41

IMMOBILIZATION OF BACTERIAL PROTEASES FOR ENVIRONMENTAL APPLICATIONS ON WATER-SOLUBLE POLYMER BY MEANS OF ELECTRON BEAM

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41.1 INTRODUCTION

Natural systems, such as enzyme preparations, are attractive as possible alternatives for pollution control. The main question is the proper choice of the enzyme with the required catalytic activity for application under complex environmental conditions. In general, the native form of the enzyme may or may not be useful for solving the ecological problem of interest.

Another serious obstacle that has restricted the ecological applications of enzymes is their unavailability on a commercial scale. This unavailability is mainly due to their relatively high cost and low efficiency. For this reason native enzyme preparations have not found widespread application in solving ecological problems and have remained within the frame of laboratory studies.

One possible solution to this problem has been the development of radiation technologies that are used to obtain enzyme preparations on an industrial scale that are relatively cheap and have a high catalytic stability. The high stability is in part due to the process of radiation immobilization on the various kinds of carriers. Enzyme preparations have been reported for decomposing oil products polluting the soil and water surface in reservoirs [1], for the decomposition of rubber in used tires, and for treating various kinds of wastewaters [2]. These enzymes are also used to treat garbage, cellulose, and timber production wastes [3–5].

This chapter covers the fundamentals of radiation technology for production of immobilized proteases from bacterial raw materials. The results of their use in treating wastewaters from the preparation of milk products and primary wool treatment are described. The application of immobilized proteases is restricted because of the lack of availability of the cost effective preparations and not their potential widespread application in areas of pollution control.

41.2 IMMOBILIZED PROTEASE PREPARATIONS

It is possible to obtain various types of immobilized enzymes with widely varying properties, quality and cost, using commercial sources. The task of creating

immobilized enzyme preparations for the solution of specific problems related to pollution control imposes specific requirements, for example, the proper enzyme–carrier combination in sufficient quantity at an affordable price and enzyme activity.

The choice of the enzyme–carrier depends on the application and the enzyme constraints such as high specificity for the substrate, required activity and stability in a wide range of the medium pH. The carriers for enzyme preparations have to be inert and subject to biodegradation.

41.2.1 Choice of Enzyme for Radiation Immobilization

One area of investigation in the field of environment protection is the application of hydrolytic enzymes, particularly proteases, for the degradation of the protein pollution of various origins. The idea of immobilized forms of proteases results from the restricted use of native forms of the enzymes in practical applications. The term *immobilized proteases* in this application refers to enzymes stabilized by means of connection with some carrier. In this case the term *immobilized* does not correspond to all variations of stabilized enzymes, and a more proper description might be the term *modified*. The *modified enzyme* is a more exact definition, but below we use the term *immobilized* as it is most commonly used in the literature.

The use of proteases obtained by methods of traditional biotechnology from bacterial raw material is well documented [6,7]. Among those are the proteases that are obtained from *Bacillus subtilis*. For example, some strains of *B. subtilis* can produce, under optimal conditions, 1 g of various proteases from 1 kg of growth medium. Therefore, the possibility of obtaining relatively cheap and highly active proteases from various strains of *B. subtilis* (in comparison with other sources) is appealing.

Proteases obtained from *B. subtilis* have a high specific activity over a wide range of pH and strong substrate specificity. For example, researches have reported the ability of *B. subtilis* to produce alkaline proteases—with pH optimum of 8.0–10.0—and neutral proteases—with pH optimum of 6.5–8.0 (references). However, proteases have an important drawback that is low thermal stability, a tendency to autolyze and quick loss of specific activity in the presence of the substrate. This temperature sensitivity is a serious obstacle for use of native forms of the enzymes in environmental applications. Radiation immobilization is one way to minimize this drawback and allow the use of the immobilized enzyme for pollution control.

41.2.2 Alkaline and Neutral Proteases for Degradation of Protein Pollution

The alkaline proteases of *B. subtilis*, known as *subtilisines*, are capable of decomposing both denatured and native proteins. The rate of decomposition of the denatured proteins is much faster than that of the native proteins. The

resistivity to decomposition of the native proteins is perhaps the result of three-dimensional structure of the native protein, when compared to the denatured proteins. It is also possible that the native proteins have a natural tendency or protection from enzymatic proteolysis. In any event, the *B. subtilis* proteases are capable of continuous action, at high concentrations of the enzyme, to decompose proteins faster and more completely than other enzymes of animal origin such as trypsin, chymotrypsin, and pepsin. Studies of the hydrolysis kinetics for various protein bonds have shown that the subtilisines preferably hydrolyze the bonds formed by carbonyl groups of the amino acids with the hydrophobic side chains of leucine, phenylalanine, tyrosine [8,9].

The general reaction describing the hydrolysis of peptide bonds is as follows:

$$R—CO—NH—R' + H_2O \rightarrow R—COOH + R'—NH_2 \qquad (41.1)$$

Highly purified subtilisines are more stable in alkaline medium than trypsin and chymotrypsin. Another advantage of subtilisines for use in pollution control is their ability to hydrolyze, to free amino acids, terminal peptide bonds as well as peptide bonds along the protein chain, of various origin.

In some cases a decrease of pH to below 5.0 leads to the loss of specific activity of enzymes and irreversible denaturing [10]. Therefore it may be preferable to use enzyme complexes that function in a wider pH range without loss of specific activity. This is another advantage of the enzyme isolates from *B. subtilis*, which have demonstrated activity over a greater pH range.

41.2.3 Neutral Proteases of *Bacillus subtilis*

Neutral proteases of *B. subtilis* represent proteolytic enzymes with Zn in the active center. Their pH optimum for specific activity is neutral (6.5–7.5). Three neutral proteases are known that destroy the peptide bonds formed in synthetic substrates and denatured proteins by amino groups of hydrophobic acids: leucine, valine, and L-phenylalanine [11,12].

The neutral proteases are characterized by a higher specific activity when compared to subtilisines and other proteolytic enzymes. The drawbacks of these enzymes are their restricted time of activity in the presence of the substrate, narrow pH range, and low thermal resistance. But the possibility of radiation immobilization of these enzymes on selected carriers minimizes these potential problems.

41.2.4 Choice of Carriers for Radiation Immobilization of Enzymes

The physical and chemical properties of the carriers that are important are the physical strength that they impart and also the potential influence on the cat-

alytic properties of the attached enzyme. These properties are important to consider during the technology development process for production of immobilized enzymes. In some cases an improper choice of the carrier results in a barrier for interaction of the enzyme with the protein substrate or in the worst-case scenario may result in the denaturization of the enzyme. An example of such a problem would be the immobilization on the carriers containing ionizable groups, for example, ion-exchange resins. In this case the diffusion of the solvent ions, H_3O cations, in the zone of enzyme's active center could be either hampered or facilitated, and as a result, the rate of enzymatic reaction changed.

The influence of the microenvironment on the catalytic properties and stability is also apparent in case of immobilization on hydrophilic and hydrophobic carriers. It has been shown that enzymes with a hydrophilic active center, for example, lactate dehydrogenase and glucose oxidase, are more stable on the hydrophobic carriers. By analogy, enzymes with hydrophobic active centers, for example, tyrosinase and alcohol dehydrogenase, are more stable when immobilized on hydrophilic carriers.

If easily activated nucelophilic groups, such as NH, SH, and OH, prevail in the structure of the carrier, the enzyme can be immobilized on such carriers by traditional methods with the formation of covalent bonds. If the carrier does not contain such groups, or has groups capable of being ionized, the immobilization methods based on physical or physicochemical interaction of the carrier with the enzyme are better. So, the structure of the carrier permits a choice based on existing immobilization methods, the optimal process determined by the interaction between the enzyme and the carrier.

Considerable experience has been gained in the area of immobilized enzymes aimed for use in biotechnology, chemical technology, analytic chemistry, and other applications. Many carriers and immobilization methods used can be adapted for obtaining immobilized enzymes for environmental protection from protein pollution [13,14]. In this regard, one has to briefly examine the existing carriers, their properties and drawbacks concerning the possibility for their use as matrices for enzymes in radiation immobilization process.

Classification of the carriers used for immobilization is not complicated and is reduced to their partition into two large groups: organic and inorganic polymer carriers. The organic carriers represent a series of natural and synthetic polymers. The polysaccharide materials (cellulose, spongy starch, algin acids and their salts, chitin) are traditionally used for chemical immobilization [15–17]. These carriers have some advantages, for example, relative compatibility with biological systems and the absence of diffusion restrictions for interaction with substrate. Highly reactive groups necessary for chemical immobilization on these carriers can be formed using reagents such as diisocyanates, epichlorhydrin, and disulfides.

The use of natural polysaccharide carriers for the immobilization of many enzymes such as trypsin, isomerases, glucoisomerases is known. The polysac-

charide carriers have two main drawbacks: (1) they are not mechanically durable, and (2) they are susceptible to microbial degradation. The linked agarose is the exception, but it is relatively costly. The natural sources of many polysaccharide carriers are restricted (starch, algin acid, chitin), and that presents an obstacle for their environmental applications [18].

Synthetic carriers suitable for immobilization are made up of synthetic organic polymers, the most common of which is polyacrylamide. Its amide groups can be easily activated by various reagents including glutaric aldehyde [19]. This activation method has found a widespread application in affinity chromatography. The use of polyacrylamide gels has made it possible to include various enzymes into the gel while retaining of 80–100% of enzyme's initial activity. The experience in obtaining polyacrylamide gels by copolymerization of acrylic monomers such as acrylo-*N*-tetrabutoxycarbonyl hydrazine, cryloamorphoilacryloxysuccinimide has been reported [20].

The ethylene glycol–methacrylate gels and also gels based on *N*-vinylpyrrolidone, *N*-vinylpyridine, and polyethylenoxide; copolymers of ethylene and maleic anhydride; polyurethane; polystyrene; nylon; and various kinds of polyamides, polyvinylchlorides, polymethylmetacrylate have been used for chemical immobilization [21–24]. These immobilization technologies are labor-intensive and characterized by a large number of process stages. The use (and consumption) of expensive toxic compounds for synthesis of active chemical groups in the carrier and in the enzyme also presents drawbacks. The process itself creates chemical pollution and an additional expense for decontamination. The experience of their use in environment protection has been limited to research projects.

The problem associated with the high cost of production has now been resolved using radiation technology. This process permits a more cost-effective solution with gels of polyacrylamide, polyethylenoxide, polyurethane, and polyvinylpyrrholidon and the inclusion of the necessary enzymes into the structure of gels, and is now a trend in obtaining the immobilized multifunctional enzymes, including the ones aimed at pollution control.

Technologies with inorganic carriers are also being developed; the most common inorganic carrier being synthetic porous glasses, silica gels, silochrome, and zeolites [25]. The advantages of inorganic carriers are the high mechanical durability, chemical resistance, and relative economic efficiency. The use of inorganic carriers also makes it possible to regenerate the surface of the carrier, after the enzyme has lost its efficiency, and immobilization of the new enzyme.

One enzyme acceptable for pollution control and in particular for the destruction of proteinaceous pollutants is the one based on proteolytic enzymes obtained from the bacterial raw materials. To apply this enzyme system it would be immobilized on the appropriate carrier by means of radiation technology. This chapter focuses on the immobilization of bacterial proteases on low molecular polyethylenoxide carriers.

41.3 TECHNOLOGICAL PRINCIPLES OF RADIATION IMMOBILIZATION OF BACTERIAL PROTEASES

41.3.1 Choice of Immobilization Method

Immobilization of an enzyme has to satisfy the following requirements:

1. That the carrier forms a relatively stable chemical complex with the enzyme and that this complex is capable of selectively reacting with one or more components of protein substrates retaining its specific activity during tens of hours or days.
2. The carrier has to be compatible with the function of immobilized proteases, including the formation of the necessary hydrophilic environmental surrounding the protease molecule.
3. The location of the protease molecules on the surface of the polymer should allow the complex access for interaction with protein substrate.
4. When the enzyme–carrier have completed the job, the complex should be biologically degradable so as to minimize additional pollutant loading to the environment.
5. The enzyme–carrier system must be cost-effective.

41.3.2 Characteristics of Proteases and Carrier

The proteolytic enzymes complex produced by the highly active industrial strain *B. subtilis* CH-15 was chosen for radiation immobilization. The strain was obtained by directed mutagenesis of known strains of *B. subtilis* [26]. Chromatographic studies have shown that this strain produces both alkaline and neutral enzymes that actively hydrolyze casein and other denatured proteins of animal origin. The final product was compared with preparations of free proteases: trypsin ("Spofa," Czec.). The proteolytic activity PA was determined by casein hydrolysis according to the formula [27]

$$\mathrm{PA} = \frac{D(280)}{V^*M^*T}$$

where $D(280)$ = optical density at wavelength of 280 mm
V = volume of analyzed sample (mL)
M = mass equivalents of the substrate in grams
T = time (h)

The acid-soluble products of hydrolysis were assayed by spectroscopic measurements at wavelength 280 nm. The quantity of the sample containing protease that, during 1 h at 37°C, transformed 1 mg of casein (substrate) into a

trichloracetic acid (solution 0.3 mol/L) soluble form was the measure of proteolytic activity.

Low-molecular weight polyethylene oxide (PEO) was used as a carrier for the enzymes, which is a relatively inert substance permitted for use in the medical and food industries. For use as a carrier the polymer must be activated by the formation of chemically active groups to which the proteases can be bound. Immobilized complexes consisting of PEO and an active center (bacterial protease) have a prolonged activity in comparison to native protease [28]. Systems using the PEO carrier can be used for washing solutions for milk production and equipment for mince-meat production and other food processing applications [29].

41.3.3 Radiation Sources for Immobilization of Enzymes

Radiation technology for the immobilization of bacterial proteases, at an industrial scale, is possible with various types of existing equipment. Some of the advantages of using radiation technology when compared to classic chemical immobilization methods are:

1. A reduction in product costs
2. A relatively higher processing rate because of the high irradiation dose rates
3. A reduction in heat generation and in chemical pollution
4. A reduction in the amount of chemical reactants necessary for immobilization
5. Higher-efficiency equipment and therefore a more effective use of electric power
6. The possibility of multitasking of the accelerator for parallel tasks, for example, the sterilization of continuous flows of solutions, mixtures and the production of gels
7. Equipment lifetime that is substantially longer than that for other processes

Other advantages are that processes that use electron-accelerators generate little or no wastes. Another consideration is that when working in the range of 2.5–4.0 MeV, the formation of radioactive isotopes is not possible. The industrial electron accelerator ILU-10 with electron energy range up to 4.0 MeV and beam power up to 40 kW would be the source used for immobilization of proteases [30].

The immobilized protease can be produced in two ways: (1) *one-stage process*, where the PEO solutions and the proteases are irradiated in the same solution resulting in the immobilization of the enzyme; and (2) a *two-stage immobilization*, in which (a) the PEO solution is transformed into a gel with radiation (radiation-induced crosslinking) and (b) the proteases are added to gel and irradiated, immobilizing them on the polymer carrier. The irradiation doses required for the immobilization of proteases are 2–10 kGy.

41.3.4 Design of Installation for Immobilization of Enzymes

A dose of 10 kGy (or 1 Mrad) corresponds to the deposition of 10 kJ of energy by the radiation source to 1 kg of treated material. Therefore, to achieve 10 kGy, a 10-kW beam (10,000 J/s) has a processing rate 1 kg/s of gel or immobilized proteases. Lower power or higher flow rates would be possible for lower doses.

The penetration depth of 3.5-MeV electrons is about 12 mm for water (unit density). In case of PEO the optimal thickness of the layer will be about 10–12 mm with relatively homogeneous dose distribution. The ILU-10 is an accelerator has an electron energy range of 3.0–4.0 MeV and an average beam power of 40 kW. The accelerator and the entire installation are controlled by a PC.

The irradiation scheme shown in Figure 41.1 is an installation designed for a continuous operation. The solution or gel to be irradiated is pumped into the feed tank. This tank is equipped with a horizontal gap and an inclined tray. The solution flows through the gap and is accelerated along the slope due to gravitation. At the end of the tray the solution falls in air and is irradiated at that time. In case of a gel (because of differences in viscosity) another tray with different design and dimensions would be used. The stream falls into a collection tank and is then pumped out. The thickness of stream was chosen at about 10 mm, similar to the penetration depth of 3.5 MeV electrons.

The irradiation zone under the extraction device of ILU-10 is about 800 mm in width. Using a thickness of the gel layer of about 10 mm the velocity of flow for the production rate 2 kg/s would be about 250 mm/s (0.25 m/s). This velocity can be achieved easily using gravitation flow.

The electrons are effectively absorbed by the falling solution or gel and the electrons that are not absorbed are absorbed by the solution in the collection tank. The x-rays (bremsstrahlung) that are formed are also absorbed by the gel in the collection tank.

The solution in this irradiation scheme is stirred vigorously, and the efficiency of the irradiation scheme approaches 100%. At a dose of 10 kGy (or 1 Mrad) a 20 kW beam (20,000 Joules/s) will result in a production rate of 2 kg/s of gel or immobilized proteases, or 7.2 tons per hour or about 55 tons per one 8-h shift. The accelerators are most effectively used if they are on 24 h per day.

The main components of the installation (accelerator and tanks with pumping system) are placed in the irradiating room, inside a concrete bunker with wall thicknesses of about 2 m. The dimensions are like the second variant (except for the conveyor). The conveyor is not needed in this irradiation scheme but it can be used for irradiation of other products such as single-use medical goods. Another underbeam transportation system can also be designed where the bunker has a removable steel door and labyrinth channels for the cables and pipes. The solutions and gels are pumped into the installation and out of it through pipes placed in labyrinth channels. All communications (electric, water, and air) are done through separate labyrinth channels. The equipment layout can be the same as in second variant (Fig. 41.2).

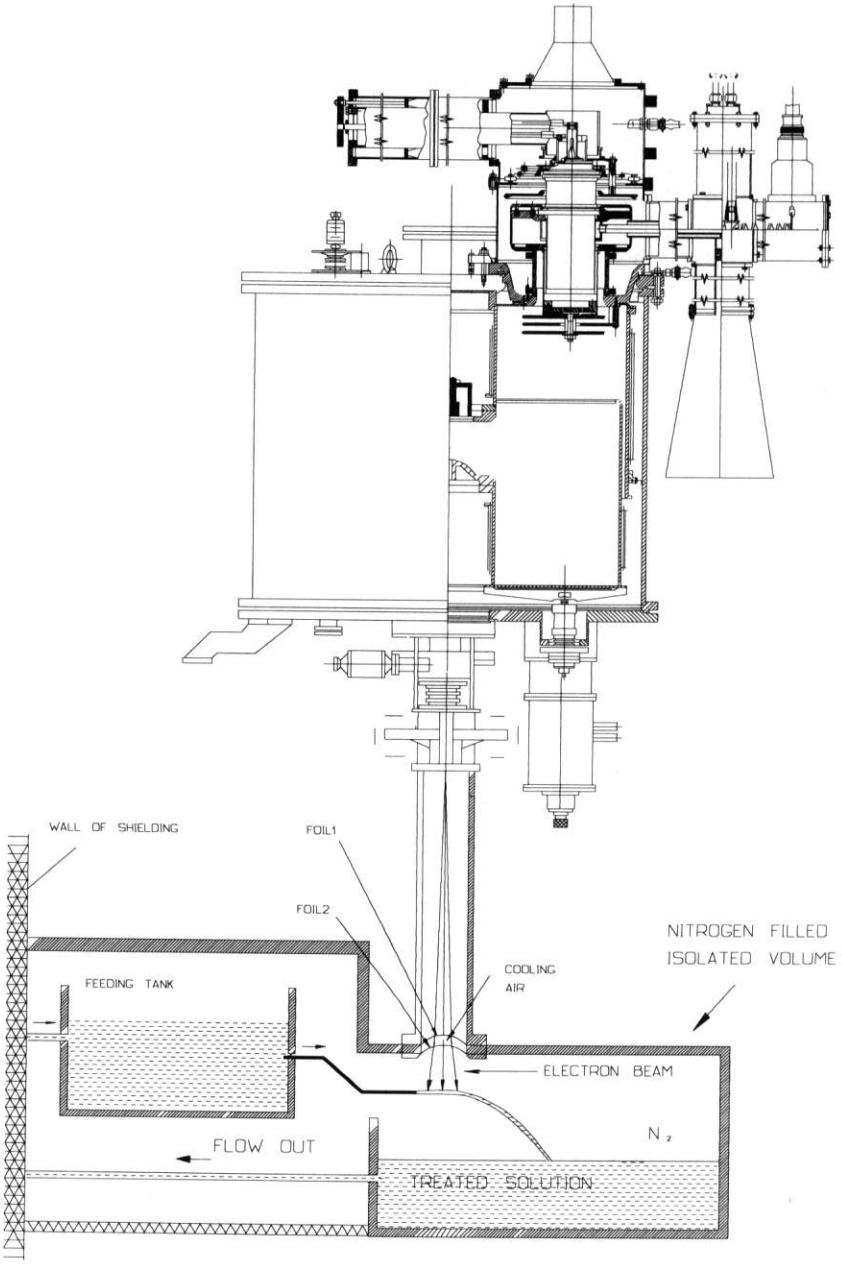

Figure 41.1 Irradiation scheme for continuous flow operation.

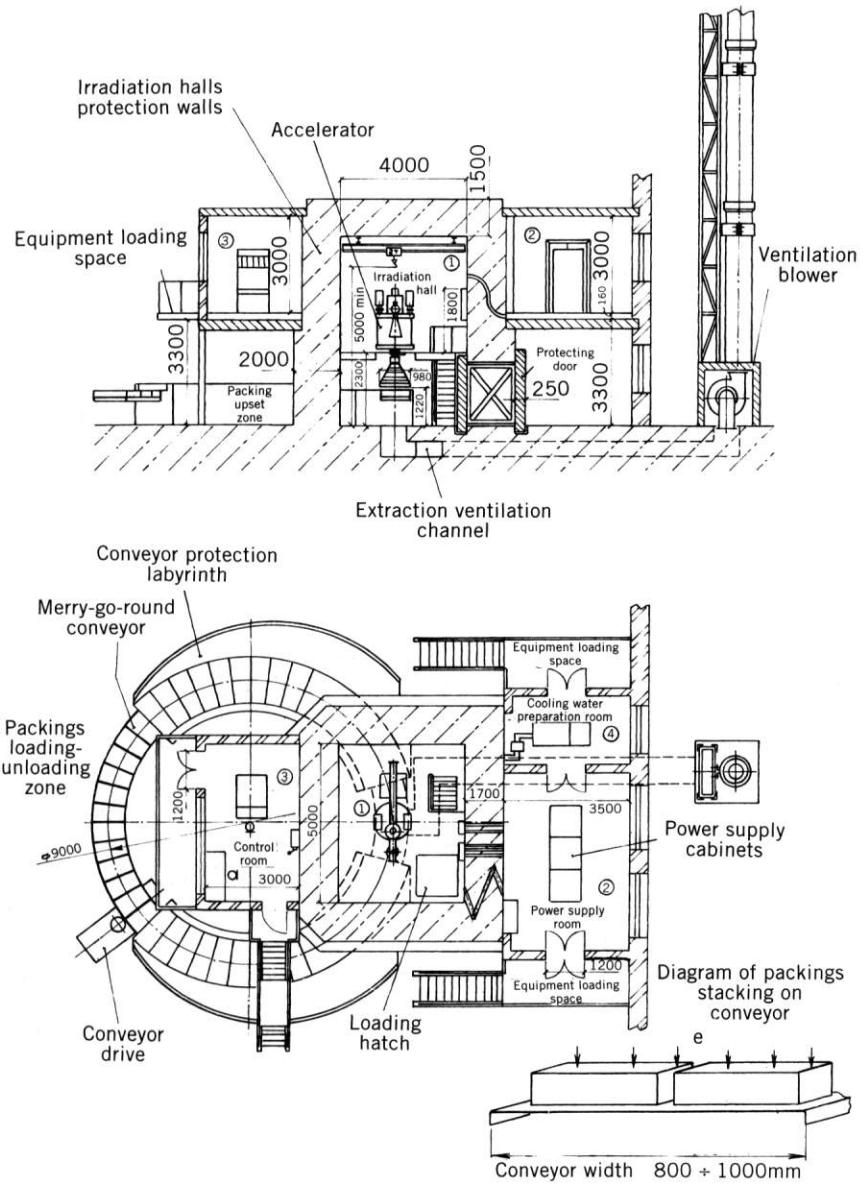

Figure 41.2 Installation for radiation treatment of immobilized enzyme preparations and sterilization of single-use medical goods based on industrial electron accelerator ILU-10.

The second design option [31] of an installation is described below. The installation provides the irradiation of batches (containing 51 of solution or gel) with horizontal dimensions of 600 × 800 mm. Oher products to be treated can also be transported by the same conveyor in the packs with compatible outer dimensions as described below.

The installation is presented on the drawing (Fig. 41.2). The system is comprised of four areas (three main and one auxiliary):

1. The irradiation hall
2. The power-supply room
3. The control room
4. The cooling water preparation room

The loading and the unloading of the batches or packs are done in the special zone, as is shown in Figure 41.2.

The merry-go-round conveyor (mountable–dismountable) with the friction electric drive and a velocity of up to 8-m/min transports the batches into the irradiation hall. The cylindrical rollers all along the conveyor provide the horizontal and the vertical stability of the motion. The input and the output conveyor passages to the irradiation hall via concrete troughs to protect them from the scattered radiation. These troughs are covered with removable steel plates. The conveyor also carries the lead partitions overlapping the cross section of passages. The distance between the partitions is determined by the dimensions of packs and batches. The variations in the radiation protection and the simple conveyor organization permits a decrease in the price of construction.

The irradiation room height is determined by the dimensions of the accelerator and the lifting-transportation gear. The radiation protection can be made from the usual concrete (d = 2.3 g/cm^2), in this case the wall thickness can be approximately 2 m. The exact thicknesses of the concrete irradiation room and through walls, ceiling, removable steel plates, and the irradiation room protective door would be designed to meet national standards for each installation. The total production area of the installation is no more than 220 m^2.

The beam efficiency in this design is about 50%. The production rate of an installation with a beam power 20 kW and a dose of 10 kGy (1 Mrad) is approximately 3 tons per hour.

41.4 PROPERTIES OF IMMOBILIZED ENZYMES OBTAINED BY MEANS OF RADIATION IMMOBILIZATION

41.4.1 Radiation-Induced Activation of Polyethylene Oxide

Among the polymers convenient for radiation immobilization are the polyethylene oxides (PEO) (polyethylene glycols) having the general formula

$$H[—O—CH_2=CH—]_n—OH$$

They are low in toxicity, compatible with biological media, easily soluble in biologic fluids and easily excreted once inside a body. For use as a carrier PEO must be activated by the formation of chemically active groups to which the enzymes can be bound.

Known chemical methods for the activation of PEO are labor-intensive multistep processes [32]. Most frequently the immobilization is accomplished by the formation of chemical "bridges," a bifunctional reagent. The molecule playing the role of "bridge" must have small dimensions and reactive groups capable of binding the PEO and the enzyme. Chlorine cyanuric acid corresponds to these requirements. It has three groups C—Cl capable to react. The activated PEO (2-*O*-methoxypolyethyleneglycol-4,6-dichloro-*S*-triazine) is obtained with the help of chlorine cyanuric, and it is capable of forming covalent bonds with various enzymes (catalase, glycosidase, galactosidase) [33,34]. The main drawback of such methods is that the bifunctional reagents often react with the active center of the enzyme causing reduction or complete inhibition of its enzymatic activity [35].

Radiation activation of PEO has some advantages over the known chemical ones. It permits reliable control of the activation level, it has only one step, and there is no need for expensive and/or toxic intermediate compounds, as above, and there is no cleaning of the final product. The entire process is waste-free minimizing environmental contamination.

The number of carbonyl functional groups has been shown to be controlled by the method of Lappin and Clark [36]. It has been shown that the irradiation of water solutions in the 2–10-kGy range results in functionality of up to 10^{-6} mol/L. The linear dependence of the functionality and dose is shown in Figure 41.3. While Figure 41.4 shows the relationship of the PEO concentration.

It is possible to speculate that the formation of functional groups on the PEO results from molecular oxygen in the water solution and at least the oxygen concentration is sufficient to account for the oxidation of PEO. Such radiation-chemical oxidations of organic matters in aqueous solutions are also known for other compounds [37]. Apparently, the hydroxyl groups of the PEO are subject to oxidation, with the formation of carbonyl compounds, similar to the radiolysis of water solutions of aliphatic alcohols [37,38].

The proportional dependence of the functionality on irradiation dose demonstrates the possibility of choosing the optimal irradiation regime, the optimum concentration of the PEO solution, thereby, permitting optimum number of active groups for binding to the proteases or other biologically active compounds, capable of reacting with carbonyl groups.

41.4.2 Influence of Irradiation on Reaction Mixture of Proteases and Carrier

Some properties of native proteases of *B. subtilis* and PEO were described in the earlier chapter. The main result of irradiation is the formation of a stable complex consisting of the enzyme and PEO. To clarify the question of whether

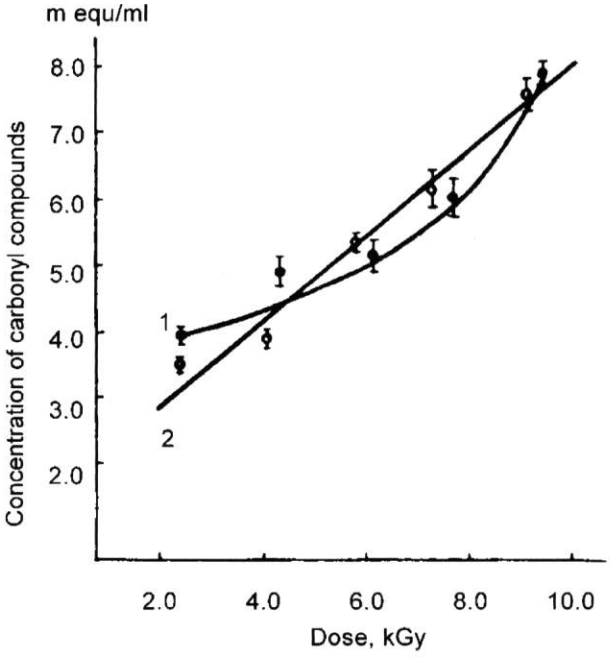

Figure 41.3 Formation of carbonyl compounds in solutions of PEO-4000 (1) and of PEO-1500 (2) depending on irradiation dose.

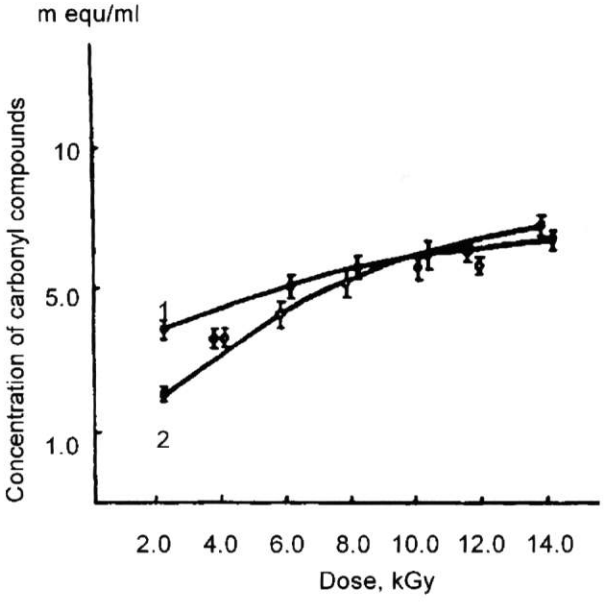

Figure 41.4 Formation of carbonyl compounds in solutions of PEO-4000 (1) and of PEO-1500 (2) depending on the concentration. Irradiation dose 6 kGy.

the linking of the enzyme with the carrier was successful, chromatographic studies of proteins before and after the irradiation were used.

Ion-exchange chromatography on DEAE cellulose (DE-52b Whatman, England) was used for this purpose. The studies were conducted using gradient elution (0–15 *M* solution of LiCl) at pH 9.5. The desorbed protein fractions were determine by means of an UV absorbance detector adjusted to 290 nm. The proteolytic activity in the various fractions was determined using the hydrolysis of (colored) casein.

The chromatography of *B. subtilis* proteases has shown that their protein content is highly heterogeneous and under the conditions the chromatogram has seven peaks. The highest proteolytic activity was concentrated mainly in the sample corresponding to the third, and fourth peaks, in other samples the activity was lower.

The addition of a 10% solution of PEO to the solution of proteases did not change the order of elution of the protein peaks. Its chromatographic profile is superimposed on the profiles of proteins, and in every sample of proteins one can find free PEO (Fig. 41.5). The chromatographic study of the mixture

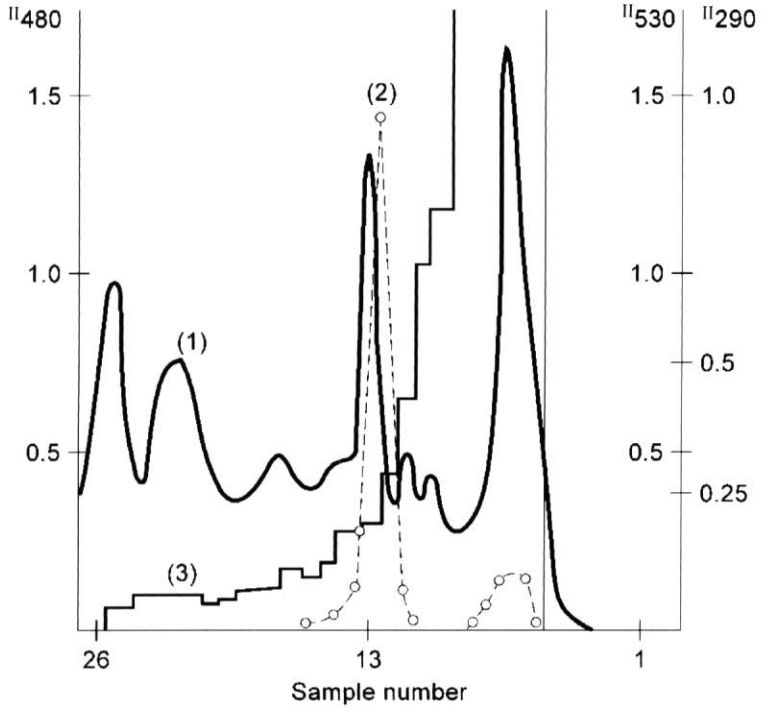

Figure 41.5 Chromatography of a mixture of cultural medium Bac. subtilis and PEO-1500 on DEAE-cellulose before irradiation. 1-protein concentration; 2-proteolytic activity; 3-PEO-1500 concentration.

of proteases and PEO after irradiation with dose 6 kGy indicates that the PEO combined with the peaks of the proteins responsible for the proteolytic activity (Fig. 41.6). He observed changes in the chromatograms of proteins allows speculation on the possible mechanism for immobilization of the proteases.

As a result of the irradiation, the PEO is activated with highly active carbonyl groups. These groups can react with various compounds containing nucleophilic groups (amine, thiol, hydroxyl) [27], and it has been shown that the proteases subjected to immobilization contain many amino groups [39].

The interaction of the amino and carbonyl groups leads to formation of azomethylene bonds between enzyme and polymer according to the formula:

$$H(OCH_2—CH_2)_n—C + H_2N \xrightarrow[-H_2O]{} C{=}N—R$$

The possibility of the formation of other kinds of bonds between the components is also possible and can not be ruled out on the basis of the experimental evidence.

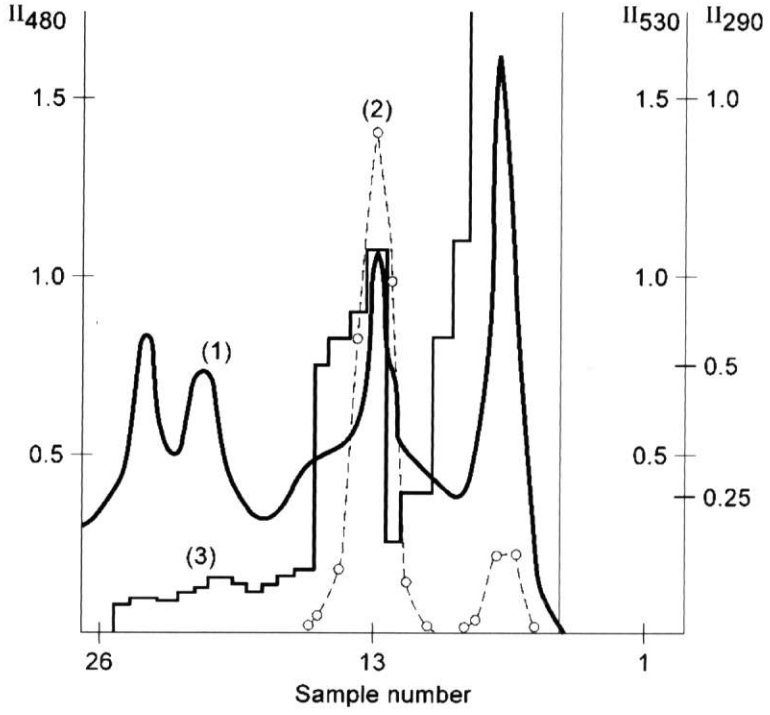

Figure 41.6 Chromatography of a mixture of cultural medium Bac. subtilis and PEO-1500 on DEAE-cellulose after irradiation with dose 6 kGy. 1-protein concentration; 2-proteolytic activity; 3-PEO-1500 concentration.

41.4.3 Thermal Stability of Immobilized Proteases

The thermal stability of enzymes is an important feature of enzymes for use in environmental applications. Enzyme preparations have to function over a wide range of ambient temperatures. For example, it is well established that heating solutions of proteases leads to decrease of specific activity. Solutions of proteases heated to 50°C has led to loss of 90% activity of trypsin and to substantially decrease of activity of native proteases.

We have shown that by immobilizing proteases their thermal stability is enhanced. The proteases immobilized by radiation technology retain 85–100% of proteolytic activity in the temperature range 35–70°C; however, at 80°C activity decreased 70–74% of maximum value. The temperature range for activity was increased to 15°C higher than for native forms (Fig. 41.7).

The exact reason for increased temperature stability using the radiation immobilization technology is connected is not known. It is likely that it is related to steric restrictions of interenzyme autolytic reactions and in some way with increased structure stabilization of enzymes by PEO.

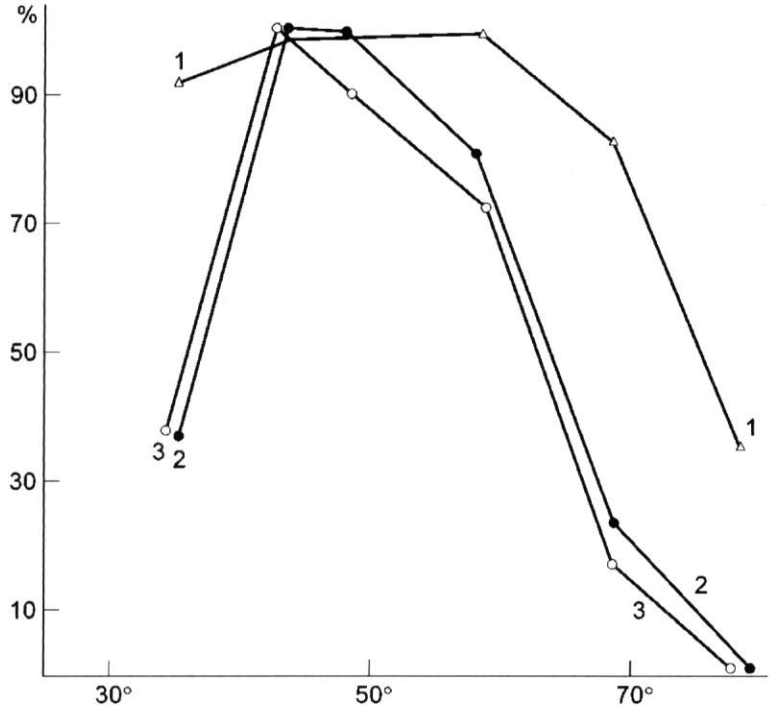

Figure 41.7 Thermal resistance of proteases in presence of substrate 1-bacterial proteases, immobilized on 1,4-PAO (immozim); 2-free bacterial proteases in 1,4-PAO solution; 3-free bacterial proteases in the water. Maximum value of ferment's activity was assumed as 100%.

41.4.4 Dependence of Proteolytic Activity of Proteases on Medium pH

The specific activity of immobilized proteases depends on the medium pH and the presence of substrate similar to that of the native forms of enzymes as shown in (Fig. 41.8). For example, the radiation immobilized enzyme had an optimal activity range of pH 6.8–11.2 while the native proteases have a pH range of 6.8–8.0.

The observed shift of the pH activity optimum for an enzyme is typical for immobilized enzymes on polymers containing groups capable of being ionized. Although PEO does not contain such groups, it is possible that the pH optimum shift in enzymatic activity of the immobilized proteases was related in some way with the specific properties of the carrier. The polyethylenoxides are known to form structures capable of chelating cations [40], and this may account for the shift in activity pH range.

41.4.5 Dependence of Irradiation Dose and Duration of Storage on Proteolytic Activity of Immobilized Proteases

It is known that the absolute proteolytic activity of the enzyme is lower, than native forms of the enzyme, after irradiation. The higher the dose the more the

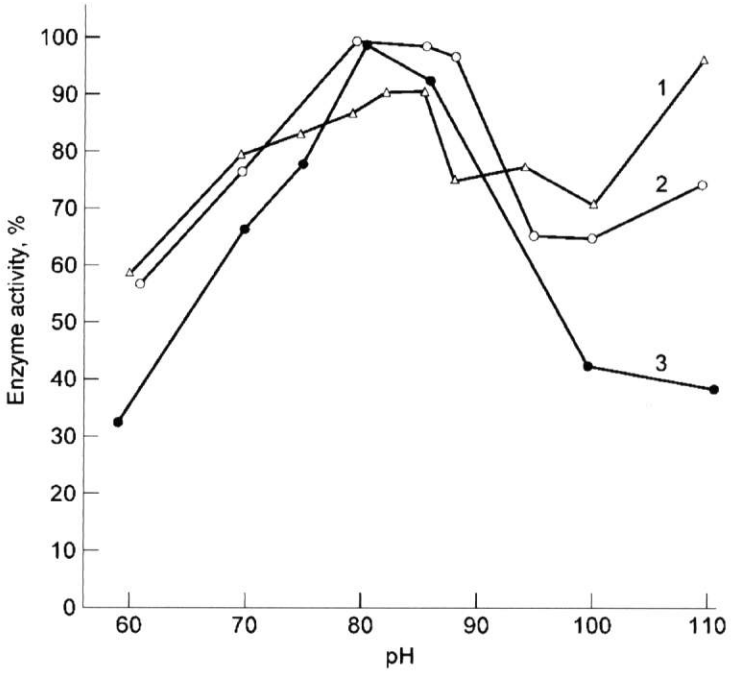

Figure 41.8 Dependence of proteolytic activity from pH of substrate. 1-bacterial proteases immobilized on 1,4-PAO; 2-free bacterial proteases; 3-trypsin. Maximum value of ferment's activity was assumed as 100%.

decrease of activity of the enzyme [41]. However, the radiation technology permits control of the activity and therefore the decrease in activity because of its predictability. The irradiation regimes chosen for this study decreased enzyme activity of the immobilized proteases up to 60–65% of the initial value, during the first weeks after irradiation, with a relative stabilization for longer periods of use. Knowing this, it is possible to prepare the irradiation reaction mixture with deliberately increased protease activity, relative to the native proteases, then the decrease of activity can be taken into account. The results are a final product with the desired activity.

The second cause of loss of activity is related to the duration of the storage. After storage of the immobilized proteases for 1.5 years at 4–8°C, the activity may decrease by 15–18% (Fig. 41.9).

41.4.6 Influence of Irradiation of Molecular Mass and Destruction of Components of Reaction Mixture for Preparation of Immobilized Enzymes

The molecular mass and concentration of PEO determine, to large extent, the structural behavior in water solutions. The low molecular PEO, chosen as a carrier for this study, is a group of nonionic surface-active structures that form micelles in the millimolar concentration range. At concentrations higher than the critical value for the formation of micellular structures, the activated form is spherical and with other thermodynamically stable structures [42].

The method of quasi-elastic laser scattering permits analysis of the formation

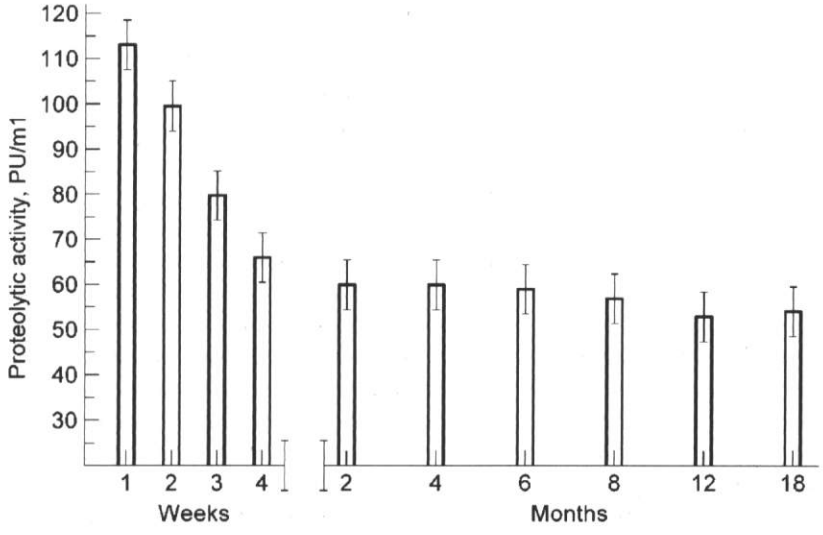

Figure 41.9 Change of the specific activity of immobilized proteases during 1.5 years at the storage temperature 4–8°C.

of the micelle-like structures from the components of the irradiated reaction mixture. The irradiated PEO solution without proteases contains micelle-like structures with dimensions of up to 270 nm. The irradiated mixture of proteases and PEO resulted in free-radical linking of components and the formation of micelle-like particles with dimensions of up to 360 nm. The data from light scattering techniques suggests complex structures for the particles. They can be thought of as relatively large micelle-like aggregates of PEO with built-in molecules of proteases.

The process of radiation linking of the polymers cannot be separated from the destruction of the substrates. The main side products formed in free-radical interaction of proteases and PEO are presumably peroxide compounds, carbonyl groups that were not utilized in the immobilization, and, long-lived free radicals. To determine the presence of free radicals and peroxide compounds in the irradiated products five different "batches" were assayed. The free radicals were determined by electron paramagnetic resonance (EPR) spectroscopy. Testing for the peroxide compounds was completed using the colorimetric method of Wagner, using iron rhodamine. This method permits the detection of the peroxide activity for the reaction with a resolution of 0.001 meq. The results showed that the product, the solutions of immobilized proteases, was free from free radicals and peroxide compounds capable for reaction. From these results one could conclude that the application of radiation technology for immobilizing proteases results in a final product of with minimal mutagenic and carcinogenic action.

41.5 APPLICATION OF IMMOBILIZED PROTEASES

41.5.1 Application of Immobilized Proteases for Cleaning of Equipment and Wastewaters in Production of Milk and Meat Products

These use of immobilized enzymes is a technology that can be used efficiently in designing and implementing closed-loop industrial water supply systems for the recovery of valuable components of industrial wastes. Enzymes could be used either as additives in the circulating solutions for washing equipment or in the immobilized form on filter elements for waste treatment. The effectiveness of the enzymes is achieved by choosing the proper immobilized enzyme with high specific activity for the given substrate to be hydrolyzed. Radiation technology provides the opportunity to form immobilized water-soluble forms of proteolytic enzymes for optimization in the waste stream and production processes.

One example of a cleaning process for solubilized enzymes is equipment used in milk production. This includes lines accessible only by means of washing using solutions. This would include nondismountable units of pipes, connecting units, and pumps. In these parts, conditions are favorable for the precipitation of proteins and calcium salts dissolved in milk and milk products. Later, crystallization of calcium salts on these surfaces and possible corrosion results in the reduction of the flow in milk pipes. Precipitation of proteins can

also increased the possibility of microbial contamination. The immobilized proteases contained in the washing solution hydrolyze the proteins precipitated on the surfaces of the equipment and serve to clean the surfaces.

Figure 41.10 shows the efficiency of casein hydrolysis by immobilized enzymes in scouring waters. The analysis of products of decomposition of milk protein showed an increase in the amino acid fraction, dipeptides, and polypeptides. Thus, the application of immobilized proteases resulted in a reduction in the amount of washing and scouring waters 1.4–2.2 times. It also increased the lifetime of the equipment and decreased the risk and value of milk contamination by microflora.

41.5.2 Application of Immobilized Enzymes as a Water-Conservation Technique in Primary Wool Treatment

Another application of immobilized proteases is in the treatment of wool. The need to reduce water consumption in processing of wool is particularly important in countries with limited water resources, while there is a need for increased wool production. The studies of raw wool have showed that the wool contaminants can be classified as easy to remove and hard to remove. Easy-to-remove contaminants are greases, both oxidized and nonoxidized, and, other organic and proteinaceous compounds. A large part (60–90%) of the contaminants on greasy wool are easily removed during scouring by conventional aqueous solu-

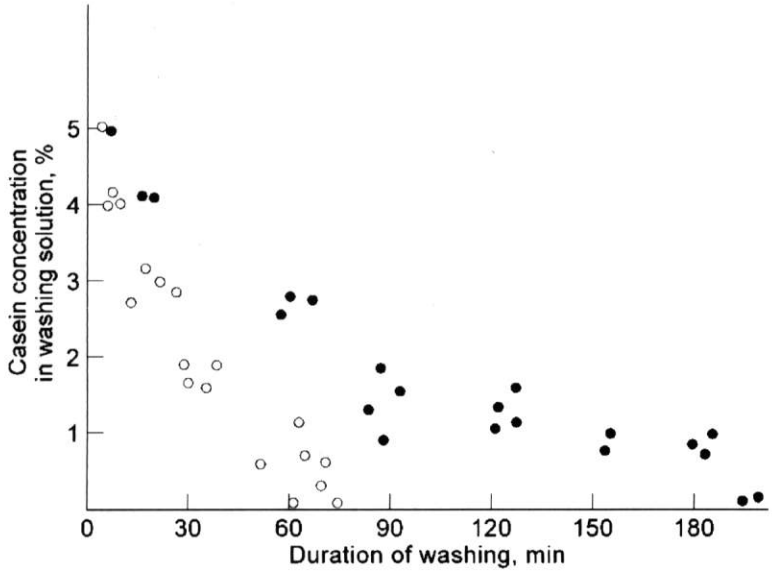

Figure 41.10 Efficiency of plant milk pipe system cleaning from residual casein by various washing solutions: ○-with immobilized ferments (experimental), disk-without ferments (standard).

tions [43,44]. The remaining hard-to-remove contaminants are grease, dirt, and some proteins adhering to the fibre surface.

It was shown that by using immobilized proteases in the washing solution resulted in rapid decomposition of protein contamining complexes on the surface of wool fibers. The preparation of immobilized proteases "Woolsib" was designed for treatment of wool in the process with simultaneous removal of easy-to-remove and hard-to-remove components.

Components of washing solution "Woolsib" forms a two-phase liquid system with the immobilized proteases. A major advantage of the process is that the components are ecologically better and safer after use than the widely used sulfonates, sodium phosphate, ferro- and polyphosphates and sodium silicate. "Woolsib" can also be recycled (Fig. 41.11).

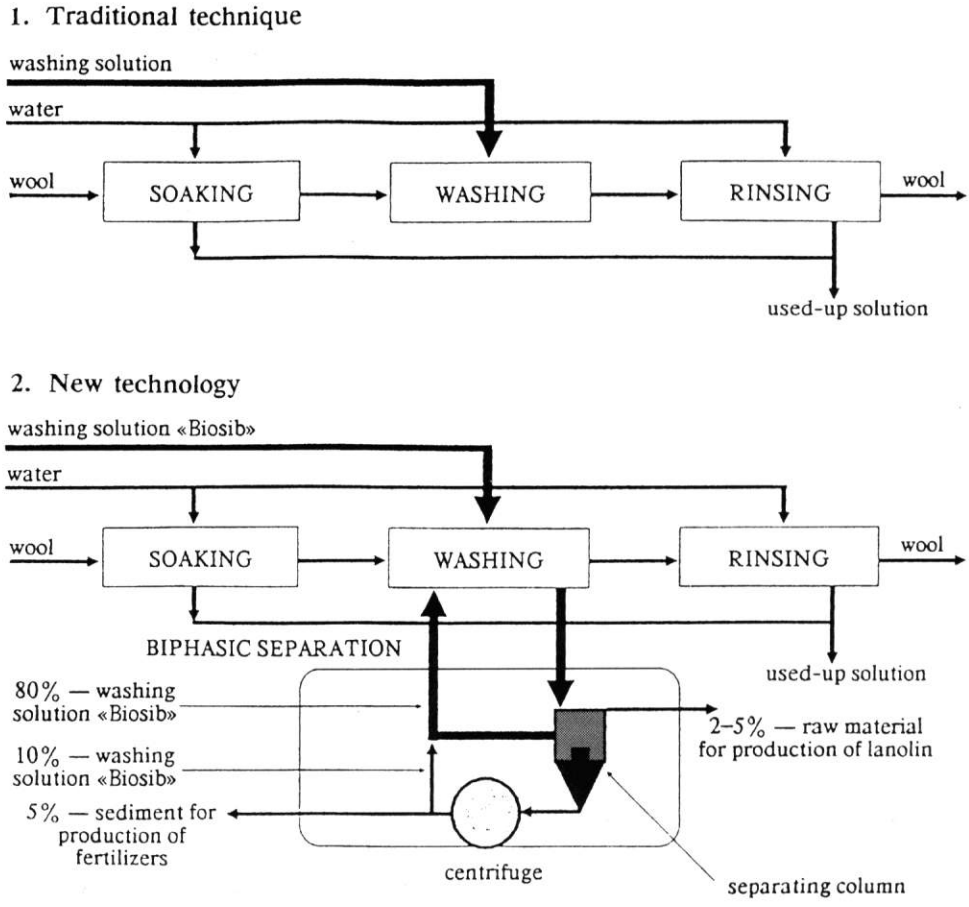

Figure 41.11 Technology of primary wool processing.

The used wash solution is pumped into a separating column, where it is separated rapidly into three layers. The top layer contains large quantity of lanolin that can be extracted for use in cosmetic industry. The middle layer (about 85% of total volume) is the pure washing solution ready for reuse. The bottom layer contains particulate matter removed from the raw wool. The recycling rate of "Woolsib" washing solution depends on the level of wool impurity.

Comparative parameters of wool before and after washing with "Woolsib" (preparation of immobilized enzymes) and traditional reagents are given below:

Main Parameters	Initial Material	"Woolsib"	Traditional
Relative tensile strength	8.7–9.3	9.6–9.9	8.0–8.4
Solubility in urea-bisulfate bisulfate	5.2–5.6%	4.5–5.1%	6.2–6.6%
Residual weight of lipid content	2.4–4.3%	0.3–0.5%	0.9–1.5%

In conclusion, the advantages of "Woolsib" process are

1. Increased production rate without any quality loss due to reduction of total duration of technological process.
2. A considerable reduction in the amount of calcium hydroxide and sodium sulfide in the wastewater, reducing treatment costs associated with their treatment.
3. An additional profit due to the reduction of expenses for sewage treatment.

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