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Curdlan as a support matrix for immobilization of enzyme

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Abstract

Curdlan, a high molecular weight extracellular β(1 → 3) glucan produced by pure culture fermentation by *Agrobacterium radiobacter NCIM 2443* contains large number of free hydroxyl groups. The reaction of hydroxyl containing supports with epichlorohydrin results in activated epoxy groups that can covalently link with available amino, hydroxyl, or sulfhydryl groups of enzymes, thereby immobilizing it. The present work reports on preparation of epoxy-activated matrix for immobilization of a model enzyme, porcine pancreatic lipase. The binding capacity of the matrix prepared by extraction of epoxy-activated curdlan by isopropyl alcohol was found to be 58.7% with about 0.6% loss of the enzyme activity during immobilization. Further, the specific activity of the enzyme increased marginally from 9.37 to 10.2. The corresponding value was 10.15 for a commercial sample of curdlan, epoxy-activated as for laboratory-isolated curdlan. Sepharose, the most widely used support matrix for the immobilization of enzymes was used for comparison in this study.

Keywords: Curdlan; Sepharose; Epoxy activation; Porcine pancreatic lipase; Support matrix; Immobilization

1. Introduction

Curdlan, a high molecular weight polymer of glucose, β-(1 → 3)-glucan produced by pure culture fermentation from a nonpathogenic and nontoxicogenic strain of *Agrobacterium biobar*1 (identified as *Alacligenes faecalis* var. *myxogenes*) or *Agrobacterium radiobacter* (Nishinari & Zhanf, 2000) has recently been approved for food use by US FDA (Pszczola, 1997). Its high molecular weight and large number of free hydroxyl groups, which are available for activation and hence for binding with the enzyme suggests it to be suitable for immobilization of enzymes.

The use of immobilized enzymes for production of sugars, amino acids, and drugs predates for more than two decades (Schuler & Kargi, 2002). The primary base supports available for protein immobilization include agarose, cellulose, dextrans, polymers such as polyvinyl chloride, acrylates, nylons and polystyrene and silica in the form of silica gel and glass beads. Hybrids of these base materials such as agarose–acryl amide and polymer-coated silica have also been used for immobilization of enzymes such as penicillin acylase for production of 6-APA from mutant strain of *Penicillium chrysogenum* (Kragl, Greiner, & Wandrey, 1999). The primary considerations for these materials as applied to biological molecules are nonspecific binding, high surface interaction areas, storage and usage stability (both mechanical and chemical), high porosity, and surface chemistry. In general, most supports will bind from 2 to 50 mg protein/g support, while some supports claim to bind as high as 170 mg protein/g (Hannibal-Friedrich, Chun, & Sernetz, 1980). Such high binding capacities may result in steric interference problems, loss of protein activity, and changes in net surface charge of the support. Even at low protein binding capacities, the amount of protein bound may not correlate with the amount of activity recovered after immobilization. The amount of the protein bound to a support will also depend largely on surface density of available linking groups and also on the conditions of coupling reaction used such as time, temperature, and pH. While the optimal coupling conditions have been determined for most commercial supports for common proteins, these conditions cannot be extrapolated to other proteins and optimization studies may be necessary for each support matrix (Taylor, 1991).

The reaction of hydroxyl containing supports with epichlorohydrin or bisoxiranes, such as 1,4-butanediol–diglycidyl ether, results in supports with active epoxy

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groups able to covalently link ligands with available amino, hydroxyl, or sulfhydryl groups. The resulting alkylated amines, ethers, or thioethers, are stable and significant ligand leakage does not occur with time or use. Coupling to epoxy-activated supports is carried out at pH 5–12 for 4–72 h, at 4–60 °C. Buffers containing up to 50% of organic solvent such as dioxane or dimethylformamide can be used in the coupling reaction. Such coupling in organic solvents is necessary for many ligands and may be necessary for the immobilization of proteolipids and hydrophobic peptides. The epoxy activation of the carrier can also be the method of choice because it offers a very high flexibility in the reaction conditions.

Although glucans such as cellulose, modified starches and gellan (Abdulhareem, Bryjak, Markiewiez, & Croch, 2002; Sankpal and Kulkarni, 2002) have been used as matrices for immobilization no detailed information is available with respect to curdlan. The present work aims to prepare curdlan matrix by epoxy activation and evaluate it with respect to immobilization of model enzyme, porcine pancreatic lipase (PPL). Sepharose and epoxy-activated sepharose has also been evaluated similarly for comparative purpose.

2. Materials and methods

2.1. Materials

PPL was obtained from M/S Advanced Biochemical's Ltd, Thane. Sepharose was purchased from M/S Sisco Labortaories Ltd, Mumbai. Curdlan was purchased from M/S Sigma Aldrich Ltd, Israel. Epichlorohydrin and tributyrin were purchased from M/S Himedia Chemicals Ltd, Mumbai. All other chemicals were of AR grade and obtained from M/S S.D. Fine Chemicals, Mumbai.

2.2. Methods

2.2.1. Fermentative production of curdlan

Curdlan production was carried out in two stages. In the first stage, cells were grown in the seed culture; and in the second, seed culture was inoculated into the fermentation medium for curdlan production. *A. radiobacter NCIM 2443* was maintained on slants of defined medium (0.5 g beef extract, 0.1 g of yeast extract, 0.5 g of peptone, 0.5 g of sucrose, 30 mg $MgSO_4{\cdot}7H_2O$ and 2% agar in 100 ml distilled water) at 4 °C and sub-cultured every 2 weeks. The cells grown at room temperature (28 °C) for 48 h were used for inoculation into seed culture medium (20 g/l sucrose, 5 g/l yeast extract, 5 g/l peptone. pH 7.0) (Lee and Lee, 2001). The slant culture was then transferred to 500 ml conical flask containing 100 ml of sterile seed culture medium. The flasks were incubated at room temperature for 24 h on a rotary shaker at 230 rpm. Ten millilitre of the seed culture was then inoculated into 100 ml of the fermentation medium (150 g/l sucrose; 2.4 g/l NH_4Cl; 1.0 g/l KH_2PO_4; 0.5 g/l $MgSO_4{\cdot}7H_2O$; 0.6 μg/ml UMP added after 50 h of fermentation; 10 ml of trace elements (5 g $FeSO_4{\cdot}7H_2O$ + 2 g $MnSO_4$ + 1 g $CoCl_2{\cdot}H_2O$ + 1 g $ZnCl_2$ in 1 l 0.1 M HCL)), as optimized previously in our laboratories (Saudagar and Singhal, unpublished work). The flasks were incubated at room temperature for 72 h on a rotary shaker at 230 rpm. The pH of the broth was regulated at 6.1 ± 0.3 during entire course of fermentation.

2.2.2. Isolation of curdlan from the fermentation broth

Curdlan was isolated from the broth by the procedure described by Harada and Misaki (1968). The recovery procedure is based on conformational transition, which occurs when the concentration of alkali exceeds 0.2N. After completion of fermentation, 100 ml of 1N NaOH was added to the fermentation broth in order to selectively dissolve the curdlan. The broth was then neutralized with 5N HCl to precipitate out the curdlan. The broth was then centrifuged at 8000*g* for 10 min at 4 °C to obtain a pellet containing curdlan and the cell debris. Ten millilitre of 0.1N NaOH was added to the pellet to selectively dissolve curdlan. The solution was vortexed for 10 min for maximal recovery of curdlan and then centrifuged at 8000*g* for 10 min at 4 °C. Curdlan so obtained was then washed three times with water and then dehydrated using 99% acetone, and then lyophilized to get a free flowing powder of crude curdlan.

2.2.3. Assay of lipase (Montenecourt, Carrol, & Lazilotta, 1984)

Lipase activity was estimated by its hydrolytic action on tributyrin as follows: 1 ml of tributyrin was mixed with 2.0 ml of 7.5% gum acacia solution (a 7.5% solution of gum acacia was prepared by suspending 7.5 g of gum acacia in 100 ml of phosphate buffer (50 mM), pH 7.0. The solution was centrifuged at 6000 rpm for 15 min and the supernatant was used) on a cyclomixer until an emulsion was formed. To this emulsion, 10 ml of phosphate buffer of pH 7.2 and 0.5 ml of enzyme sample were added. The mixture was kept in an incubator shaker at 40 °C. At 0 and 10 min, 0.5 ml of aliquots were removed and the liberated butyric acid was titrated against 0.01 M NaOH solution using phenolphthalein as an indicator, after the reaction was quenched using 1 ml methanol. From this, the micromoles of fatty acid liberated (as butyric acid) was determined as follows:

$$\text{Micromoles of butyric acid produced} = (b - a) \times 0.01 \times 1000 \times 13/0.05 \times 1/10$$

where a, b are burette reading at 0 and 10 min, respectively.

Lipolytic activity is expressed in terms of lipase units (U) per unit weight of enzyme sample. One unit of lipase activity was expressed as the amount of enzyme producing 1 μmol of butyric acid liberated by hydrolysis of tributyrin per minute.

Table 1
Immobilization of PPL on curdlan matrix prepared by various methods

Sample	Enzyme activity (micromoles of butyric acid formed/ml)			%Enzyme bound			Amount of protein bound mg/ml			Specific activity (lipase units/mg of protein)		
	A	B	C	A	B	C	A	B	C	A	B	C
Crude enzyme	78.0 ± 2.0			0			8.32 ± 0.5			9.37 ± 0.2		
After immobilization												
Supernatant	19.5 ± 0.2	20.5 ± 0.6	21.5 ± 0.6	25.0 ± 0.1	26.30 ± 0.6	27.56 ± 0.6	2.12 ± 0.2	2.01 ± 0.2	2.12 ± 0.1	9.20 ± 0.1	10.20 ± 0.1	10.16 ± 0.2
1 wash	12.0 ± 0.1	6.0 ± 0.2	9.0 ± 0.1	15.40 ± 0.3	7.70 ± 0.1	11.53 ± 0.1	0.81 ± 0.2	0.44 ± 0.1	0.51 ± 0.1	14.18 ± 0.3	13.61 ± 0.3	17.67 ± 0.2
2 wash	3.0 ± 0.3	2.0 ± 0.1	1.80 ± 0.1	3.80 ± 0.4	2.60 ± 0.1	2.30 ± 0.2	0.60 ± 0.3	0.27 ± 0.1	0.46 ± 0.2	5.00 ± 0.4	7.41 ± 0.2	3.91 ± 0.3
3 wash	0	0	0	0	0	0	0.12 ± 0.1	0.02 ± 0.1	0.20 ± 0.1	0	0	0
Curdlan matrix	35.0 ± 0.2	45.0 ± 0.3	45.0 ± 0.5	44.87 ± 0.1	57.70 ± 0.3	57.70 ± 0.5	4.16 ± 0.1	4.51 ± 0.3	4.11 ± 0.2	8.40 ± 0.2	9.98 ± 0.1	10.15 ± 0.1

Results are mean ± SD of three determinations. A, Curdlan matrix prepared by using method 1; B, Curdlan matrix prepared by using method 2; C, Curdlan matrix prepared by using method 3.

2.2.4. Preparation of curdlan matrix for the immobilization of PPL

Three methods were being used for preparation of epoxy-activated curdlan.

2.2.4.1. Method 1. In 5 ml of 0.5N NaOH, 100 mg of curdlan was dissolved. One millilitre of epichlorohydrin was added and then stirred on a magnetic stirrer for 8 h at room temperature. The epoxy-activated curdlan was then recovered from the solution by pouring it in 10 ml of 5 M acetic acid solution. Being insoluble in acetic acid, it separated out, and was then recovered by centrifugation at 4000 rpm for 10 min at 8 °C. The precipitate (average particle size: 0.164) was washed with water and used as such.

2.2.4.2. Method 2. In this case, initially the curdlan beads were formed by dissolving 100 mg of curdlan in 3 ml of 0.5N NaOH and then adding this solution drop wise through an insulin syringe into a bath containing cold alcohol. The beads formed (0.606 mm) were then air dried and incubated with epichlorohydrin at 100 rpm and 25 °C for 12 h after which the beads were recovered by centrifugation at 4000 rpm for 10 min at 8 °C and held at room temperature until use.

2.2.4.3. Method 3. In 5 ml of 0.5N NaOH, 100 mg of curdlan was dissolved. One millilitre of epichlorohydrin was added and then stirred on a magnetic stirrer for 8 h at room temperature. The epoxy-activated curdlan was then recovered by pouring the emulsion in bath containing cold alcohol. The precipitated epoxy-curdlan (average particle size: 0.108) was then centrifuged at 4000 rpm for 10 min and at 8 °C. It was washed with water and used as such.

2.2.5. Preparation of sepharose matrix for the immobilization of PPL

One hundred milligrams of sepharose was directly incubated with 1 ml of epichlorohydrin and 5 ml NaOH for 8 h. Epoxy-activated sepharose was then recovered by centrifugation at 4000 rpm for 10 min at 8 °C. The epoxy-activated sepharose was then washed with water and used as such.

2.3. Immobilization of PPL on curdlan and sepharose matrix

One millilitre of crude PPL solution was added to the curdlan matrix prepared by each of the three methods and then stirred at 100 rpm at 30 °C for 8 h. The supernatant was separated; the matrix was washed for three times with phosphate buffer, pH 7.2. The supernatant and the three wash solutions were analyzed for lipase activity as described above. The matrix was also analyzed for the immobilization of lipase by adding 1 ml of tributyrin and determining the lipase activity. The supernatant and

the matrix were also checked for the protein immobilized by measuring the extinction at 280 nm. Similar procedure was adopted for sepharose matrix.

2.4. Determination of molecular weight of curdlan and the particle size of the beads

Molecular weight of curdlan produced at the end of the fermentation was determined by using the method developed by Futatsyama, Yui and Ogawa (1999). Molecular weight was determined by substituting the value of intrinsic viscosity $[\eta]$ in Sakurada–Houvink equation, $[\eta] = KM^a$, where $K = 1.6 \times 10^{-4}$ and $a = 0.74$. Particle size of the epoxy-activated curdlan formed by different methods was determined by image analysis (Packer & Thomas, 1990) using Biovis image analyzer.

3. Results and discussion

Table 1 shows the results for the immobilization of PPL on standard curdlan matrix prepared by using methods 1, 2 and 3. The total binding capacity of the curdlan matrix produced by method 1 was 44.9, i.e. 44.9% of the added enzyme was immobilized on the matrix, and there was 10.9% loss of the enzyme activity during immobilization. Further, about 4 mg/ml of the total protein (8.3 mg/ml) was bound to the curdlan matrix in 8 h. The specific activity of the lipase was found to decrease from 9.4 to 8.4 units/mg of protein. The possible reason for the decrease in the specific activity of the enzyme may be due to denaturation of the enzyme under the reaction conditions. Further, the enzyme used was not pure and contained large amount of other proteins, which may compete with the enzyme for binding to the substrate resulting in decreased specific activity.

The binding capacity of the beads prepared by method 2 was 57.7% with almost 6% loss of the enzyme activity during immobilization. The specific activity of the lipase enzyme increased from 9.4 to 10 units/mg of protein, which indicate efficient binding of the enzyme to the epoxy-activated curdlan beads. The matrix prepared using method 2 was found to be more efficient for immobilization of PPL than that prepared using method 1 as it showed increased enzyme binding as well as increased specific activity of the enzyme. The binding capacity of the beads prepared by this method was 57.7% with approximately 1% loss of the enzyme activity during immobilization. The specific activity of the lipase increased from 9.4 to 10.15 units/mg of protein. The possible reason for the increase in the specific activity of the enzyme may be due to an increase in

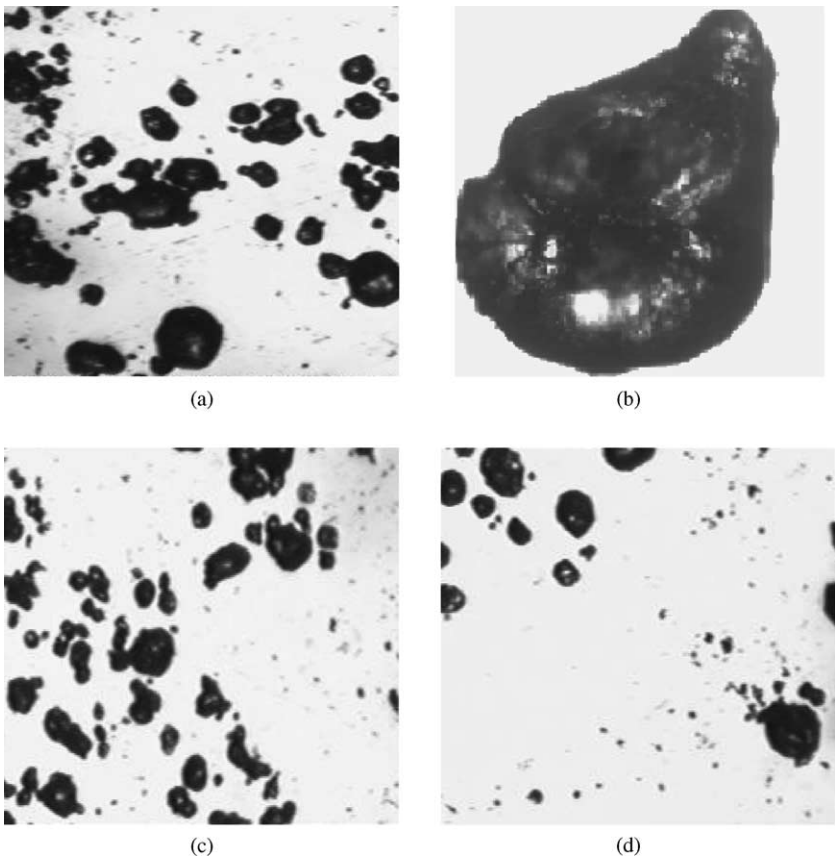

Fig. 1. Particle size analysis of (a) epoxy-activated standard curdlan prepared using method 1 (surface area 0.085 mm^2), (b) epoxy-activated standard curdlan produced by using method 2 (surface area 1.155 mm^2), and (c) and (d) epoxy-activated standard and laboratory prepared curdlan produced by using method 3 (surface areas of 0.0370 and 0.0376 mm^2, respectively).

Table 2
Immobilization of PPL on curdlan (prepared in laboratory) matrix prepared by using method 3

Sample	Enzyme activity (micromoles of butyric acid formed/ml)	%Enzyme bound	Amount of protein bound (mg/ml)	Specific activity (lipase units/mg of protein)
Crude enzyme	78.0 ± 2	0	8.32 ± 0.2	9.37 ± 0.4
After immobilization				
Supernatant	22.5 ± 0.1	28.85 ± 0.1	2.22 ± 0.3	10.15 ± 0.1
1 wash	8.0 ± 0.1	10.26 ± 0.1	0.52 ± 0.2	15.36 ± 0.2
2 wash	1.0 ± 0.2	1.30 ± 0.2	0.56 ± 0.1	1.80 ± 0.1
3 wash	0	0	0.20 ± 0.1	0
Curdlan matrix	46.0 ± 0.3	58.74 ± 0.3	4.51 ± 0.3	10.21 ± 0.2

Results are mean ± SD of three determinations.

the amount of enzyme bound to the matrix under the reaction conditions. This probably may be due to increase in the number of epoxy-activated hydroxyl groups on curdlan matrix, which are available for binding to the enzyme.

Method 3 was found to be the best for preparation of curdlan matrix for immobilization of PPL as it resulted in greater enzyme binding, increased specific activity and decreased loss of enzyme under reaction conditions. The greater enzyme binding in method 3 could be explained on the basis of higher surface area per 100 mg of the curdlan sample and the lower particle size of the epoxy-activated curdlan. Fig. 1(a)–(c) show the particle size analysis with respect to the available surface area of standard curdlan prepared by methods 1–3. The size of the particles prepared by methods 1 and 3 were 0.164 and 0.108 mm having a surface area of 0.085 and 0.037 mm^2, while that of the beads prepared by method 2 was 0.606 mm with a surface area of 1.155 mm^2.

Since method 3 gave the best results for immobilization of PPL on standard curdlan procured from M/S Sigma Aldrich, the same method was also used for the preparation of the curdlan matrix from the laboratory prepared curdlan. Table 2 documents the results of the same. It can be seen that the laboratory prepared curdlan was comparable to the standard curdlan obtained from M/S Sigma Aldrich for the immobilization of PPL. The binding capacity of the matrix from laboratory prepared curdlan was found to be 58.8%, i.e. 58.8% of the total amount of the added enzyme was immobilized whereas there was approximately 0.6% loss of the enzyme activity during immobilization. The specific activity of the enzyme increased marginally from 9.4 to 10.21 as compared to 10.15 obtained with the standard curdlan. The molecular weights of standard and laboratory prepared curdlan were estimated and found to be almost similar at 2.5×10^6 and 2.6×10^6, respectively.

Sepharose is amongst the most widely used support matrix for the immobilization of enzymes, and was used for comparison in this study. Sepharose is a bead-formed gel prepared from agarose cross-linked with either epichlorohydrin or cyanogens bromide. The agarose used to make sepharose is obtained by purification process, which removes the charged polysaccharides to give a gel with very small number of residual charged groups. Cross-linked sepharose, i.e. sepharose CL gel has substantially the same porosity as the parent gel, but with greatly increased thermal and chemical stability. After cross-linking, the gel is desulphated by alkaline hydrolysis under reducing conditions to yield a gel with an extremely low content of ionizable groups. Thus, it requires activation of the charged groups to make sepharose a suitable matrix for immobilization of enzymes. Table 3 documents the results obtained on immobilization of PPL on sepharose without activation, and that prepared by cross linking by method 3 for curdlan as above. It was seen that only 3% of the added enzyme

Table 3
Immobilization of PPL on sepharose matrix prepared using different methods

Sample	Enzyme activity (micromoles of butyric acid formed/ml)		%Enzyme bound		Amount of protein bound mg/ml		Specific activity (lipase units/mg of protein)	
	A	B	A	B	A	B	A	B
Crude enzyme	78.0 ± 2.0		0		8.32 ± 0.5		9.37 ± 0.2	
After immobilization								
Supernatant	58.0 ± 0.1	19.0 ± 0.2	74.35 ± 0.1	24.35 ± 0.1	5.87 ± 0.2	4.82 ± 0.2	10.0 ± 0.1	10.16 ± 0.1
1 wash	10.0 ± 0.2	8.0 ± 0.3	12.82 ± 0.3	10.25 ± 0.3	0.81 ± 0.4	1.61 ± 0.4	12.5 ± 0.1	9.85 ± 0.1
2 wash	5.0 ± 0.3	1.0 ± 0.3	6.41 ± 0.1	1.28 ± 0.3	0.56 ± 0.2	1.00 ± 0.2	10.0 ± 0.2	1.78 ± 0
3 wash	0	0	0	0	0.20 ± 0.3	0.50 ± 0.3	0	0
Sepharose matrix	3.0 ± 0.1	49.0 ± 0.1	3.84 ± 0.1	62.82 ± 0.1	0.80 ± 0.1	4.80 ± 0.1	3.75 ± 0.2	10.21 ± 0.2

Results are mean ± SD of three determinations. A, Immobilization of PPL on sepharose matrix prepared without activation; B, immobilization of PPL on epoxy-activated sepharose matrix.

was immobilized on the matrix without epoxy activation. This may be due to less number of free groups on sepharose available for binding to the enzyme. Therefore, it was essential to first activate the groups on sepharose to use it as a matrix for immobilization. The binding capacity of the activated sepharose beads is 62.8% with 1.3% loss of the enzyme during immobilization. In comparison, the binding capacity of epoxy-activated curdlan prepared by method 3 was 59% with 0.6% loss of the enzyme activity during immobilization. Further, the specific activity of 10.21 of the enzyme immobilized on sepharose was similar to that obtained with curdlan beads.

4. Conclusion

Curdlan can serve as an efficient matrix for immobilization of enzymes. Work on immobilization of other enzymes using this matrix is warranted.

References

Abdulhareem, J. H., Bryjak, J., Markiewiez, B. J., & Croch, W. P. (2002). Immobilization of wood rotting fungi laccases on modified cellulose on modified cellulose and acrylic carriers. *Process Biochemistry*, *37*(12), 1387–1394.

Futatsyama, H., Yui, T., & Ogawa, K. (1999). Viscometry of curdlan, a linear (1 → 3)-β-D-glucan in DMSO or alkaline solutions. *Bioscience, Biotechnology and Biochemistry*, *63*(8), 1481–1483.

Hannibal-Friedrich, H., Chun, M., & Sernetz, M. (1980). Immobilization of β-galactosidase, albumin, and γ-globulin on epoxy activated acrylic bids. *Biotechnology and Bioengineering*, *22*, 157–161.

Harada, T., & Misaki, A. (1968). Curdlan: A bacterial gel forming â(1,3)-glucan, *Archives of Biochemistry and Biophysics*, *124*, 292–298.

Kragl, U., Greiner, L., & Wandrey, C. (1999). Enzymes, immobilized, reactors. In M. C. Flickinger, & S. W. Drew (Eds.), (pp. 1064–1074). *Encyclopedia of bioprocess technology: Fermentation, biocatalysis and bioseparation*, (*Vol. 2*). New York: Wiley.

Lee, J. H., & Lee, I. Y. (2001). Optimization of uracil addition for curdlan â(1,3)-glucan production by *Agrobacterium species*, *Biotechnology letters*, *23*, 1131–1134.

Montenecourt, B. S., Carrol, J. O., & Lazilotta, R. P. (1984). Assay of industrial microbial enzymes. In M. Y. Young (Ed.), (pp. 346–347). *Comprehensive biotechnology*, (*Vol. 4*). Oxford: Pergamon press.

Nishinari, K., & Zhanf, H. (2000). Curdlan. In G. O. Phillips, & P. A. Williams (Eds.), *Handbook of food hydrocolloids* (pp. 268–286). England/Florida: Woodhead Publishing Limited/CRC Press.

Packer, H. L., & Thomas, C. R. (1990). Morphological measurements on filamentous microorganisms by fully automatic image analysis. *Biotechnology and Bioengineering*, *35*, 870–881.

Pszczola, D. (1997). Curdlan differs from other gelling agents. *Food Technology*, *51*(4), 30.

Sankpal, N. V., & Kulkarni, B. D. (2002). Optomization of fermentation condition for gluconic acid production using *Aspergillus niger* immobilized on cellulose microfibrils. *Process Biochemistry*, *37*(12), 1343–1350.

Schuler, M. L., & Kargi, F. (2002). *Enzymes in: Bioprocess engineering basic concepts* (2nd ed.). New Jersey: Prentice-Hall, pp. 57–104.

Taylor, R. (1991). *Covalent immobilization. In Protein immobilization fundamentals and applications*, New York: Marcel Dekker, pp. 56–67.