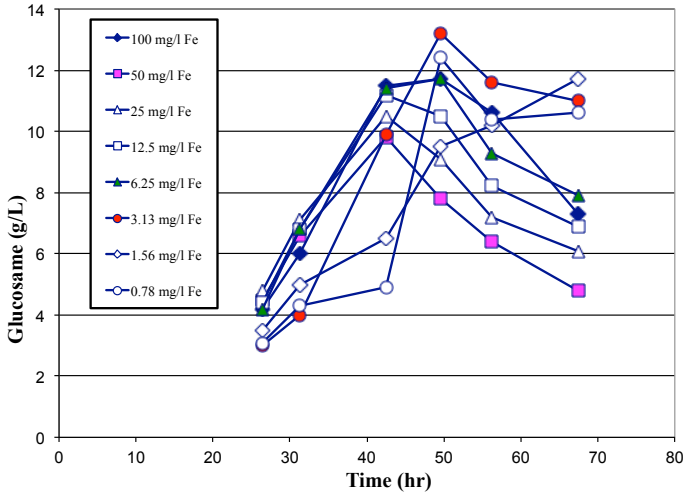


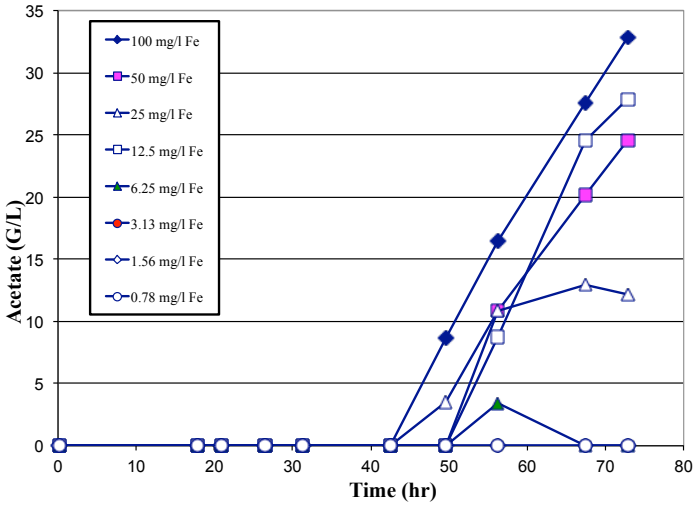
Low phosphate: 2.8 g/L KH_2PO_4, 3.2 g/L K_2HPO_4
High phosphate: 14 g/L KH_2PO_4, 16 g/L K_2HPO_4

- Fed-batch fermentation: growth and production phases, separated by glucose feeding period for induction
- Fe feeding is required, but feeding levels had to be optimized
- High phosphate level strongly promoted glucosamine production at the expense of biomass

GlcN Process at Different Iron Levels in Fermentors

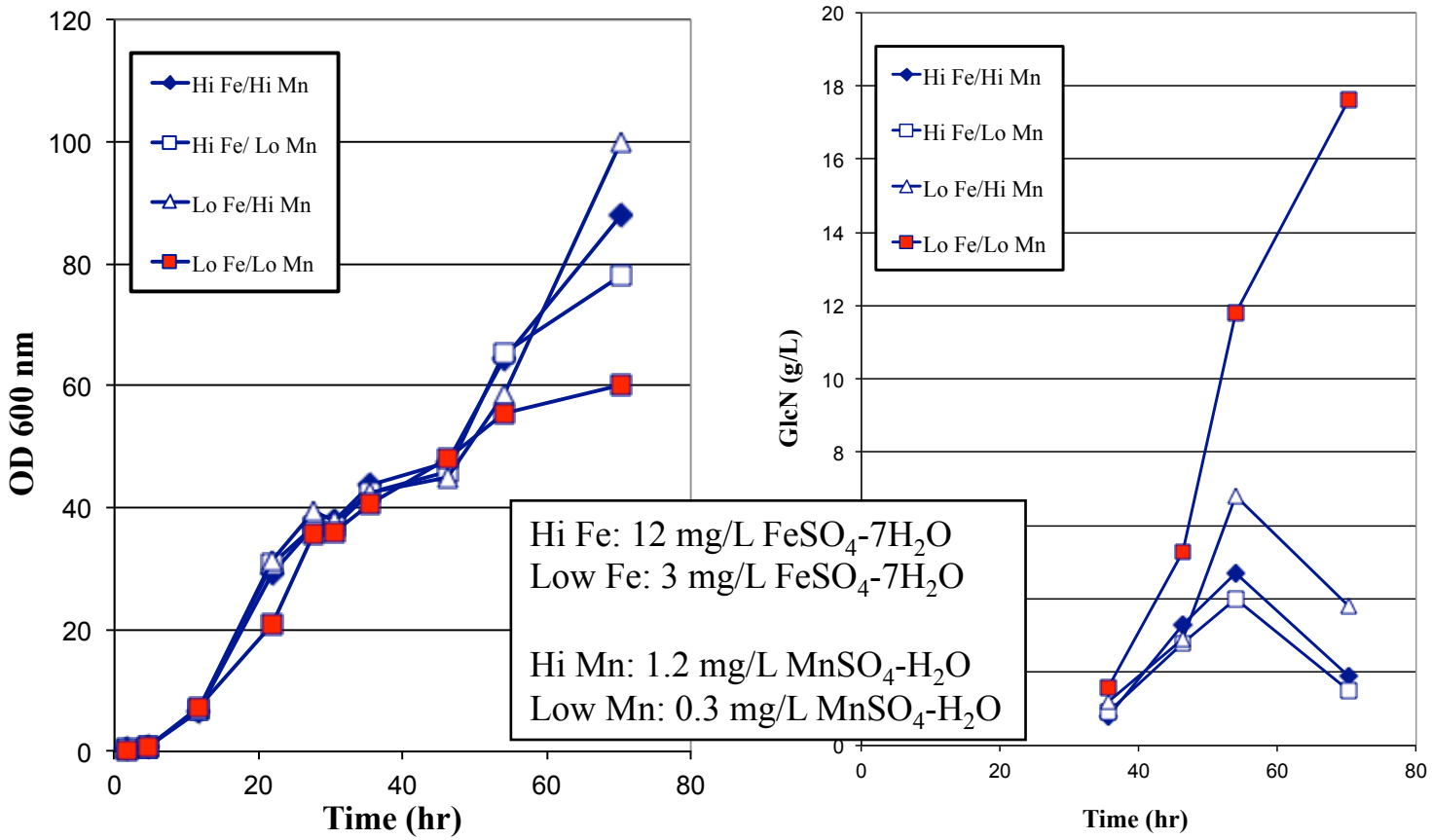






- Acetate accumulation: a significant problem, affecting both growth and GlcN production.
- As little as 5 g/L acetate slowed growth of induced culture; higher levels increasingly inhibitory.
- Acetate issue mitigated by lowering temperatures to 25C, glucose limiting feeding, and strict limitation of iron and other important trace elements
- Shown: evaluation of different iron levels

GlcN Process: Fe and Mn in Fermentors

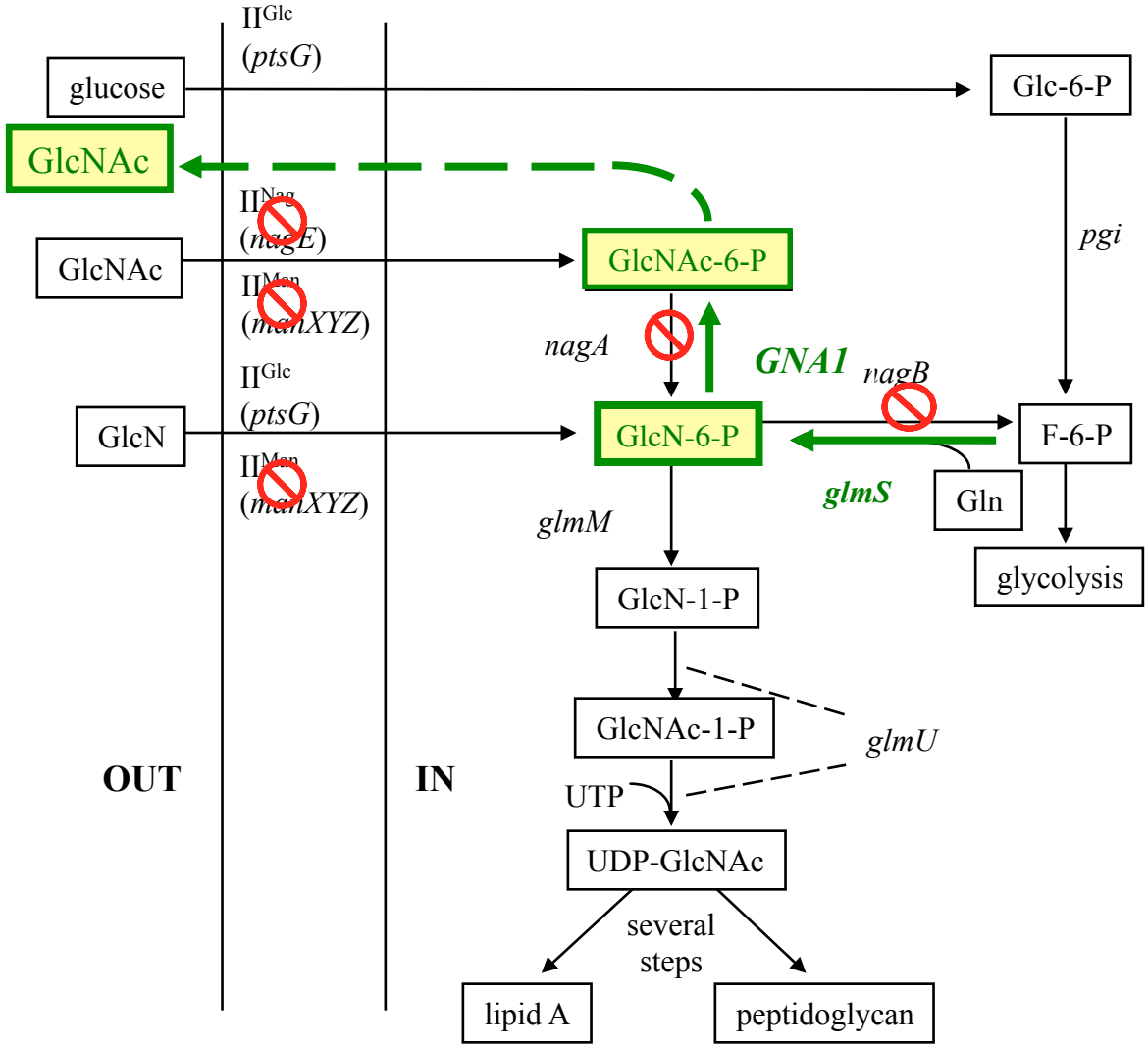


- Iron (Fe), manganese (Mn) and zinc (Zn) had significant effects on glucose uptake rate, acetate, and GlcN production. Fe had the most significant effect.
- Too much Fe and Mn were detrimental to the GlcN process
- GlcN titer reached over 17 g/L under Low Fe/Low Mn conditions

Metabolic Engineering to Overcome Fermentation Process Challenges

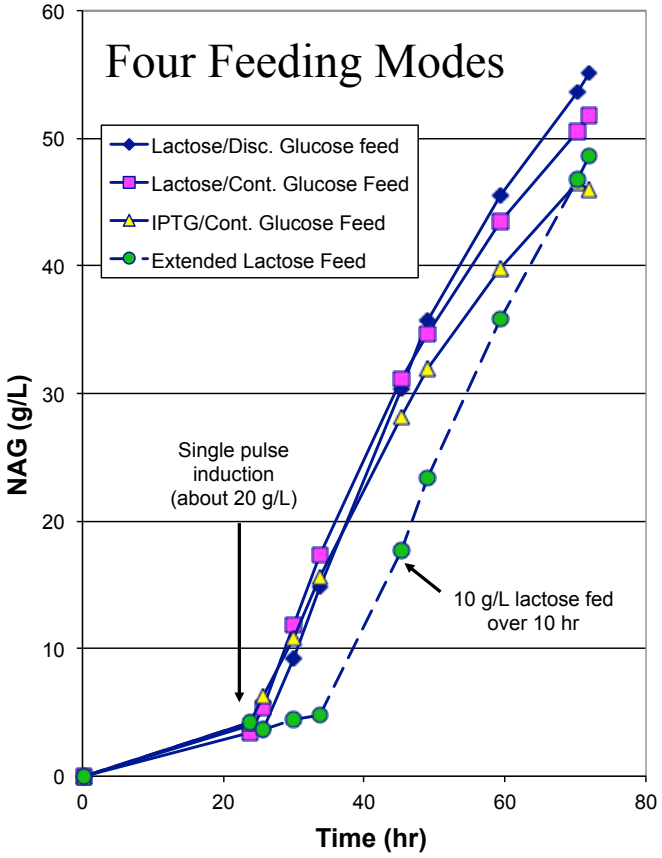
- **GlcN titers up to 18 g/L were achieved, but further improvement was difficult**
  - GlcN is labile at neutral pH.
  - GlcN and its degradation products are inhibitory to the host.
  - Lowered pH during production offered only marginal benefit.
- **Over-expression of GNA1 (acetyltransferase gene) extended the pathway to *N*-acetylglucosamine (NAG)**
  - NAG: stable,
  - NAG: non-inhibitory to the host.
  - NAG: easily hydrolyzed to GlcN.
  - Other process advantages discovered later

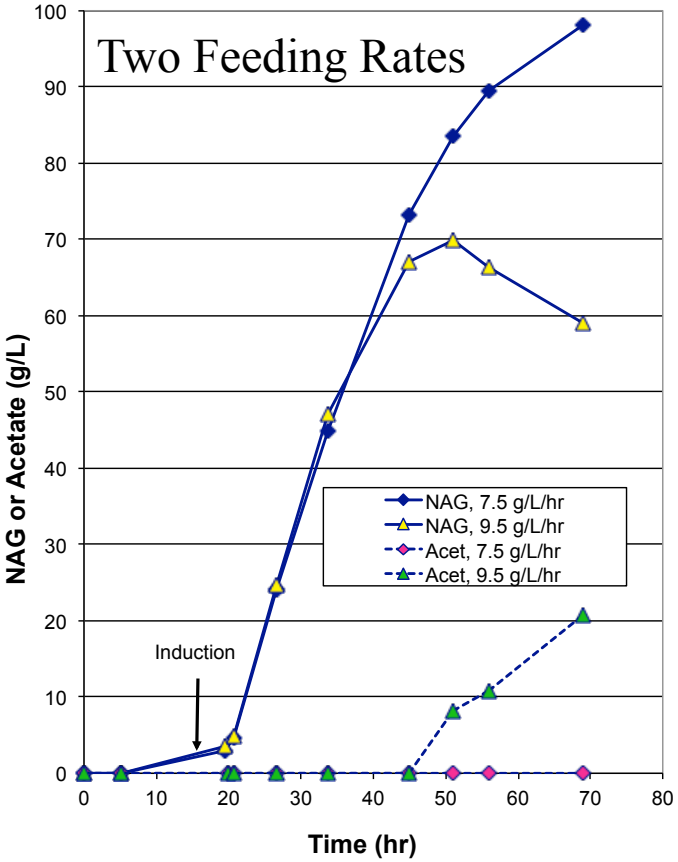
Metabolic Engineering to Produce *N*-Acetylglucosamine



- GlcNAC: the only amino sugar accumulated at high levels
- GlcN: not detectable

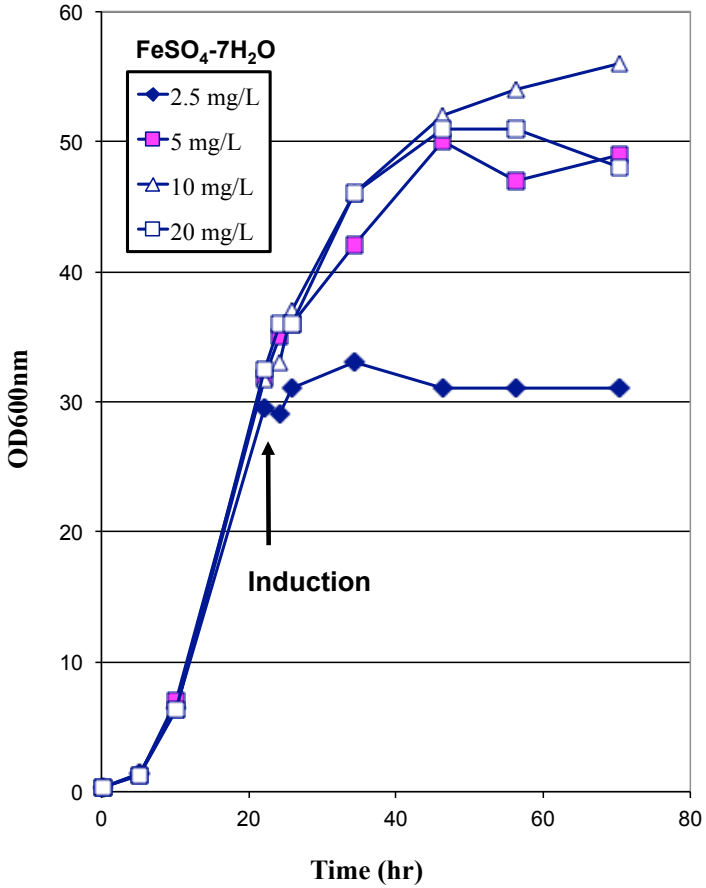
NAG Process: Modes of Induction and Post-Induction Glucose Feeding

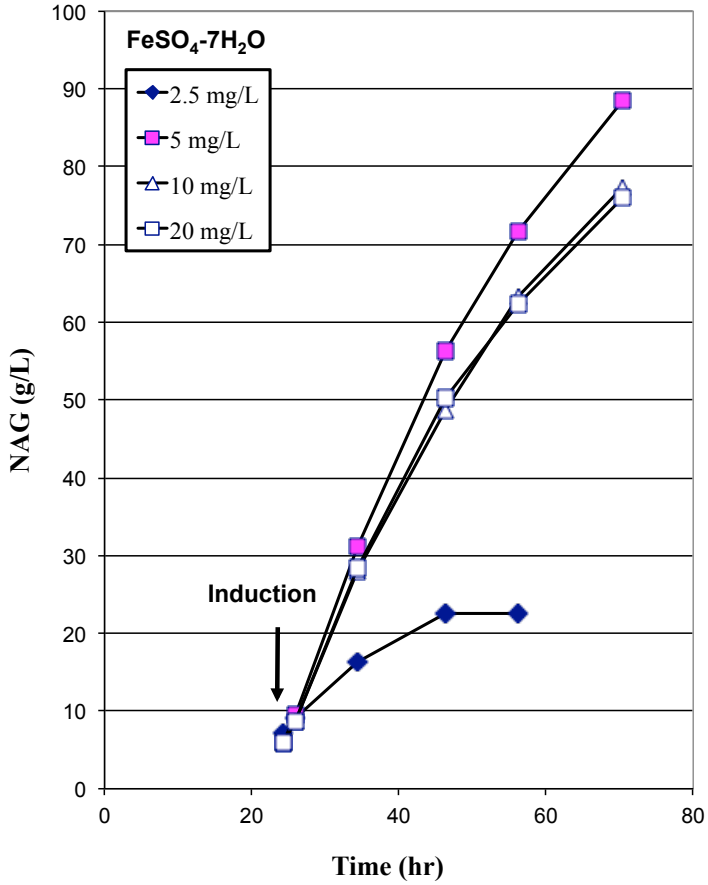




- Early induction at inoculation: near total inhibition of growth for 24 hr & total inhibition of NAG production
- Good induction timing: OD of ~25
- Optimal glucose feeding at 7.5 g/L; feeding at 9.5 g/L caused acetate accumulation)

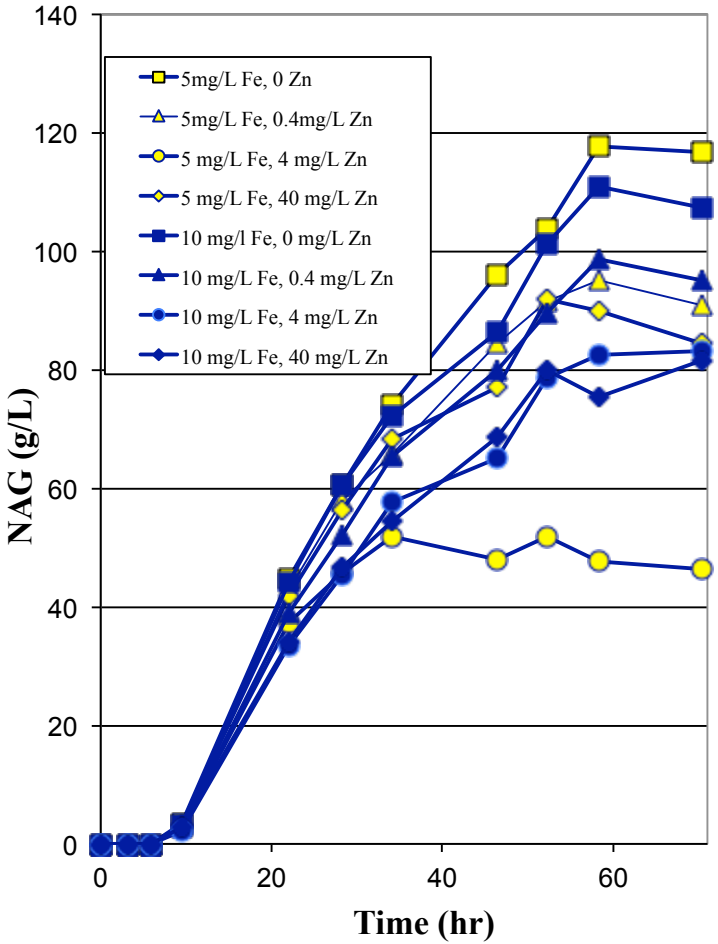
NAG Process: Fe level is Critical

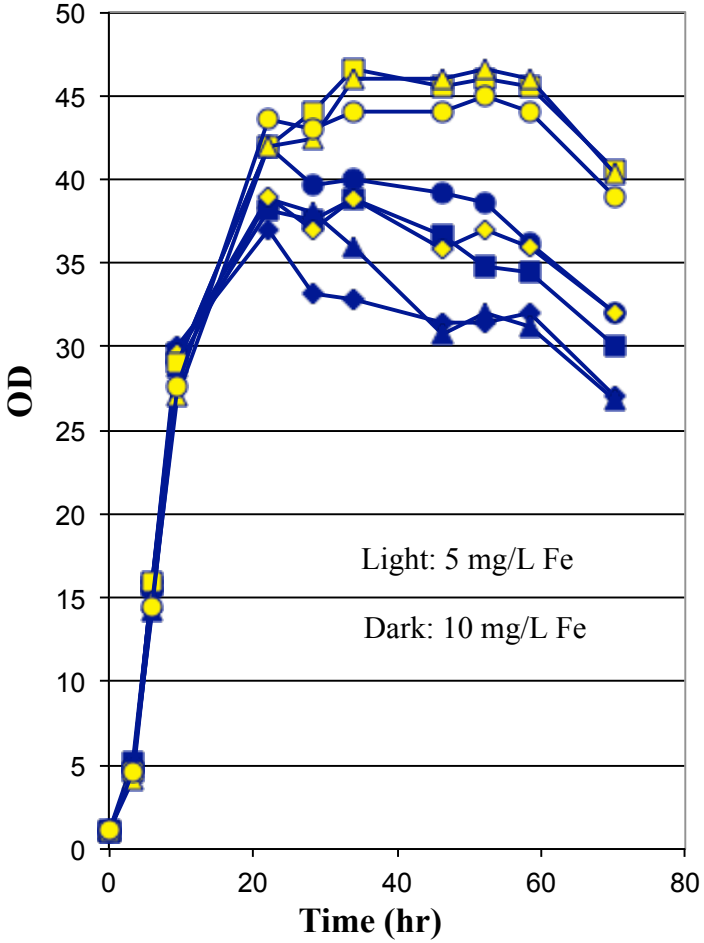




- Medium improvement included lowering phosphate level and eliminating certain trace elements.
- NAG process was more tolerant of higher Fe levels.
- Growth and NAG production were severely limited by Fe deficiency

NAG Process: Fe and Zn



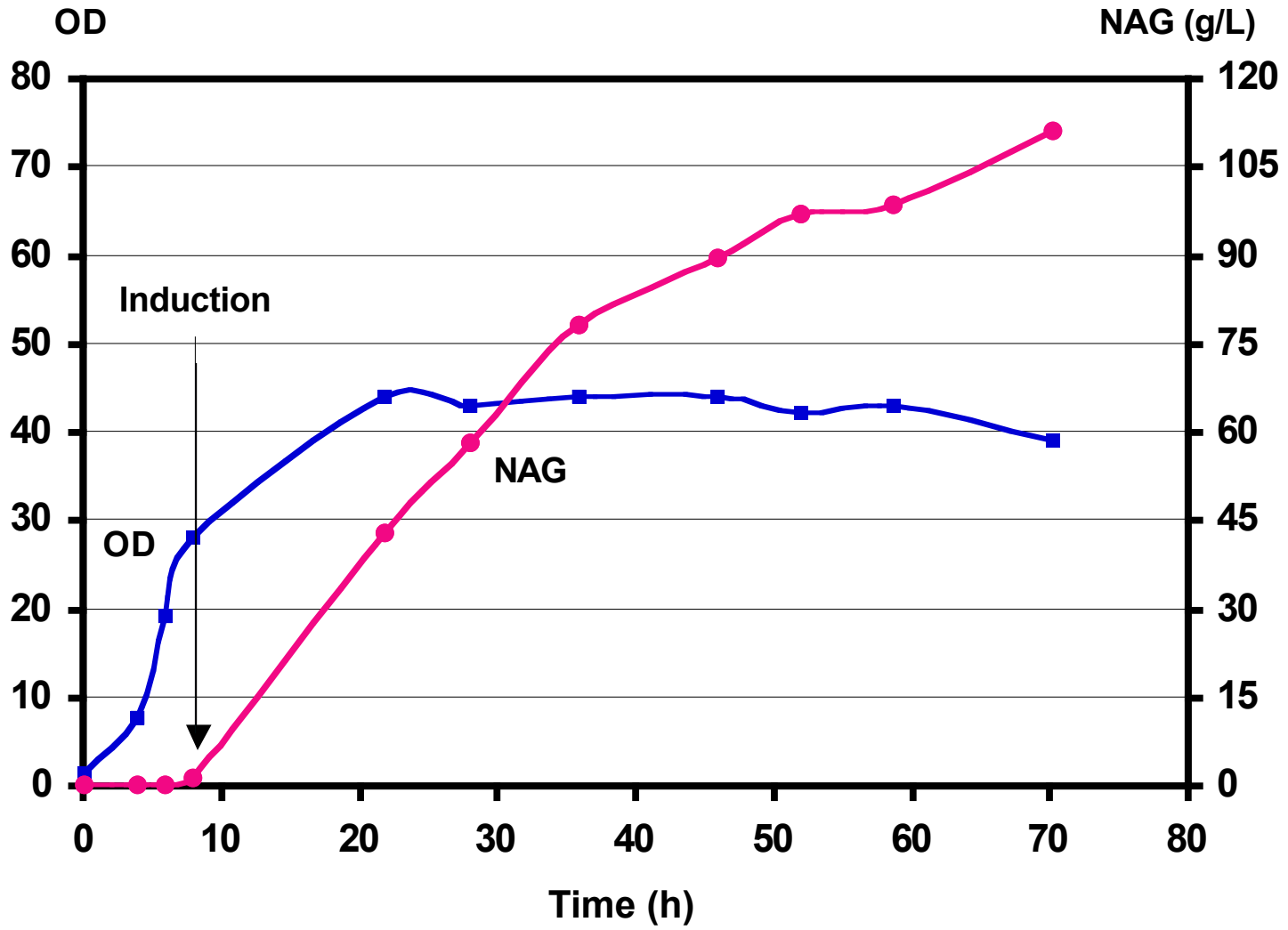


- 5 mg/L Fe is appropriate; doubling the amount stimulated growth but negatively affected NAG production
- Zn levels (0 to 40 mg/L) negatively correlated with NAG titer.

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, a single dose of 5 g/L
  - Cell Density: 25 g/L

Lactose-Induced *N*-Acetylglucosamine Production



Conclusions

A fermentation process to produce glucosamine by recombinant *E. coli* was developed successfully, with titers up to 18 g/L. Further metabolic engineering to the strain, coupled with fermentation development, led to a process to produce *N*-acetylglucosamine over 110 g/L within 60 hrs.

- Glucosamine production culture is sensitive to acetate accumulation. Glucose-limiting feeds and lower production temperature (25 C) helped to reduce acetate levels.
- Trace metals addition promoted growth, but iron and manganese had a significant effect on glucose uptake and acetate accumulation. Restriction of these key trace elements, especially iron, further reduced acetate formation and made the fermentation more robust.
- Glucosamine is labile at pH values optimal for *E. coli*. This factor, coupled with culture sensitivity to glucosamine (and its degradation products) limited potential for glucosamine production. It was determined that *N*-acetylglucosamine (NAG), stable, non-inhibitory and easily hydrolized to glucosamine, is an ideal alternative fermentation product.

Conclusions (continued)

- The NAG fermentation process allowed for a higher growth and production temperature (37 C), resulting in a more efficient, more robust and a more overall economical process.
- The NAG fermentation is more tolerant over a higher range of iron levels, but a threshold level is still required for optimal growth and induction. The NAG process also require much lower phosphate, reducing salts and further improving economics.
- A single pulse of a relatively low level of lactose provides an efficient induction, further simplifying the process.

Reference:

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)