

Microorganisms and Processes Involved In The Production of Enzymes and Vitamin B12





Major industrial enzymes from bacteria, molds and

yeasts

Enzymes	Producer organisms
Asparaginase	Aspergillus spp. and and Penicillium spp
Amylase	Aspergillus niger, Aspergillus. oryzae
Catalase	A. niger, Penicillium spp.
Cellulase	A. niger, Trichoderma reesei, T. viride, Penicillium finiculosum
Dextranase	Penicillium spp.
ß-Glucanase	A. niger, Penicillium emersonii, T. reesei, T. viride
Glucoamylase	A. niger, A. oryzae
Glucose oxidase	A. niger, Penicillium spp.
Hemicellulase	A. niger, A. oryzae, T. reesei, T. viride, P. emersonii
Laccase	Pyricularia oryzae
Lipase	Several species including A.niger, A. oryzae
Pectinase	Several species including A. niger, Rhizopus oryzae
Protease	Several species including A. niger, A. oryzae
Rennet	Mucor miehei, Endothia parasitica
Tannase	A.niger, A. oryzae
Xylanase	A. niger, T. reesei



Chymosin

- Also known as rennet/chymase and is used in the manufacture of cheese.
- Over 90% of chymosin used today is produced by *E. coli, Kluyveromyces lactis* and *Aspergillus niger*.
- Genetically engineered chymosin is
 preferred as, it is purer than calf
 chymosin and is more predictable and
 preferred by vegetarians.



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- Highly specialized cells within tissues (mucosal layer of abomasum), frequently contain large amounts of mRNA that codes for pre prochymosin.
- This provides basis for isolation of specific mRNA to make cloning easier.
- Total RNA from mucosal layer of abomasum is isolated and mRNA is fractionated by oligo(dT) cellulose affinity chromatography.
- mRNA is reverse transcribed to cDNA and subsequently cloned in suitable vector and expressed in suitable bacterial or yeast host.
- Gene for chymosin is successfully cloned and expressed using plasmid or modified plasmid vectors and host organisms such as *E. coli, Bacillus subtilis, Saccharomyces cerevisiae, Kluyveromyces lactis, Aspergillus* spp.

 Several forms of chymosins have been expressed, including preprochymosin, met-prochymosin, and met-chymosin as heterologous proteins made in *E. coli*.

 Chymosin is suited for expression as a fusion protein which is auto-catalytically reactivated to chymosin during product recovery, ensuring that final product has proper amino terminus.





Shaping Lives... Empowering Communities... Method for Chymosin A produced from *Escherichia coli* K-12 containing calf prochymosin A gene

- Construction of production strain *E. coli* K12
 JA198 strain was subjected to several genetic
 manipulations to construct the recipient strain for
 expression plasmid carrying prochymosin A gene.
- Expression plasmid was derived from widely used cloning vector pBR322.
- cDNA coding for bovine chymosin A was previously cloned and characterized.
- Prochymosin gene divided into three sections, each terminated by a unique restriction endonuclease recognition site.
- Each section was assembled from several synthetic oligonucleotides synthesized in an automated DNA synthesizer.



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- Each assembled section was subcloned into a pBR322 vector, transformed into *E. coli*, and amplified.
- All three subcloned sections were assembled together in the correct order to reconstruct prochymosin gene, which was inserted into pBR322 vector.
- Gene was attached to vector DNA through ribosomal binding site and *E. coli* tryptophan (trp) promoter.
- Created expression vector was transformed into recipient strain GE81.
- Plasmid carries ampicillin resistance gene as a selective marker for bacterial transformants carrying prochymosin gene.

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- Production strain is grown in an aqueous solution
 containing carbohydrates, nitrogen, mineral salts
 and miscellaneous inorganic and organic
 compounds.
- Recovery :
- Solid prochymosin is liberated from the producing organism by cell disruption and harvesting of "inclusion bodies" by centrifugation or membrane concentration.
- Harvested inclusion bodies are washed with phosphate buffer solution containing 1-4 M urea.
- Residual *E. coli* are inactivated by holding at pH less than 2.0 for at least one hour



- Inclusion bodies are then dissolved by addition of urea to conc. of 7-9 M and pH adjustment to 10.0 - 11.0.
- Solution containing prochymosin is diluted
 with buffer, and pH is reduced to 8.5- 9.5,
 followed by 2-h period to allow renaturing of
 prochymosin, which is subsequently activated
 to chymosin by adjusting pH to 1.8- 2.2 and
 holding for 1 h.
- Following readjustment of pH to 5.5 6.0, chymosin is purified via absorption on a suitable anion-exchange resin followed by elution with a buffer containing 1 M sodium chloride.



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Vitamin B₁₂:

- Maintain healthy red blood cells and nerve cells, as well as aid in production of DNA.
 - Person with B₁₂ deficiency is susceptible to anaemia,
 low blood pressure, dementia, muscle weakness,
 Alzheimer's disease, breast cancer, fatigue and heart
 disease.
- Synthesized by wide range of bacteria and *Streptomycetes*.
- Processes using *Propionibacterium* sp. are most productive and widely used commercially.
- Select microbial species which make the 5, 6-dimethyl benzimidazolylcobamide exclusively.
- Several manufacturers have been led astray by organisms that gave high yields of related cobamides including pseudo-vitamin B12 *Streptomyces* cells.



- Vitamin B₁₂ activity is released from cells by acid, heating, cyanide or other treatments.
 Addition of cyanide solutions decomposes coenzyme form of vitamin in and results in formation of cyanocobalamin.
- Natural form of vitamin is Barker's Coenzyme where a deoxyadenosyl residue replaces cyano group found in the commercial vitamin.
- Cobamides formed in fermentation are retained in cells, and first step is separation of cells from fermentation medium.
- Large high speed centrifuges are used to concentrate bacteria to cream, while filters are used to remove.

- Cyanocobalamin is adsorbed on ion exchange resin IRC-50 or charcoal, and is eluted.
- Then purified further by partition between Phenolic solvents and water.
- Vitamin is finally crystallized from aqueous acetone solutions.
- Most commercial sound procedure produces B12 produced industrially by microbial fermentation, using almost exclusively *Pseudomonas denitrificans* and *Propionibacterium* spp.
- **Propionibacteria** are food-grade.
- Processes using *Propionibacterium* sp. have advantage that they allow to formulate natural vitamin B₁₂ together with biomass in which it is produced.





- Such processes avoid conversion of natural vitamin B₁₂
 into the cyanocobalamin form by chemical processes
 including cyanidisation followed by extraction and
 purification steps using organic solvents.
- Production process to be expensive, unsafe to operators and environmentally unfriendly.
- Several *Propionibacterium* sp. are capable to produce vitamin B₁₂ in large scale fermentation processes.
- Process is two-stage fermentation with 72-88 h anaerobic fermentation followed by 72-88 h aerobic phase.
- Vitamin B₁₂ concentration in cells rapidly increases in aerobic phase, with typical values of 25-40 mg vitamin B12/1.
- Anaerobic growth followed by an aerobic phase with limited growth is important for economic production of vitamin B₁₂ using *Propionibacterium* sp.
- Attempts have been made to overcome propionic acid toxicity to increase biomass and yield of vitamin B₁₂.

- Formulation of medium Step- Sterilization of the medium Making starter culture Step- Anaerobic Fermentation Step-4 Aerobic Fermentation Step-5 Recovery of cyanocobalamine Step-6
- Alternated anaerobic-aerobic phases e.g. suggested to reduce amount of acids.

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- In aerobic phase, propionic acid is converted to less toxic acetic acid, with simultaneous formation of vitamin B₁₂.
- Relative yield of vitamin B₁₂ increase, but final titre is rather low.
 - This is probably due to inhibition early in synthesis of vitamin B_{12} and/or other oxygen related products limiting synthesis of vitamin B_{12} .
- Final vitamin B₁₂ produced with this method is 9 mg/l compared to 4.5 mg/l with fully separated anaerobic and aerobic phases.