

# Dextranase enzyme production



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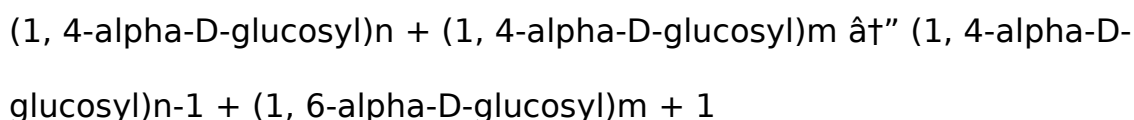
## **Dextranase definition and its uses**

Dextran is a collective name given to a large class of homopolysaccharides composed of D-glucans with contiguous  $\alpha$ -1, 6 glycosidic linkages (95%), with minor secondary linkages such as  $\alpha$ -1, 2,  $\alpha$ -1, 3 and  $\alpha$ -1, 4 [74]. It is produced by microorganisms such as *Leuconostoc mesenteroides*, *Streptococcus* sp., *Acetobacter capsulatus* and *Acetobacter viscus* [44]. Dextrans are well soluble in water, have low toxicity, and relative inertness. These properties make dextrans effective water-soluble carriers for dyes, indicators, and reactive groups in a wide variety of applications. They are widely used in the pharmaceutical and biochemical fields. Dextrans of low molecular weight are used as an alternative to blood plasma. They are also used for clinical purposes such as drug delivery [82], and by cross-linking for the production of the chromatographic matrix Sephadex. They are also widely used as both anterograde and retrograde tracers in neurons [94]. On the other hand microbial synthesis of dextrans in damaged cane and beets or other products containing sucrose is a serious problem in sugar and food industry. Dextran is also a structural component of dental plaque which causes the development of dental caries [78], [85].

Dextranases are enzymes that cleave the  $\alpha$ -1, 6 glycosidic linkages of dextran to yield either glucose or isomaltose (exodextranases) or isomalto-oligosaccharides (endodextranases), and are only produced as extracellular enzymes by a small number of bacteria and fungi, including yeasts and perhaps some higher eukaryotes [44].

Enzymes in many groups can be classified as dextranases according to function: dextranhydrolases, glucodextranases, exoisomaltosehydrolases, exoisomaltotriohydrolases, and branched-dextran exo-1, 2- $\alpha$  glucosidases.

In particular the chemical reaction catalyzed is as follows:



These enzymes belong to the family of glycosyltransferases, specifically the exosyltransferases. The systematic name of this enzyme class is: 1, 4- $\alpha$ -D-glucan: 1, 6- $\alpha$ -D-glucan 6- $\alpha$ -D-glucosyltransferase. Other commonly used names include dextrin 6-glucosyltransferase and dextrin dextranase.

Many microorganisms are known to produce dextranase, including filamentous fungi belonging to the genera *Penicillium*, *Aspergillus*, *Spicaria*, *Fusarium* and *Chaetomium*, bacteria, e. g. *Lactobacillus*, *Cellvibrio*, *Flavobacterium* etc. The only yeasts reported to produce dextranases are members of the family *Lipomycetaceae*. Only *Lipomyces kononenkoae* [104] and *Lipomyces starkeyi* dextranases have been characterized [47].

Potential commercial uses of dextranases include:

- The synthesis of potentially valuable oligosaccharides [30]
- Potential mouthwash ingredients since isomaltose may be of significant importance for the prevention of dental caries [40], [41]
- Clearance of dextran contamination in cane sugar processing [25]

Dual-stimuli-responsive drug release as in biodegradable polymer-structured hydrogels of gelatin and dextran [55]. Hydrogels are used for a wide range of

biomaterials applications such as: contact lenses, drug delivery vehicles and tissue adhesives. Dextranase are polymers that mimic biological sugars found on tissue surfaces. The dextran hydrogel system with tunable mechanical and biochemical properties appears promising for applications in cell culture and tissue engineering [58]

Drug delivery device suitable for delivering drug to the colon [7], [8].

Brondsted et al. studied the application glutaraldehyde dextran as a capsule material for colon-specific drug delivery. The dextran capsules were challenged with a dextranase solution, simulating the arrival of the drug delivery to the colon, so they broke and the drug was released as a dose pump. The outcome highlights the dextran capsules as promising candidates for providing a colon-specific drug delivery

Also in site-specific drug delivery systems with the use of antibodies [69]

The improvement of brewing yeast strain for beer industry. Due to the rising demand for low-calorie beverages, including beer, recombinant strains of *Saccharomyces cerevisiae* have been produced by integrating LSD1 gene of *Lipomyces starkeyi* [101]. *S. cerevisiae* lacks the ability to produce extracellular depolymerising enzymes that can efficiently liberate fermentable sugar from abundant, polysaccharide rich substrates [75]. By introducing the gene mentioned above, adding an exogenous enzyme during beer fermentation to achieve starch hydrolysis and oligosaccharide reduction can be avoided

Carbohydrase activity produced can also be exploited in sensitive chromogenic bioassays for toxicity: a mycotoxin bioassay using the

intracellular  $\beta$ -galactosidase activity of *Kluyveromyces marxianus* has been developed [20]

## **Classification of dextranase based on amino acid sequence**

Dextranases are dextran-degrading enzymes that form a diverse group of carbohydrases and transferases. The more recent classification divides dextranases into two classes: endodextranases ( $\alpha$ -1, 6-glucan-6-glucanohydrolase; also referred to as dextranase) and exodextranases ( $\alpha$ -1, 6-glucan-6-glycosidase; also referred to as dextran glucosidases). The Nomenclature Committee of the International Union of Biochemistry and Molecular Biology (IUB-MB) provides a system of classification depending on the type of reaction catalyzed and product specificity (Table 1). Contrary to that system, the Carbohydrate Active Enzymes (CAZy) database describes the families on terms of structural and mechanical features of these enzymes; enzymes with different substrate specificities are placed in the same family and those that hydrolyze the same substrate are sometimes placed in different families. In another classification system, Henrissat and Bairoch [33] have divided glucosylhydrolases and glycosyltransferases into five families on the basis of the similarities in the amino acid sequences (Table 2).

Table 1: The IUB-MB classification system

EC3. 2. 1. 11

Dextranases

EC3. 2. 1. 70

Glucan-1, 6-glucosidases

EC3. 2. 1. 94

Glucan-1, 6-isomaltosidases

EC3. 2. 1. 95

Dextran-1, 6-isomaltotriosidases

EC3. 2. 1. 115

Branched-dextran exo-1, 2-glucosidases

Table 2: Classification of dextran hydrolysing enzymes, based on amino acid sequences.

Dextran-glucosidases

Families 13 & 15

Isomaltodextranase

Family 27

Isomaltotriosidase

Family 49

Endodextranases

Families 49 & 66 (no sequence similarities between the two families)

Aoki and Sakano (1997) came up with 4 families [2]. They isolated and sequenced the isopullulanase gene (*ipuA*) from *Aspergillus niger* ATCC 9642. The gene shows significant amino acid similarity to the dextranase produced by *Penicillium minioluteum* (PEMDEX) and *Arthrobacter* sp. (ARTDEX). Since ASNIPU shows great similarity to PEMDEX and ARTDEX, they can be classified as Family 1. In the same fashion, the researchers compared the amino acid sequences of dextranases and dextran-hydrolysing enzymes, including ASNIPU.

## **Lipomyces species and Lipomyces starkeyi**

*Lipomyces starkeyi* and *Lipomyces kononenkoae* belong to the Lipomycetaceae family and are the only yeasts reported to produce dextranases. The first *Lipomyces* species was identified by Robert Starkeyi in 1946 during a study of nitrogen-fixing bacteria: it was then that he discovered *L. starkeyi*, a fat-producing, ascospore-forming soil yeast. The family Lipomycetaceae was proposed later, in 1952 by Lodder and Kreger von Rij. *Lipomyces* species can utilize starch as a sole source of carbon. Both species contain highly efficient amylolytic systems, permitting growth on starch with very high biomass yields [97].

The family Lipomycetaceae is known to utilize certain heterocyclic compounds, such as imidazole, pyrimidine, and pyrazine and their derivatives, as sole nitrogen sources [92]. Information on the genome organization and molecular genetics of this group of yeasts is very limited.

The ascospore-forming soil yeast *L. starkeyi* has been reported to produce commercially useful extracellular dextranase activity [97], [52], [53], and it

can utilize a variety of other compounds, like hexoses, pentoses, alcohols and organic acids, as sole sources of carbon and energy [46]. The strains of *L. starkeyi* currently used are NCYC 1436, IGC 4047, ATCC 12659 and its de-repressed mutant ATCC 20825.

### ***L. starkeyi* dextranases**

Commercial use of dextranase began in 1940s, mainly by producing low-molecular-weight clinical dextran. Therefore, industrially practical mixed culture fermentation of *L. starkeyi* and *Leuconostoc mesenteroides* was capable of producing controlled-size dextrans in order to satisfy clinical use, in which dextranase produced by *L. starkeyi* hydrolyzed the high molecular weight dextran produced by *L. mesenteroides* to a controlled size [46]. The enzyme production system of *L. starkeyi* needs an inducer. Dextran is its normal inducer but it is a relatively expensive carbon source for large-scale fermentations. Also, *L. starkeyi* is reported to have slow growth and difficulty of avoiding contamination from other microorganisms during growth. With that in mind D. W. Koenig and D. F. Day (1989) undertook to establish conditions which would minimize the cost of the inducer for producing an enzyme by using a de-repressed mutant of *L. starkeyi* ATCC 12659 grown on glucose. Thus the mutant ATCC 20825 is capable of hyperproducing dextranase at low pH to provide biologically contaminant-free supernatant liquid containing dextranase.

*Lipomyces starkeyi* (IGC 4047), when grown on dextran as a sole carbon source produced a dextranase able to hydrolyse blue dextran and Sephadex G-100. The molecular weight was 23kDa and the isoelectric point was 5.4 [97]. The dextranase of *L. starkeyi* (ATCC 20825) studied by Koenig and  
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Day (1988, 1989a, 1989b) was analysed by SDS-PAGE and produced four bands, of molecular weights 65 kDa, 68 kDa, 71 kDa, and 78 kDa. Millson and Evans (2007) have isolated extracellular dextranase of *L. starkeyi* NCYC 1436 and have found that for their strain the enzyme occurs as three molecular weight species and seven isoelectric forms [68].

### **L. starkeyi nutrients (YPDex / YPD)**

The main ingredient in the chosen media is yeast extract. Yeast extract is a dried autolysate which facilitates rapid and luxuriant growth when used in various media or fermentation broth. It is a good source of amino-nitrogen and vitamins, especially the water-soluble B-complex vitamins. However, yeast extract is reported to enhance glucose metabolism to lipids, but inhibit lipolysis [18]. The metabolic pathway consists of converting glycerol into pyruvate or glucose and then hydrolysis by a phosphatase gives glycerol again. The disruption of this metabolic pathway, could account for the seemingly truncated numerous bands that SDS gives after prolonged storage of the yeast. Mycological peptone is incorporated in the media and discourages bacterial growth because of its acidity.

### **Environment that dextranases favour**

Dextranase activity is affected by temperature, pH, metal ions and nutrients. According to Lin Chen et al (2007), dextranase activity is optimized between temperatures of 10°C and 60°C at pH of 6.0 [12]. In the particular study, the effect of pH on enzyme activity was determined by varying the pH between 3.5 and 8.5 under the temperature of 30°C. The pH of 3.4-4.5, 5.0-7.5, and 8.0-8.5 were maintained by sodium acetate buffer (20mM), citrate and phosphate buffer (20mM) and sodium phosphate buffer (20mM)

respectively. The effects of metal ions ( $\text{AlCl}_3$ ,  $\text{CaCl}_2$ ,  $\text{CoCl}_2$ ,  $\text{CuSO}_4$ ,  $\text{FeCl}_3$ ,  $\text{KCl}$ ,  $\text{MgCl}_2$ ,  $\text{NaCl}$ ,  $\text{NiSO}_4$ ,  $\text{MnCl}_2$  and  $\text{ZnCl}_2$ ) and SDS on dextranase activity were assayed by incubation of dextranase with 1mM metal ions or 1 mM SDS at pH 4.5 for 3h at 37°C, and then the enzyme activity of dextranase was determined.

Ravi Kiran Purama and Arun Goyal (2008) in a study for optimization of nutritional factors, estimated dextransucrase activity in the cell free extract of *Leuconostoc mesenteroides*. They analysed the regression coefficients and t-values of six ingredients: yeast extract, sucrose, intercept,  $\text{K}_2\text{HPO}_4$ , beef extract, peptone and Tween 80. Yeast extract, sucrose, beef extract, and  $\text{K}_2\text{HPO}_4$  displayed a positive effect for enzyme production whereas, peptone and Tween 80 had a negative effect on enzyme production. The variables with confidence levels greater than 90% were considered as significant. Sucrose was significant at 99.99% confidence levels for dextransucrase production.  $\text{K}_2\text{HPO}_4$  and yeast extract were found significant about 94% level for dextransucrase production. Beef extract was significant 91% for dextransucrase production. Peptone and Tween 80 were found insignificant with negative coefficients for enzyme activities.

### **Methods used for enzyme activity measurement**

Enzymatic activity is measured with the help of laboratory methods called enzyme assays. All enzyme assays measure either the consumption or production of product over time. Enzyme assays can be split into two groups according to their sampling method: continuous assays, where the assay gives a continuous reading of activity, and discontinuous assays, where

samples are taken, the reaction stopped and then the concentration of substrates/products determined [11], [20].

Continuous assays:

Spectrophotometry in which you follow the course of the reaction by measuring a change in how much light the assay solution absorbs

Fluorimetric assay in which we make use of the difference in the fluorescence of substrate from product to measure enzyme reaction. These assays are in general much more sensitive than spectrophotometric assays, but can suffer from interference caused by impurities and the instability of many fluorescent compounds when exposed to light

Calorimetric assay in which the heat released or absorbed by chemical reactions is measured

Chemiluminescence in which the light emitted by some enzyme reactions is measured so as to detect product formation. The detection of horseradish peroxidase by ECL is a common method of detecting antibodies in western blotting

Discontinuous assays:

Radiometry in which the incorporation of radioactivity in substrates is measured

Chromatographic assays measuring product formation by separating the reaction mixture into its components. This is usually done by high-performance liquid chromatography (HPLC), but thin layer chromatography  
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can also be used. Although this approach needs a lot of consumables its sensitivity can be increased by labelling the substrates/products with a radioactive or fluorescent tag

## **Methods and assays for dextranase activity measurement**

The large variability of available substrates makes it difficult to estimate the enzyme activity, because the reaction product is often an undefined mixture of sugar polymers. The existing assays try to compromise convenience, speed and accuracy [44]

Viscosimetric analysis was among the first to be used [31], [35], [36]. This method measured the amount of enzyme which reduced the specific viscosity of the dextran solution by half in 10min. and it is more suitable when dextranase hydrolyses the dextran molecule at random, producing long oligosaccharides.

Reducing-sugar assay or saccharogenic methods measure the rate of increase in reducing sugar as measured with the Somogyi assay, the 3, 5-dinitrosalicylic acid method (DNS) [102], thiourea borax-modified O-toluidine colour reagent (35) and alkaline potassium ferricyanide solution (225). These methods test the presence of free carbonyl group (C=O). It is a simple method commonly used to analyze for reducing sugars produced from enzymatic hydrolysis of substrates such as starch and sucrose [67]. The most common substrates applied are Dextran T2000, 47 T-260, 3 and T110 [54], [72]. A number of substances have been reported as interfering with DNS colour development and citrate is one of them. Acetate and citrate are reported to enhance colour development and the true antagonist in this

reaction is the proton (H<sup>+</sup>) [96]. This method is based on the release of short coloured products from polymeric blue dextran and their selective colorimetric detection at 610-650nm after precipitation of the polymer. DNS colorimetric assays reported in literature are often modifications of the method of Webb and Spender-Martins (1983). E. F. Khalikova and N. G. Usanov (2001) developed a dextranase assay using an insoluble substrate, namely, Sephadex G-200 with Remazol Brilliant Blue dye [45]. The action pattern of dextranase was then, studied by means of exclusion chromatography. Overall, this assay was reported as convenient for quantitative dextranase detection, relatively independent of the enzyme source, and is proposed as an inexpensive alternative to the known procedures utilizing coloured substrates.

The dextranase substrates can be either dye-releasing or fluorogenic. The assay procedures based on these substrates are accurate, fast and can be recommended for dextranase-producing microbial screening and enzyme purification.

Other assay procedures worth mentioning include a spectrophotometric method with the use of Blue Dextran developed by Kauko K. Makinen and Illika K. Paunio (2004) who recommend it for column chromatography [62], and a method based on simple titration, developed by Eggleston and Gillian (2005) for easy use at the sugar cane factory [19].

Fluorometric assays are based on measuring the fluorescence of the samples and the results are often compared to a series of standards of *Penicillium* sp. A very sensitive fluorometric assay using amino-dextran-70 coupled with

fluorescent dye BODIPY (4, 4-difluoro-5, 7-dimethyl-4-bora-3a, 4a-diazasindacene-3-propionic acid, succinimidyl ester) as the substrate was described by M. Zhou et al. (1998). The BODIPY FL dye-labelled dextranase substrate is an amine-containing dextran derivative that is labelled with the pH-insensitive, green fluorescent BODIPY FL dye, resulting in almost total quenching of the conjugate's fluorescence. The increase of the fluorescent degradation products of BODIPY FL dextran is proportional to the amount of dextranase activity [102].

A suspension of Sephadex in a buffer is supplemented with agar, sterilized, and poured in Petri dishes, and after the wells are filled with the test solution, they are left to incubate. The dextranase activity can be evaluated by the extent of halos around the holes due to the opalescence of Sephadex. Milson and Evans (2007), measured dextranase activity using SDS PAGE as described by Laemmli (1970), using both mini-gel and Protean II electrophoresis systems, and stained using Coomassie Blue [68], [56]. Molecular weight markers were used to construct a calibration curve, from which molecular weights of dextranase were determined. Native gel electrophoresis was performed, but the loading buffer and the gel lacked SDS and  $\beta$ -mercaptoethanol and the samples were not heated prior to loading on the gel. In the same study, dextranase activity was estimated in SDS gels, without extraction, by a plate modified from the method of Lawman and Bleiweis (1991) [57].

### **FL versus DNS assay method**

The classic method (DNS) for measuring glycosidases through release of reducing activity is simple and inexpensive and, as cited above, has been

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modified in several studies so as to suit the researchers' needs. It may, however, have some pitfalls. The reaction taking place is the following:

aldehyde group ———oxidation———> carboxyl group

3, 5-dinitrisalicylic acid ———reduction———> 3-amino, 5-nitrosalicylic acid

(Nam Sun Wang, University of Maryland)

The above reaction scheme shows that 1 mole of sugar reacts with 1 mole of 3, 5-dinitrisalicylic acid. However, it is suspected that there are many side reactions, and the actual stoichiometry is more complicated than that previously described. Different reduced sugars yield different colour intensities; thus it is necessary to calibrate for each sugar. Apart from the oxidation, other side reactions may compete for the availability of 3, 5-dinitrisalicylic acid. Consequently, the calibration curve may be affected and the intensity of the developed colour may be enhanced. Therefore, the method has low specificity and one must run blanks diligently if the colorimetric results are to be interpreted correctly and accurately [96].

Another obstacle to be dealt with when using DNS is non-linearity. One cause of non-linearity could be the common practice of diluting reaction products before quantification of reducing compounds and another is the insufficiency of substrates.

The fluorometric assay (FL), seems to gain ground in the most recent studies as faster and more accurate and it seems to leave space for modifications and combined use with other methods (see §1. 3. 1). A standard curve is

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constructed from *Penicillium* sp. and then compared with the one derived from *Lipomyces starkeyi*.

As described in the previous paragraph dextranase activity is estimated by the increase of the fluorescent products of dextran degradation. However, if too many fluoro are conjugated to the dextran molecule undesired may come up.

Molecular Probes TM seems to overcome this problem by removing as much of the free dye as possible and then assaying the fluorescent dextran by (TLC) to ensure that it is free of low molecular weight dyes. So, in general, FL seems to yield accurate curves. Millson and Evans (2007), used an assay of dextranase activity which was a variation on that reported by Zhou et al. (1998). In that study, fluorescence vs. dextranase activity produced a linear log [68], [102].

## **Purification of *L. starkeyi* dextranase**

### Dialysis tubing

Dialysis tubing is typically used for changing the buffering solution of a protein and is also a method for concentrating protein solutions by dialysis against a hygroscopic environment (e. g. PEG, Sephadex). The protein solution is contained within a membrane which permits solute exchange with a surrounding solution and whose pore size prevents the protein from escaping. Except for small volumes, this method is time-consuming [11].

### Filtration – Ultrafiltration



Ultrafiltration (UF) is a variety of membrane filtration in which hydrostatic pressure forces a liquid against a semi-permeable membrane. Suspended solids and solutes of high molecular weight are retained, while low molecular weight solutes pass through the membrane. UF is not fundamentally different from microfiltration or nanofiltration, except in terms of the size of the molecules it retains. [11], [77].

## SDS-PAGE

Purification of *Lipomyces starkeyi* dextranase is carried out mainly by running a SDS-PAGE (sodium dodecyl sulphate-polyacrylamide gel electrophoresis) analysis. The solution of proteins to be analyzed is first mixed with SDS, an anionic detergent which denatures secondary and non-disulfide-linked tertiary structures, and applies a negative charge to each protein in proportion to its mass. SDS binds in a ratio of approximately 1.4g SDS per 1.0g protein. The size of the protein is directly related to the distance it migrates through the gel. Dextranase molecules migrate as bands based on size. Each band can be detected using stains such as Coomassie blue dye [77].

Modifications to the polypeptide backbone, such as N- or O- linked glycosylation, however have a significant impact on the apparent molecular weight. Thus, the apparent molecular weight is not a true reflection of the mass of the polypeptide chain.

In most cases, SDS-polyacrylamide gel electrophoresis is carried out with a discontinuous buffer system in which the buffer in the reservoirs is of a different pH and ionic strength from the buffer used to cast the gel. After

migrating through a stacking gel of high porosity the SDS-polypeptide complexes are deposited in a very thin zone (or stack) on the surface of the resolving gel. The discontinuous buffer system that is most widely used was originally devised by Orstein (1964) and Davis (1964) [77]. The sample and the stacking gel contain Tris Cl (pH 6.8), the upper and lower buffer reservoirs contain Tris-glycine (pH 8.3) and the resolving gel contains Tris Cl (pH 8.8). All components of the system contain 0.1% SDS [56].

## **Precipitation methods of proteins**

Precipitation is widely used in downstream processing of biological products, especially proteins. It serves to concentrate and fractionate the target product from various contaminants, as in biotechnology industry where precipitation helps to eliminate contaminants commonly contained in blood. The underlying mechanism of precipitation is to alter the solvation potential of the solvent and thus lower the solubility of the solute by addition of a reagent.

Precipitation is usually induced by any of the following methods [11]:

- Salting out
- Isoelectric point precipitation
- Precipitation with organic solvents
- Non-ionic hydrophilic polymers
- Flocculation by polyelectrolytes
- Polyvalent metallic ions

Salting out

This is the most common type of precipitation. Normally a neutral salt is added, such as ammonium sulphate, which compresses the solvation layer and increases protein – protein interactions. As the salt concentration of a solution is increased, more of the bulk water is associated with the ions. Consequently, less water is available to partake in the solvation layer around the protein, which exposes hydrophobic interactions, aggregate and precipitate from solution.

#### Isoelectric point precipitation

The isoelectric point (pI) is the pH of a solution at which the net primary charge of a protein becomes zero. At a solution pH that is above the pI<sup>™</sup> the surface of the protein is primarily negatively charged and therefore like-charged molecules will exhibit repulsive forces. At a solution pH that is below the pI, the surface of the protein is primarily positively charged and repulsion between proteins occurs. At the pI, the negative and positive charges cancel, repulsive electrostatic forces are reduced and the dispersive forces predominate, and will, therefore, cause aggregation and precipitation. The pI of most proteins lies in the pH range of 4-6. Mineral acids, such as hydrochloric and sulphuric acid are used as precipitants. The greatest disadvantage to isoelectric point precipitation is the irreversible denaturation caused by the mineral acids. For this reason isoelectric point precipitation is most often used to precipitate contaminant proteins, rather than target protein.

#### Precipitation with organic solvents

Ethanol or methanol, if added to a solution may cause the proteins of the solution to precipitate. As the organic solvent gradually displaces water from the surface of the protein and binds it in layers around the organic solvent molecules, the solvation layer around the protein decreases. In that state, the protein can aggregate by attractive electrostatic and dipole forces.

Parameters to consider are temperature (should be less than 0°C to avoid denaturation), pH and protein concentration of the solution. Miscible organic solvents decrease the dielectric constant of water, which in effect allows two proteins to come together. At the pI the relationship between the dielectric constant and protein solubility is given by:

$$\log S = k/e^2 + \log S_0$$

$S_0$  is an extrapolated value of  $S$ ,  $e$  is the dielectric constant of the mixture and  $k$  is a constant that relates to the dielectric constant of water [98].

#### Non- ionic hydrophilic polymers

Dextrans, polyethylene glycols and other polymers are used in precipitation of proteins due to their low flammability and are less likely to denature biomaterials compared to pI precipitation. These polymers attract water molecules away from the solvation layer around the protein, which enforces protein-protein interactions and induces precipitation. For the case of polyethylene glycol, the following equation models precipitation:

$$\ln(S) + pS = X - \hat{\pm}C$$

$C$  is the polymer concentration,  $P$  is a protein-protein interaction coefficient,  $\hat{\pm}$  is protein- polymer interaction coefficient and

$$X = (\hat{\mu}_i - \hat{\mu}_i^0) RT$$

$\hat{\mu}_i$  is the chemical potential of component I, R is the universal gas constant and T is the absolute temperature [98].

### Flocculation by polyelectrolytes

Polyelectrolytes form extended networks between protein molecules in solution. These include alginate, carboxymethylcellulose, polyacrylic acid, tannic acid and polyphosphates. The pH of the solution determines the effectiveness of these polyelectrolytes. Anionic polyelectrolytes are used at pH above the pI. Cationic polyelectrolytes are used at pH below the pI. The precipitate may dissolve back into the solution if an excess of polyelectrolytes is used.

### Polyvalent metallic ions

Enzymes and nucleic acids are precipitated with the use of metal salts at low concentrations. Most frequently polyvalent metallic ions used are Ca<sup>2+</sup>, Mg<sup>2+</sup>, Mn<sup>2+</sup> or Fe<sup>2+</sup>.

### Precipitation reactors

Industrial scaled reactors that are used to precipitate large amounts of proteins, such as recombinant DNA polymerases from a solution include:

#### Batch reactors

The agent is slowly added to the protein solution under mixing, so the aggregating particles tend to be regular in shape. The protein particles are

exposed to a wide range of shear stresses for a long period of time and become mechanically stable.

### Tubular reactors

The precipitating reagent and the feed protein solution are contacted in an area of mixing and then added into elongated tubes where precipitation occurs. Plug flow is approached by the elements as they move along the tubes. The tubular reactor is inexpensive to be constructed but can become long and slow in case that aggregation of the particles occur slowly.

### Continuous stirred tank reactors

CSTR reactors also known as vat or back mix reactors, run at steady state with a continuous flow of reactants and products in a well-mixed tank. It is a type of reactor mainly used in chemical engineering. A CSTR often refers to a mathematical model which is used to estimate the key unit operation variables when using a continuous agitated-tank reactor to reach a specified output. Perfect mixing is demanded.

## **Precipitation of *L. starkeyi***

The most common precipitation methods in the case of *L. starkeyi* cited in literature are:

### Isoelectric focusing

Koenig and Day (1988) used precast IsoGel agarose isoelectric focusing plates, pH 5.0-8.5. A standard mixture of proteins was applied in the lane next to each sample and the protein profile was quantified by densitometer

scans. The enzyme activity in the gel was determined by slicing an unstained gel into 0.9 mm sections. Each section was placed in a test tube with 1.0 ml 0.05 M citrate/phosphate (pH 5.5) buffer, allowed to elute overnight at 4°C and assayed for enzyme activity. This method separated the protein mixture into five isoelectric bands. All five forms were found to have dextranase activity and exhibited the same  $K_m$  values.

### Organic solvents

Polyethylene glycol precipitation is often used. Nishimura et al. (2002) used this method in an effort to prepare total DNA from *L. starkeyi* for taxonomy analysis. They added phenol solution (phenol: chloroform: isoamyl alcohol = 25: 24: 1) to a test tube of Tris-SDS. The