ELSEVIER

PII: S0141-3910(97)00185-7

Polymer Degradation and Stability **59** (1998) 93–99

Printed in Northern Ireland
0141-3910/98/$19.00

Improved production of bacterial cellulose and its application potential

E. J. Vandamme,[a]* S. De Baets,[a] A. Vanbaelen,[b] K. Joris[a] & P. De Wulf[a†]

[a]*Laboratory of Industrial Microbiology and Biocatalysis, University Gent, Gent, Belgium*
[b]*Scientific and Technical Centre of the Belgian Textile Industry (Centexbel), Gent, Belgium*

(Accepted 2 July 1997)

Bacterial cellulose, produced by *Acetobacter* species, displays unique properties, including high mechanical strength, high water absorption capacity, high crystallinity, and an ultra-fine and highly pure fibre network structure. It is expected to be a new commodity biochemical with diverse applications, if its mass production process could be improved, especially via submerged fermentation technology. It has already found application as a food matrix (nata de coco) and as dietary fibre, as a temporary dressing to heal skin burns, as an acoustic or filter membrane, as ultra-strength paper and as a reticulated fine fibre network with coating, binding, thickening and suspending characteristics. A wet spinning process for producing textile fibres from bacterial cellulose has also been developed, and applications as a superconducting and optical fibre matrix are under study. We have been able to improve bacterial cellulose production in surface culture (up to 28 g/l), as well as in submerged culture (up to 9 g/l) via strain selection, mutation, medium composition optimization and physico-chemical fermentation parameter control. Glucose and fructose as the carbon source and acetic acid as the energy source, combined with a precise control of pH and dissolved oxygen levels, results in highly improved cellulose yields. An internal pH control in stationary surface cultures was achieved by an appropriate choice of the ratio of fructose/glucose/acetic acid. It was also demonstrated that cellulose formation could be enhanced by adding insoluble microparticles such as diatomaceous earth, silica, small glass beads and loam particles to submerged, agitated/aerated *Acetobacter* cultures. This microcarrier-enhanced cellulose synthesis could be the result of the formation of microenvironments with locally lowered dissolved oxygen levels because of the attachment of *Acetobacter* cells as a biofilm on the particles. As such, less glucose is lost as gluconate, saving it for cellulose formation and maintaining the pH profile within the desirable range. We have also developed a UV-mutation and proton enrichment strategy, which allows the selection of *A. xylinum* mutants, which are highly restricted in (keto)gluconate synthesis and produce cellulose more efficiently, even under oxidative culture conditions. Combining these nutritional, genetic and bioprocess-technological improvements, very high levels of bacterial cellulose have been attained. Further improvements are needed to arrive at an economical fermentation process for mass production of bacterial cellulose.

1 BACTERIAL CELLULOSE

Cellulose (β-1,4-glucan) is one of the most abundant polymers in nature. It is found as a structural component, often bound to other polymers (pectin, lignin, arabinan, etc.) in the cell wall of plants, algae and also of some lower animals (*Tunicata*) and of some bacterial genera (e.g. *Rhizobium*, *Agrobacterium*, *Alcaligenes*). Interestingly, only a few bacterial species, taxonomically related to the genus *Acetobacter xylinum* (acetic acid bacteria), extracellularly secrete the synthesized cellulose as fibres.

*To whom correspondence should be addressed at: Laboratory of Industrial Microbiology and Biocatalysis, University Gent, Coupure links 653, B-9000 Gent, Belgium; Fax: +32-9-264 62 31; e-mail: erick.vandamme@rug.ac.be
†Present address: Department of Microbiology and Molecular Genetics, Harvard Medical School, Boston, USA.

Plant-derived cellulose is being applied intensively by the paper and textile industries, leading to a significant demand on wood biomass; hence the production of cellulose by *Acetobacter* spp. could be an interesting alternative for the plant-derived material, especially since bacterial cellulose is produced in a pure (free of other polymers) and crystalline form, which makes its recovery relatively simple.

Due to its purity and unusual physico-chemical characteristics, a wide range of speciality applications of bacterial cellulose can be envisaged in the food and medical field, while it could also be used in bulk in the paper and textile sector. Unfortunately, the current price of bacterial cellulose remains too high to make it commercially attractive. Indeed, bacterial cellulose production is a fairly inefficient process so far. Improved fermentation processes, based on an increased insight into the biochemical and genetic background of cellulose biosynthesis, might lead to a breakthrough towards an economical process for bacterial cellulose production.

2 BIOCHEMISTRY OF BACTERIAL CELLULOSE SYNTHESIS

2.1 Carbon metabolism in *A. xylinum*

Two main amphibolic pathways are operative in *A. xylinum* bacteria (Fig. 1): the pentose phosphate cycle for the oxidation of carbohydrates and the Krebs cycle for the oxidation of organic acids and related compounds.[1] Phosphofructokinase is absent[1] or weakly present,[2] resulting in no or only a weak glycolytic activity, indicating that glucose cannot be metabolized anaerobically. However,

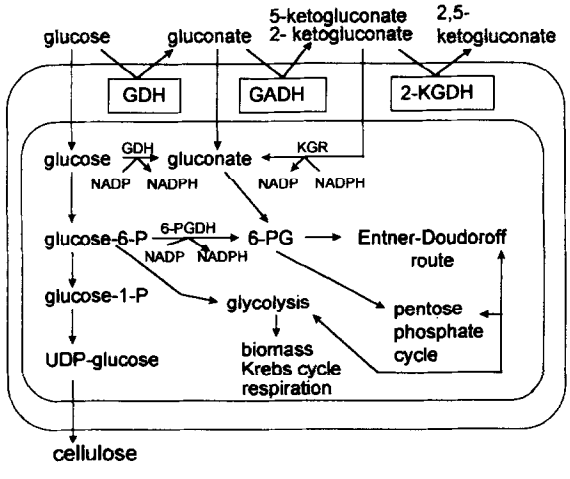

Fig. 1. Pathways of carbon metabolism in *A. xylinum* (De Wulf *et al.*, 1996[15]).

Tonouchi *et al.*[3] detected phosphofructokinase activity in *Acetobacter xylinum* subsp. *sucrofermentans* BPR2001. These researchers also investigated several specific enzyme activities involved in cellulose synthesis, and demonstrated the existence of a phosphotransferase system for fructose uptake in cellulose-producing *Acetobacter* cells. Gluconeogenesis can occur from oxaloacetate via pyruvate by means of the enzymes oxaloacetate decarboxylase and pyruvate phosphate dikinase.

2.2 Cellulose synthesis in *A. xylinum* (Fig. 2)

Uridine diphosphoglucose (UDPG), obtained from glucose-1-phosphate through the activity of UDP-glucose pyrophosphorylase, is used in the β-1,4-glucan polymerization reaction.[4] The activity of this enzyme is not affected by the presence of glucose or fructose in the *Acetobacter* culture medium, but a marked difference in activity is observed between cellulose producers and non-producers (approximately 100× more active in cellulose producers).[3] During this reaction cyclic diguanylic acid (c-di-GMP) acts as a key regulatory element. It functions as an allosteric activator of the membrane-bound cellulose synthase (UDP-glucose: 1,4-β-D-glucosyltransferase). In the absence of c-di-GMP, cellulose synthase is inactive. The activator binds directly to the enzyme in a reversible way at a regulatory site, different from the catalytic or the substrate-binding site.[1,5]

The synthesis of c-di-GMP is catalysed by diguanylate cyclase, from two molecules of GTP via the linear intermediate pppGpG.

The activation of cellulose synthase is stopped by the action of two enzymes, c-di-GMP phosphodiesterases A and B (PDE-A and PDE-B). PDE-A cleaves the c-di-GMP to form pGpG, which is then rapidly degraded to produce two molecules of 5′-GMP. The activity of PDE-A is selectively inhibited by Ca^{2+} ions.[1,5–7]

3 SURFACE VERSUS SUBMERGED FERMENTATION PROCESSES FOR BACTERIAL CELLULOSE

When certain acetic acid bacteria (e.g. *Acetobacter xylinum* strains) are grown in a suitable statically incubated surface culture medium, cellulose is produced and forms a thick, leather-like white pellicle at the air–liquid interface of the culture. During growth and cellulose production, the bacterial cells

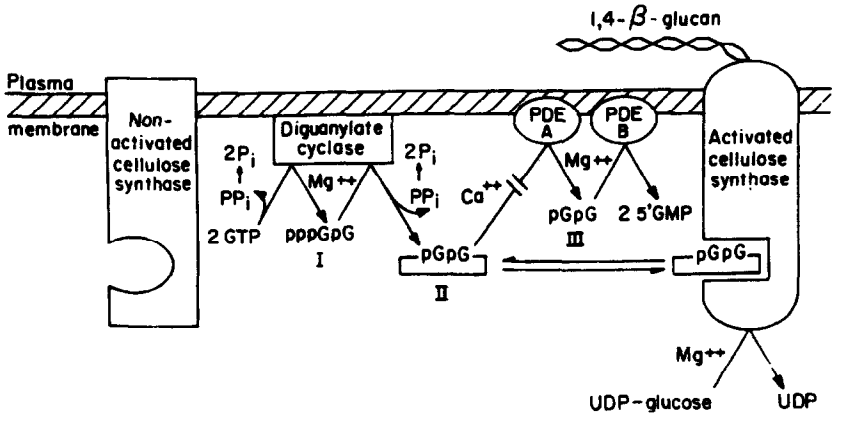

Fig. 2. Model for regulation of cellulose biosynthesis in *A. xylinum* (Ross *et al.*, 1987[7]).

gradually become entrapped in the pellicle. It is a generally accepted hypothesis that cellulose synthesis and secretion by the highly aerobic *Acetobacter* spp. help the cells to float and reach the oxygen-rich surface.[8] Masaoka *et al.*[9] confirmed experimentally that only cells occurring close to the air–liquid interface of the medium could produce cellulose. However, studies concerning the production of cellulose by *Acetobacter pasteurianus* on a silicone membrane revealed that cellulose production can also occur on an oxygen-permeable synthetic membrane placed at the bottom of the culture vessel.[10] When the membrane used was not oxygen-permeable or was too thick, the bacterial cellulose was not produced or the rate of production was decreased, respectively.

Other researchers optimized a two-stage fermentation process, with cell growth in a first stage in agitated culture and cellulose formation in a second stage in static surface cultures.[11] In agitated submerged cultures, which are more convenient from an industrial point of view, the oxygen transfer to the submerged cells is sufficient (so there is no need to float to the oxygen-rich surface), such that the bacterial cellulose productivity dramatically drops.

During the last decade, attention has been focused on the development of fermentation technology to overcome limitations in cellulose production under large-scale and submerged process conditions. Furthermore, specific fermentation media have now been developed which lead to increased cellulose levels, especially in combination with the use of cellulose overproducing mutant strains.

This paper deals with the development of an improved fermentation methodology for bacterial cellulose and with the screening for gluconate-deficient cellulose-overproducing *Acetobacter xylinum* mutants.[12–15]

4 IMPROVED PRODUCTION OF CELLULOSE WITH *Acetobacter* SP. LMG 1518

4.1 *In situ* pH control in static surface culture via an optimized fermentation medium design

Because the precursor in cellulose synthesis is uridine diphosphoglucose (Fig. 2), bacterial cellulose production is generally performed with sucrose or glucose as a carbon source for growth and polysaccharide formation. However, many other carbon substrates such as mannitol,[16] fructose,[17] sucrose,[18] arabitol,[19] glycerol,[9] etc., can also be applied for cellulose production.

The importance of the medium composition, and more particularly the type of carbon source, becomes clear when the carbohydrate metabolism of *A. xylinum* is considered (Fig. 1). D-Glucose as a carbon source is—apart from acting as an energy source and cellulose precursor—actively converted by membrane-bound *Acetobacter* dehydrogenases into (keto)gluconic acids. This feature not only lowers the overall cellulose yield, but also lowers the medium pH to suboptimal levels for cell viability and cellulose synthesis. Mainly due to this fact, applying a high initial glucose concentration with the aim of obtaining higher cellulose production levels does not increase the cellulose production proportionally, because the excess glucose is converted into (keto)gluconic acids with a concomitant lowering of the pH.[9,20] In this respect, the use of a pH-controlled fermentation process is inevitable, but this is indeed hard to achieve in static surface cultures. We have developed a successful alternative, consisting of an *in situ* control of the medium pH by using acetic acid as a co-substrate for the strain *Acetobacter* sp. LMG 1518. The rationale for this is that *Acetobacter* spp. can oxidize

acetic acid into CO_2 and water, thereby generating extra ATP and favouring a desirable pH range. Although acetic acid itself does not directly lead to cellulose formation, acetic acid-derived ATP may save part of the D-glucose, normally used to synthesize ATP, leading to a more efficient glucose incorporation into cellulose. Interestingly, the catabolism of acetic acid simultaneously leads to a pH increase, which may counteract the pH decrease due to (keto)gluconic acid formation (pKa acetic acid = 4.75). After a step-wise medium optimization procedure, the combined use of fructose (70 g/l), glucose (35 g/l) and acetic acid (7.5 g/l) in a static surface culture gave rise to 28.4 g/l of cellulose (productivity of 6.7 g/l cellulose/ day).[12] The initial medium pH 5.5, which we found optimal for cellulose synthesis, could be kept constant throughout the fermentation period, illustrating the beneficial 'buffering' capacity of acetic acid, concomitant with a decreased formation of (keto)gluconic acids. This or similarly designed media allow an improved production of cellulose in (static) surface culture. The cellulose pellicle formed by our strain LMG 1518 was easily recovered, freed of bacterial cells and dried. The cellulose fibre material was characterized by a degree of polymerization of 793 ± 9, corresponding to a molecular weight of approximately $142\,730 \pm 1660$ Da. Figure 3 shows the unique network structure of ribbon-shaped fibrils excreted by the *Acetobacter xylinum* LMG 1518 cells in surface culture.

Through response surface methodology, Embuscado *et al.*[18] predicted a yield of 13.24 g/l (experimental results: 12.67 g/l) starting from 24.8 g/l fructose, 76.5 g/l sucrose, a pH of 4.49 and an incubation temperature of 29.3°C with *Acetobacter xylinum* in non-agitated culture. Matsuoka *et al.*[21] observed stimulation of the cellulose production by addition of lactate to the medium. They explained this positive effect as a result of a promotion of the TCA cycle and generation of energy by the oxidation reaction from lactate to pyruvate.

4.2 Improved cellulose yield in agitated submerged fermentations using microparticles

Bacterial cellulose production via submerged fermentation poses specific problems. As pointed out above, an increased dissolved oxygen level in agitated cultures diminishes the cellulose yield as compared to static surface culture. Moreover, the spontaneous selection of cellulose-negative variants also contributes to a reduced cellulose yield. Two major approaches were tested to restore the cellulose yield to levels comparable to those obtained in static fermentations. One is to control the dissolved oxygen concentration in the fermentation medium at a preset, low level (± 30% saturation), and the other is to select mutant strains which are insensitive to high aeration or agitation rates. By maintaining the dissolved oxygen concentration at

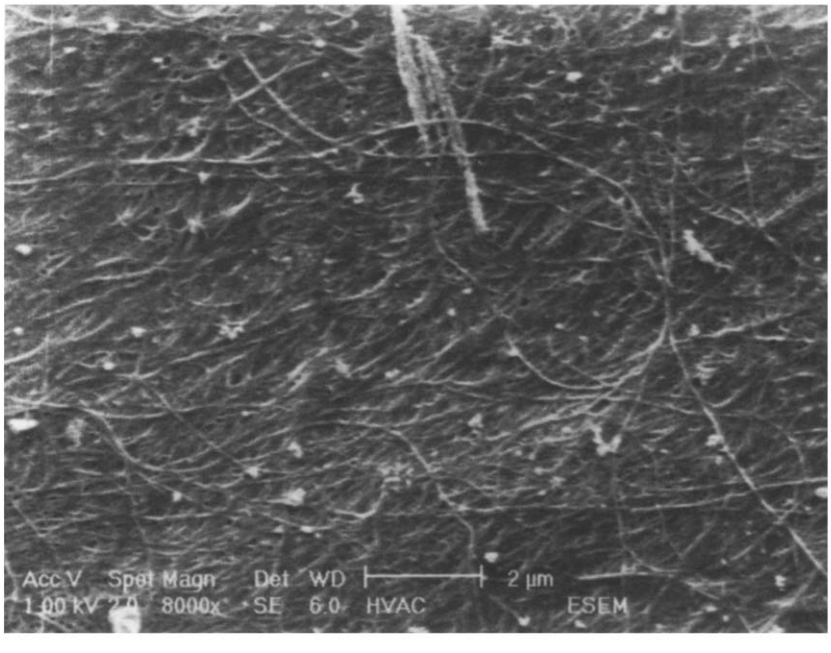

Fig. 3. Network structure of ribbon-shaped fibrils, excreted by *Acetobacter xylinum* LMG 1518 (8000×).

about 30% of the saturation level, a very low biomass and cellulose yield was obtained, *Acetobacter* being highly aerobic.[14]

Toyosaki *et al.*[17] developed an efficient screening, which allowed them to isolate 2096 bacterial cellulose-producing strains. By using shake flasks with slanted baffles (upper ends in the same direction as that of rotation) combined with a medium containing corn steep liquor and fructose, they isolated a superior strain which was capable of producing 4.4 g/l cellulose in agitated culture. In a jar fermentor, this strain reached production levels of 7.7 g/l. The strain was later identified as *Acetobacter xylinum* subsp. *sucrofermentans*.[22]

An alternative technical approach we developed arose from the observation that cellulose synthesis occurred more rapidly when the *Acetobacter* cells were attached to the static parts inside the fermentor (electrodes, baffles, stirrer). In analogy, we introduced 'multiple adhesion sites' in the culture vessels by supplying the medium with water-insoluble microparticles, ranging from diatomaceous earth, silica gel, sea sand, small glass beads and loam up to pulverized plant cellulose (CFF 90).[13] The concentration of the added microparticles was optimized, as well as the agitation rate, both at shake flask level.

The cellulose yield in the particle-provided media was triple (from 1.1 to 3.6 g/l) the level normally obtained in the corresponding reference cultures (no addition). This microcarrier-enhanced cellulose synthesis is believed to be the result of the development of an oxygen-limiting biofilm around these particles.[14] These observations led us to reconsider the still generally accepted hypothesis of Schramm and Hestrin,[8] which states that cellulose production by *Acetobacter xylinum* is essential for the cells to reach the oxygen-rich air–liquid interface. Indeed, upon agitation/aeration in the presence of microparticles, the high dissolved oxygen concentration in the bulk medium did not lead to a decrease in cellulose formation. Consequently, the role of oxygen may be more correctly linked to an increased synthesis of (keto) gluconic acids, rather than to affect cellulose synthesis directly. The positive effects of adding microparticles on cellulose production were apparently caused by the artificial creation of local oxygen-deprived niches around the particle surfaces, resulting in a favoured shift of glucose incorporation into cellulose, rather than oxidation into (keto)gluconic acids.

4.3 Improved cellulose formation by *Acetobacter* LMG 1518 mutants, deficient in (keto)gluconate synthesis

In order to limit the conversion of D-glucose into the oxidative metabolites D-gluconic acids, 2-ketogluconic acid, 5-ketogluconic acid and 2,5-ketogluconic acid (Fig. 1), and to enhance the conversion of glucose into cellulose, the isolation of (keto)gluconate-negative *Acetobacter* mutant strains was attempted.[15] To derive (keto)gluconate non-producing mutants from *Acetobacter xylinum* LMG 1518, UV-mutagenesis was applied, and the mutagenized culture was subsequently enriched, based on the proton suicidal method.[23] The rationale consists of plating the culture onto a medium that contains NaBr and $NaBrO_3$ in a 5:1 ratio. The formation of (keto)gluconic acids from glucose gives rise to toxic bromine ($5Br^- + 1BrO_3^- + 6H^+ \rightarrow 3Br_2 + 3H_2O$), which kills the acid-producing strains. Mutants that are defective in (keto)gluconate synthesis survive this plating step, and become enriched on subsequent solid medium. Several mutant strains were successfully isolated, of which one, *A. xylinum* KJ 33, produced an amount of cellulose, double that of the wild-type (from 1.8 to 3.3 g/l).[12] Such mutant strains may be very helpful in developing an efficient industrial cellulose fermentation process, as well as for studying bacterial cellulose and (keto)gluconate synthesis by *Acetobacter* spp.

Breeding mutants for improved cellulose synthesis has also been described by Ishikawa *et al.*[24] They noticed that cellulose production by *Acetobacter xylinum* subsp. *sucrofermentans* BPR2001 was linked with the growth of this strain. Since *p*-aminobenzoic acid (PABA) stimulated biomass formation of this strain, they isolated mutants resistant to sulfaguanidine (SG), a PABA analogue, which showed increased cell growth and a concomitant higher productivity of cellulose (9.7 g/l, 40% higher than the wild-type strain).

5 PERSPECTIVES

Bacterial cellulose offers a wide range of applications due to its high purity and special physicochemical characteristics. Because of its high water absorption capacity,[25] wet cellulose can be used as a temporary artificial skin to treat severe skin burns.[26] Moreover, cellulose somehow seems to enhance the growth of human skin cells. The use of

bacterial cellulose as a temporary skin has already been patented and commercialized as BioFill®.

Because dried cellulose pellicles have a uniform fibre distribution and a high tensile strength, they can serve as acoustic membranes (e.g. Fraunhofer Institute, Stuttgart, Germany; Ajinomoto Co., Tokyo, Japan) or conductive membranes (Ajinomoto Co., Japan).

The large-scale production of bacterial cellulose by Weyerhaeuser Co. (Tacoma, Washington, USA) and Cetus Co. (Emeryville, California, USA) has led to the development of Cellulon®, a bulking agent with a broad spectrum of applications. Examples include its use in the mining sector, in binding and coating applications, to stabilize membranes and as a food ingredient to act as a thickener, texturizer and/or calorie reducer.[27] The application of bacterial cellulose for the immobilization of microorganisms has been described by Fiedler *et al.*[28] Biopolymer Research Co., Ltd, also in Japan, was established for the purpose of furthering the industrial production technology of biopolymers, and is now mainly focusing on bacterial 'Bio-Cellulose'. Properties of other useful polymers of microbial origin have been reviewed by the authors.[29]

Recent improvements of the cellulose productivity relate to the addition of water-soluble chitosan to the Hestrin–Schramm medium[30] and the addition of small amounts of endoglucanase to the production culture.[31] Recently, it has been described that a cellulose-producing *Acetobacter xylinum* KU-1 produces an endo-β-glucanase in the culture broth.[32] Bacterial cellulose is expected to be a new commodity biochemical with many interesting applications, if its mass production can be further improved. The introduction of an efficient submerged fermentation methodology will be crucial in this respect. Further research into the genetic and metabolic basis of bacterial cellulose synthesis, as well as the construction of highly efficient *Acetobacter* (mutant or recombinant) strains, shall be nccessary to arrive at an economic production process for bacterial cellulose.

REFERENCES

1. Ross, P., Mayer, R. and Benziman, M., *Microbiol. Rev.*, 1991, **55**, 35.
2. De Ley, J., Gillis, M. and Swings, J., in *Bergey's Manual of Systematic Bacteriology, I, Family VI, Acetobacteraceae*, ed. R. R. Krieg and J. G. Holt, Williams and Wilkins, London, 1984, p. 267.
3. Tonouchi, N., Tsuchida, T., Yoshinaga, F., Beppu, T. and Horinouchi, S., *Biosci. Biotech. Biochem.*, 1996, **60**, 1377.
4. Ross, P., Mayer, R., Amikam, D., Huggirat, Y., Weinhouse, H., Benziman, M., de Vroom, E., Fiddler, A., de Paus, P., Sliedregt, L. A. J. M., van der Marel, G. A. and van Boom, J. H., *J. Biol. Chem.*, 1990, **265**, 18933.
5. Volman, G., Ohana, P. and Benziman, M., *Carbohydr. Europe*, 1995, **May**, 20.
6. Benziman, M., Ross, P., Amikam, D., Mayer, R., Weinhouse, H., Ohana, P. and Michaeli, D., in *Cellulose and Wood-chemistry and Technology*, ed. C. Schuerch, Wiley and Sons Inc., New York, 1989, p. 519.
7. Ross, P., Weinhouse, H., Aloni, Y., Michaeli, D., Ohana, P., Mayer, R., Braun, S., de Vroom, E., van der Marel, G. A., van Boom, J. H. and Benziman, M., *Nature (London)*, 1987, **325**, 279.
8. Schramm, M. and Hestrin, S., *J. Gen. Microbiol.*, 1954, **11**, 123.
9. Masaoka, S., Ohe, T. and Sakota, N., *J. Ferment. Bioengng*, 1993, **75**, 18.
10. Yoshino, T., Asakura, T. and Toda, K., *J. Ferment. Bioengng*, 1996, **81**, 32.
11. Okiyama, A., Shirae, H., Kano, H. and Yamanaka, S., *Food Hydrocoll.*, 1992, **6**, 471.
12. Joris, K., PhD thesis, University of Gent, Belgium, 1993.
13. Joris, K., Billiet, F., De Wulf, P. and Vandamme, E. J., in *Cellulosics: Materials for Selective Separations and Other Technologies*, ed. J. F. Kennedy, G. O. Phillips and P. A. Williams, Ellis Horwood, New York, 1993, p. 240.
14. Joris, K. and Vandamme, E. J., *Microbiol. Europe*, 1993, **May/June**, 27.
15. De Wulf, P., Joris, K. and Vandamme, E. J., *J. Chem. Tech. Biotechnol.*, 1996, **67**, 376.
16. Oikawa, T., Ohtori, T. and Ameyama, M., *Biosci. Biotech. Biochem.*, 1995, **59**, 331.
17. Toyosaki, H., Naritomi, T., Seto, A., Matsuoka, M., Tsuchida, T. and Yoshinaga, F., *Biosci. Biotech. Biochem.*, 1995, **59**, 1498.
18. Embuscado, M. E., Marks, J. S. and Bemiller, J. N., *Food Hydrocoll.*, 1994, **8**, 419.
19. Oikawa, T., Morino, T. and Ameyama, M., *Biosci. Biotech. Biochem.*, 1995, **59**, 1564.
20. Schmauder, H.-P., Geyer, U., Einfeldt, L., Klemm, D. and Marsch, S., *Med. Fac. Landbouww. Univ. Gent*, 1994, **59/4b**, 2411.
21. Matsuoka, M., Tsuchida, T., Matsushita, K., Adachi, O. and Yoshinaga, F., *Biosci. Biotech. Biochem.*, 1996, **60**, 575.
22. Toyosaki, H., Kojima, Y., Tsuchida, T., Hoshino, K.-I., Yamada, Y. and Yoshinaga, F., *J. Gen. Appl. Microbiol.*, 1995, **41**, 307.
23. Winkelman, J. W. and Clark, D. P., *J. Bacteriol.*, 1984, **160**, 687–690.
24. Ishikawa, A., Matsuoka, M., Tsuchida, T. and Yoshinaga, F., *Biosci. Biotech. Biochem.*, 1995, **59**, 2259.
25. Okiyama, A., Motoki, M. and Yamanaka, S., *Food Hydrocoll.*, 1993, **6**, 493.
26. Fontana, J. D., de Souza, A. M., Fontana, A., Torriani, I. L., Moreschi, J. C., Gallotti, B. J., de Souza, S. J., Narcisco, G. P., Bichara, J. A. and Farah, L. F. X., *Appl. Biochem. Biotechnol.*, 1990, **24/25**, 253.
27. Okiyama, A., Motoki, M. and Yamanaka, S., *Food Hydrocoll.*, 1992, **6**, 479.
28. Fiedler, S., Schnurra, I. and Sattler, K., *Zentralbl. Mikrobiol.*, 1990, **145**, 427.

29. Vandamme, E. J., Bruggeman, G., De Baets, S. and Vanhooren, P., *Agro-Food Industry Hi-Tech*, 1996, **September/October**, 21–25.
30. Tajima, K., Fujiwara, M., Takai, M. and Hayashi, J., *Mokuzai-Gakkaishi*, 1996, **42**, 279.
31. Tonouchi, N., Tahara, N., Tsuchida, T., Yoshinaga, F., Beppu, T. and Horinouchi, S., *Biosci. Biotech. Biochem.*, 1995, **59**, 805.
32. Oikawa, T., Kamatani, T., Kaimura, T., Ameyama, M. and Soda, K., *Curr. Microbiol.*, 1997, **34**, 309.