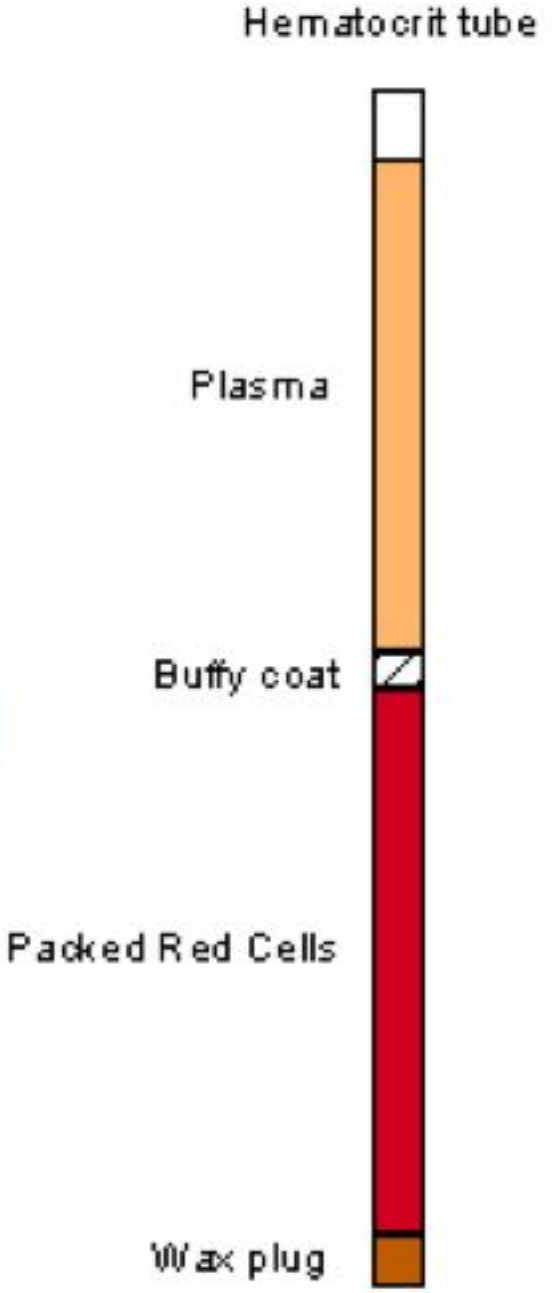
HSA, Pichia pastoris, and the AOX1 Promoter

Human Serum Albumin

- Most abundant protein in human blood plasma
- It is synthesized in the liver
- 67 kDa
- Maintains osmotic balance of blood plasma
- Serves as a transport protein for large organic anions such as fatty acids, bilirubin, and many drugs; it also carries hormones such as cortisol and thyroxine
- Decreased serum albumin (*hypoalbuminemia)* occurs in protein malnutrition, active inflammation, and serious hepatic and renal disease.

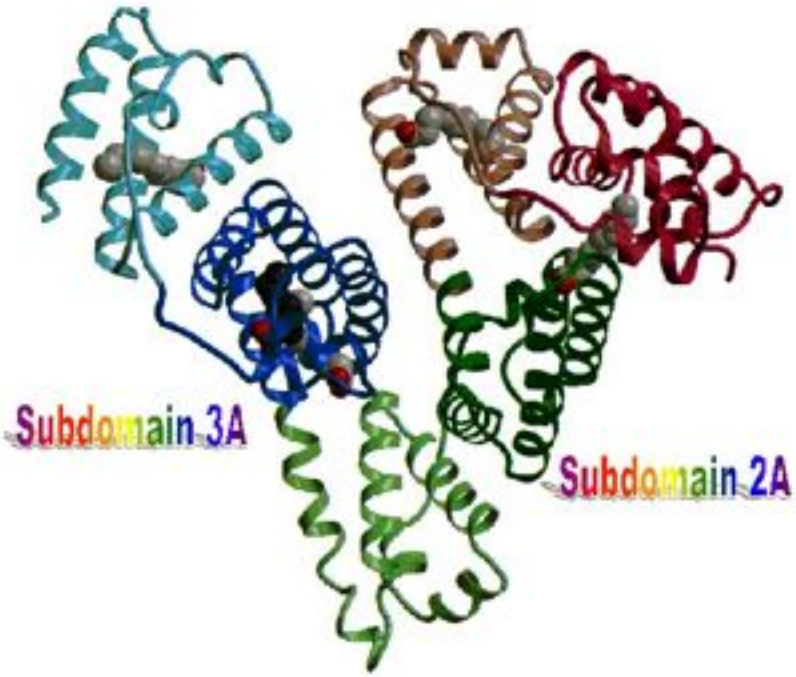
Medicinal uses of HSA

- Carrier of active ingredients of pharmaceuticals
  - Ex: Taxol - HSA
- Hard to get enough of it from blood plasma, and it is often virus infected
- Used in the treatment of shock
- Burns



Recombinant HSA

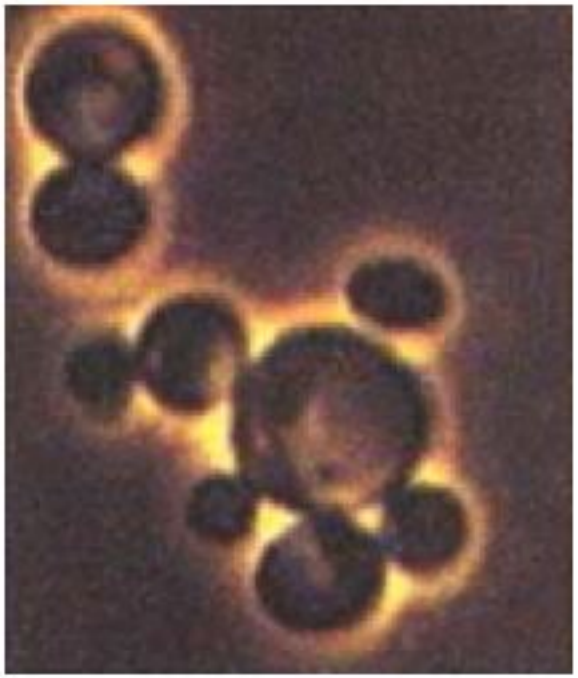
- Now mass produced in Japan
- Uses the methanol induction
- Made for the Japanese market
- Can be mass – produced, is identical to the natural protein, and is virus free!



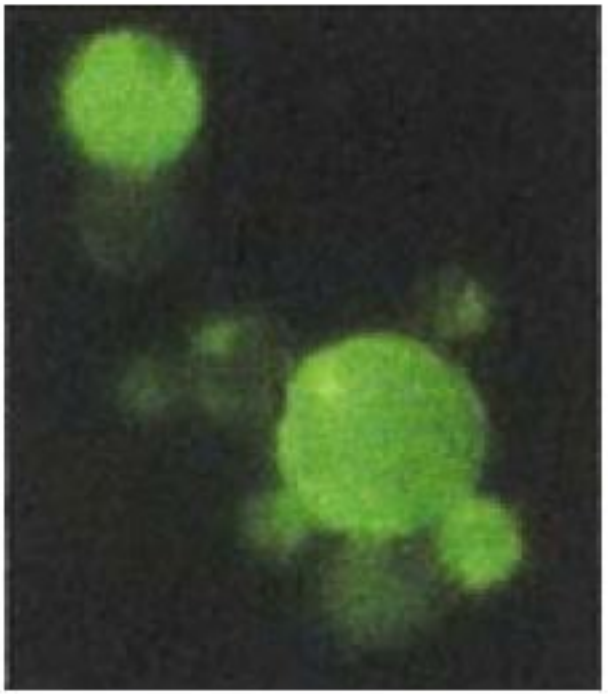
Why use *Pichia pastoris* and not *E.coli?*

What is *Pichia Pastoris?*

- Budding Yeast



phase-contrast picture of *Pichia pastoris*



GFP expressing *P. pastoris* cells: GFP triggered fluorescence is induced by UV light

Why use *Pichia pastoris?*

- Eukaryotic cell, more similar to human post translational modification than S. cerevisiae
- Similar to the heavily studied S. cerevisiae
- It can reach a high biomass-density 10 x greater than S. cerevisiae
- Easily adaptable to large scale purification in fermentors

S. cerevisiae

Pichia pastoris

- Extremely high achievable product concentrations comparing to the traditional recombinant strains (Eschericia coli, Saccharomyces cerevisiae)
- Extracellular protein expression up to 65 kDa molecular weight.
- Usually the carbohydrate structures of the glycopeptides are equivalent with the original native ones.

DNA Cloning Definitions

- Vector = the plasmid or phage chromosome used to carry the cloned DNA segment. Vectors introduce foreign DNA (gene for HSA) into host cells (P. pastoris), where the gene is replicated autonomously in large quantities.
- Expression Vector = a vector that has been designed to express cloned genes in a particular cell type.
- Repressor = any molecule that can reversibly inactivate a gene.

DNA Cloning Definitions, cont.

- Promoter = the region of an operon that acts as the initial binding site for RNA polymerase.
- Operon = A unit of genetic material that functions in a coordinated manner by means of an operator, a promoter, and one or more structural genes that are transcribed together.

Gene Expression in Yeast

- In Saccharomyces cerevisiae, few proteins are secreted. But signal sequences from any secreted protein's gene can direct a protein to the secretory pathway in yeast.
- With the right leader sequence, our HSA can be secreted into the medium.
- Our example is Pichia pastoris used in secreting the protein HSA to the fermentation medium

An Expression System for Proteins

- In 1993 the Phillips Petroleum Company released the *P. pastoris* expression system to academic research laboratories
- Since 1995, the use of *Pichia pastoris,* an expression system for a variety of proteins has grown exponentially due to the presence of a strong, tightly regulated, and easily manipulated promoter –the *AOX1* promoter derived from the alcohol oxidase *(AOX)* gene.
- Pichia is a eukaryote and capable of many of the post-translational modifications performed by higher eukaryotic cells such as proteolytic processing, folding, disulfide bond formation and glycosylation.

Pichia pastoris

- Pichia is similar to S. cerevisiae, but:
  - **Pichia can give 10 -100 fold higher levels of expression for a foreign gene.**
  - **Pichia may be more like higher eukaryotes in its glycosylations, sugar additions. Glycosylation in Pichia adds 8-14 mannose sugars per amino acid chain, compared to 50-150 in S. cerevisiae**
  - **The terminal linkages in S. cerevisiae are alpha 1,3 glycan linkages. This is not true in Pichia.**
  - **S. cerevisiae expressed proteins are hyper-antigenic (cause allergic reactions) and are not suitable for therapeutic use.**

Pichia pastoris, cont.

– Pichia can use methanol as a sole carbon source by:

Methanol –alcohol oxidase→ formaldehyde & H_2O_2 inside peroxisomes.

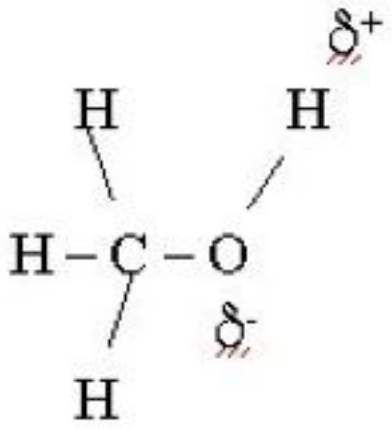
- Pichia expression vectors use the AOX1 promoter to drive expression of foreign genes. These foreign genes can be induced by methanol.
- Glucose represses the AOX1 gene. Not even a trace of mRNA for alcohol oxidase can be seen in the presence of glucose. So for expression of our HSA, we grow Pichia in glycerol to derepress the gene, then add methanol to induce production of HSA.

Why use *Pichia pastoris?*

- Efficient secretion
  - Few proteins are naturally secreted
  - Recombinant proteins fused to the native signal directed into the medium purification
  - *P. pastoris* grows on a simple mineral media and does not secrete high amounts of endogenous protein
  - Therefore the recombinant protein secreted into the culture is relatively pure and purification is easier to accomplish

Why use *Pichia pastoris?*

- Very stable host
- Has 2 very strong promoters-both inducible and constitutive promoters drive high level protein expression.
- Methyltropic
  - Can rely on methanol metabolism



Pichia pastoris

- Company in Japan is making human serum albumin using *Pichia pastoris* and methanol induction.
- Best growth is seen when we first feed with glycerol and then change to methanol.
- Too much methanol is actually lethal to the *Pichia pastoris.*

Methanol Metabolism

- The first step in the utilization of methanol is the oxidation of methanol to formaldehyde and hydrogen peroxide
- This step is catalyzed by the enzyme alcohol oxidase
- The expression of this gene is tightly regulated
- When the yeast are grown on glucose or ethanol, alcohol oxidase is not detectable in the cells
- When the yeast are grown on methanol, alcohol oxidase can make up to thirty-five percent of the total cellular protein

AOX1 and AOX2

- The control of the amount of alcohol oxidase is largely transcriptional
- There are two alcohol oxidase genes: AOX1 and AOX2.
- The protein coding regions of the genes are largely homologous, 92 percent and 97 percent at the nucleotide and amino acid sequence levels respectively
- The promoters share very little homology. No mRNA of the two genes is detectable when the yeast are grown in glycerol.
- The promoter region for AOX2 has a repressor region the leads to the inhibition of gene expression, and an activation region that leads to the enhancement of gene expression.
- The AOX1 gene promoter probably has a similar mechanism

AOX1 promoter

AOX1 and AOX2 induced by methanol, AOX1 is much stronger.

- AOX1 when induced will produce alcohol oxidase (30% of the protein in the cell).
- This promoter does not make any transcript when another food source (C-source) is present.
- Why we will starve yeast culture first.

Alternative promoter

- Can also get constitutive expression in pichia pastoris- using the GAP promoter.

- GAP from glyceraldehyde-3-phosphate

pPIC9 vector

- AOX1 promoter
- HIS4 gene
- 3' AOX1 gene for targeted integration into *Pichia* genome.
- Transformed into GS115 his4 mutant.

pPIC9

Standard resolution | High resolution

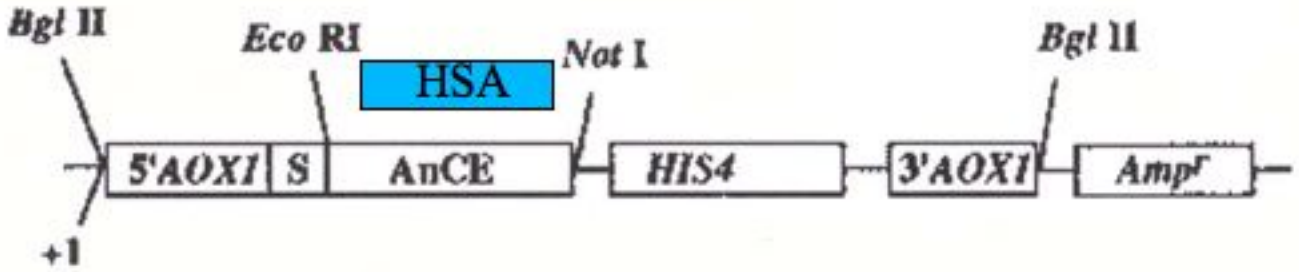


Figure 2 Transfer vector construction (pPIC9AnCE) for spheroplast transformation

A unique *Eco*RI site, introduced into the AnCE cDNA by PCR-mediated mutagenesis, allowed the fusion of the AnCE cDNA, minus the sequence encoding the signal peptide, in the same open reading frame as the *Saccharomyces cerevisiae* α-factor secretion signal peptide and prepro sequence (denoted by S) thus producing *S–AnCE*. The *HIS4* gene product is histidinol dehydrogenase which acts as a selectable marker by growth of transformants on histidine-deficient plates. *S–AnCE* and *HIS4* are flanked by sequences specific to the alcohol oxidase gene (*AOX1*) which allow replacement of the *AOX1* gene in the *Pichia* genome. The *AOX1* gene product (alcohol oxidase) enables the utilization of methanol as a source of carbon; thus recombinants in which the *AOX1* gene has been replaced by the desired *S–AnCE* sequence exhibit slow or no growth on plates containing methanol as the sole carbon source. The *Bgl* II sites allow linearization of the plasmid construct for maximum transformation efficiency [44] and *Amp*^{r} is the ampicillin resistance gene.

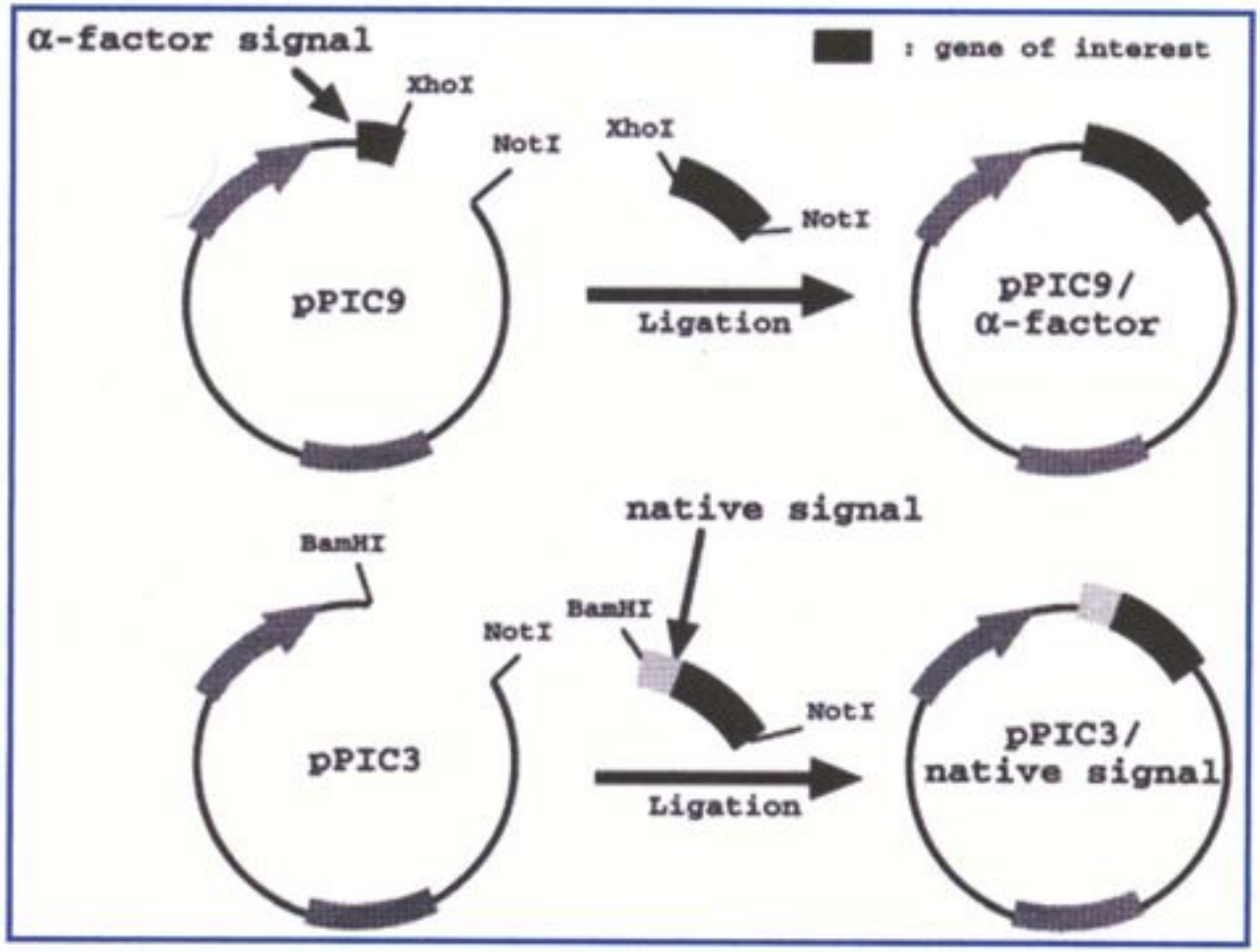


Fig. 1. Diagram of the expression vector for the *P. pastoris* system. pPIC9 is the vector constructed for the expression using the α-factor leader sequence as the secretion signal and this vector contains the α-factor leader sequence. PIC3 is the vector that has no signal sequence.