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The use of microorganism cells immobilised in carrageenan for the synthesis of organic substances

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ABSTRACT. The present state of the problem of the use of immobilised cells of microorganisms for the synthesis of organic substances is analysed. The physicochemical properties of carrageenan and the characteristic features of the immobilisation of microorganism cells in carrageenan are examined and a systematic account is given of the results of treating carrageenan and the immobilised preparations with stabilising reagents.

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I. Introduction

It is generally known that all biochemical reactions in nature and approximately 80% of industrial processes are effected by virtue of catalysis. Biocatalysts (especially the cells of microorganisms), which possess a high catalytic activity, specificity, and the ability to effect processes at the usual temperatures, have become the objects of close attention in recent years. The advances in the chemistry of steroids, where biocatalysts are widely used, clearly demonstrated their advantages over chemical reactions. However, the use of biocatalysts for the specific transformation of organic substances has been significantly restricted by the specific features of experiments involving them, by the fact that particular classes of compounds were studied, by their lability, by the difficulty of separating them from the reaction products, and in many cases by their low economic effectiveness because they are used only once.

The employment of biocatalysts developed extensively following the discovery of the possibility of immobilising them, as a result of which during the last 10–15 years they have been deliberately prepared in the form immobilised on a carrier or in a gel.[1–15] Special attention has been devoted to the creation of highly effective biocatalysts based on immobilised cells of microorganisms.

About 100 laboratory procedures for the use of immobilised microorganism cells in fine organic synthesis for the preparation of organic acids, solvents, methane, hydrogen, ethanol, enzymes, antibiotics, aminoacids, pantothenic acid, glutathione, and steroids and also for the purification of waste water are now known. Several of these processes have found practical application on an industrial scale: in the preparation of aspartic and malic acids, of lysine from caprolactam, and of leucine and ethanol.[16] A factory manufacturing phenylalanine with an annual output of 600 tonnes has been working in the USA since 1984, while in Japan not only aminoacids but also ethanol and acrylonitrile are produced with the aid of immobilised microorganism cells.

This can be explained by a whole series of advantages of the use of immobilised cells compared with the use of immobilised enzymes and ordinary cells.[17–19]

1. The lack of expenditure on the isolation and purification of enzymes.
2. The higher stability and activity compared with immobilised enzymes and free cells, since immobilisation protects the cells against external influences.
3. The reduction of expenditure on the isolation and purification of reaction products, since they are isolated from the biomass and the metabolic products of the microorganism.
4. The possibility of creating continuous automated manufacturing processes in which inhibition by both the substrate and by the reaction products is diminished.
5. The ability of polyenzyme systems to function for a long time and the regeneration of cofactors.
6. The possibility of reducing the volumes of the reactions effected by immobilised cells compared with free cells.
7. The improvement of the possibility of monitoring the reaction.
8. The likely significant diminution of the contamination of the environment.
9. A significant decrease in the cost of the reaction products.

However, despite the increasing number of publications on the immobilisation of microorganism cells, the results of this process and of the use of immobilised microorganism cells are unpredictable and are determined primarily by the ability of the cell to retain on immobilisation and in the immobilised state a specific activity and to operate stably in a specific process. They depend on a very large variety of factors. Virtually all the existing immobilisation methods have definite advantages and disadvantages. The commonest method involves incorporation in a gel. It ensures repeated use without the separation of the cells and repeated sowing of the medium, a high concentration of cells in the carrier, and the employment of highly productive reactors, but it is also distinguished by an increased resistance to mass transfer, because the substrates and products have to diffuse into the gel and out of the gel. The

physicochemical parameters of the microenvironment (for example, the pH) in the carrier particles can differ significantly from the corresponding characteristics of the medium outside the particles. Microorganisms can elute from the particles in the course of the experiments. This method is also restricted by the need to use substrates with a low molecular weight. The growth of the microorganism cells and the formation of CO_2 frequently disrupt the gel.

For incorporation in a gel, the most suitable physicochemical properties are those of polyacrylamide gel,[4,20,21] polyurethane,[3] collagen,[6] agar,[7,8] gelatine,[9,10] cellulose,[22] alginates,[12,23] and carrageenan.[13–15]

The characteristic features of polyacrylamide gel are the difficulty of obtaining regular spherical particles and the low activity of the resulting preparations, usually owing to the toxic effect on the cell by the gel components: acrylamide, *NN'*-methylenebisacrylamide, and $K_2S_2O_8$. However, the effective mechanical properties and the possibility of regulating the gel network together with data on the multiplication of the cells in the gel have aroused an increased interest by investigators in the use of this gel.[1,16,24–26]

The advantage of agar consists in the rapid growth of new cells in the gel, but its disadvantage is its brittleness. The strength of the gel rapidly falls as cells grow and CO_2 is formed.[27]

The problem with calcium alginate is its instability in the presence of phosphate buffers and certain cations: Mg^{2+}, K^+.[28,29] The gel breaks down fully in 0.1 M K_2HPO_4/KH_2PO_4 solution after 15 min,[29] while phosphate is one of the principal nutrients of microbial cells.

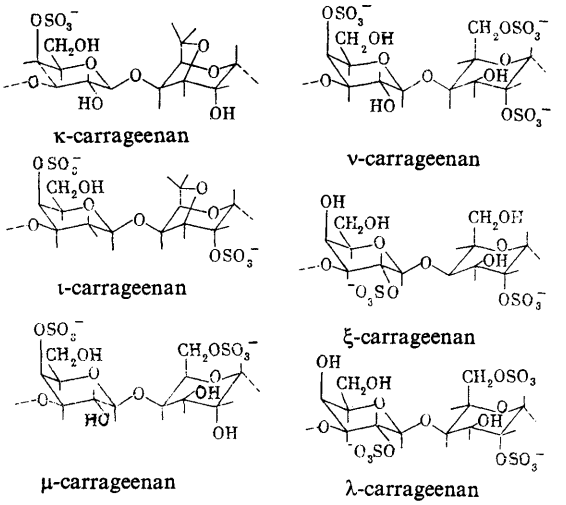
So far there is no universal carrier for the immobilisation of microorganism cells nor is there a universal immobilisation method. However, analysis of data on immobilisation in carrageenan showed that its practical use is promising, since the Japanese research laboratory of the Tanabe Seiyaku Company reported the successful incorporation of more than 50 microorganisms in this gel.

In the present review, an attempt is made to survey and describe systematically the results of experimental studies which make it possible to justify the employment of carrageenan as an immobilisation matrix, as well as data on the use of microorganisms immobilised in carrageenan for the synthesis of organic substances.

II. Physicochemical properties of carrageenan and the characteristic features of the immobilisation of microorganism cells in carrageenan

Carrageenans are sulphate derivatives of polysaccharides obtained from the red seaweed *Phodophyceae* and *Ginartinaceae*.[30] The annual manufacture of carrageenans is on a scale not smaller than that of agar. The principal consumer of these polysaccharides is the food industry, where they are used as gel-forming agents for the preparation of confectionery, milk products, dough, and meat preserves.[17,30,31]

In contrast to agar, carrageenans include, apart from D-galactose, also 3,6-anhydro-α-D-galactose and a much larger amount (20–30%) of sulphate groups[32–34] (MW = 100 000–800 000). The limiting structures of carrageenans can be represented by the following formulae:



The real polysaccharides of the carrageenan group can be very close to one of the formulae represented in the scheme above, but they are usually molecular hybrids—combinations of two and more limiting structures. Only biopolymers characterised by the limiting "kappa" and "iota" structures are able to form gels. Their characteristic feature is the formation of thermoreversible gels under certain conditions. This distinguishes the polysaccharides of the carrageenan group from neutral gel-forming polysaccharides of the agarose group.

According to modern ideas, on extraction of carrageenans from seaweed, their ordered structure breaks down with formation in aqueous solutions of a disordered conformation, which is subsequently restored on formation of a gel and drying until solid. Intermolecular packed structures close to or identical to those *in vivo* are then formed.[35]

The specific nature of the ordered structure depends on the geometry of the covalent bonds between each residue of the primary structure.[36] Carrageenans are made up of six-membered (pyranose) rings having a geometry of the chair type[37] and connected by equatorial bonds. However, the bonds between the carbohydrate rings are not parallel, which leads to the formation of a "bend" and the ordered conformation is in this case helical.[35] X-Ray diffraction data and the temperature dependence of the optical rotation confirm the formation of a double helix for κ- and ι-carrageenans. Despite the existence of hypotheses which tend to explain the nature of intermolecular interactions inducing gel formation, at the present time this question has not been finally resolved. Studies designed to elucidate the causes of the increased stability of carrageenan gels have demonstrated the necessity for the optimum ratio of the length of blocks of the regular structure which are capable of association (and their optimum distribution along the polymer chain) to the length of sections with a masked regularity.[38] The gel formation inducing agents are metal ions—Ca^{2+}, K^+, and Rb^+ for ι-carrageenan and K^+ for κ-carrageenan (other ions exhibit a weaker effect). An X-ray diffraction study of the Ca^{2+} salt of ι-carrageenan showed that in this case the double helix is more extended than in agarose, as a result of which it has no central cavity in which bound water could be inserted. The factor stabilising the ordered structure is the hydrogen bond between the O(2) and O(6) residues of D-galactose in different chains. Since the only unsubstituted hydroxy-groups are present here, the double helix

of ι-carrageenan is structurally completely bound by hydrogen bonds, in contrast to κ-carrageenan. Bearing in mind the large distance between the chains, it is unlikely that the van der Waals forces play an important role in the formation of the helix. However, the ionic interaction is undoubtedly important, each Ca^{2+} ion being linked to two sulphate groups in different helices.[39]

On substitution of Ca^{2+} by univalent cations, a similar structure can be formed in κ-carrageenan, in which there is no second sulphate group. Direct spectroscopic proof of the interaction of K^+ with the sulphate groups of κ-carrageenan has been obtained from the IR spectra of the solutions.[35] Whereas under severe conditions the formation of the double helix in κ- and ι-carrageenans can be induced by ions which are bound non-specifically, under normal conditions cations of suitable size are necessary. The reason for such cationic specificity is evidently the conformational and configurational features of ι- and κ-carrageenans, although it has still not been fully explained.

The polysaccharides from the Black Sea phyllophora can also be classified as carrageenans, since their carbohydrate skeleton is made up of only β-D-galactose and 3,6-anhydro-α-D-galactose joined by alternating α(1) → (3) and β(1) → (4) bonds. The contents of suphate groups in the polysaccharides vary significantly as a function of the phyllophora species (from 17.8 to 27.2%). The different contents of 3,6-anhydro-α-D-glactose and different distributions of the sulphate groups along the carbohydrate skeleton are also characteristic of polysaccharides from different phyllophora species.[38,40] Carrageenans from different phyllophora species show significant differences in their structure and therefore also differ in their physicochemical properties.[41,42]

Table 1. Immobilisation of enzymes and microorganism cells in carrageenans.[48]

Enzymes and microogranisms	Enzyme activity[a]		Yield, %
	before immobilisation	after immobilisation	
Aminoacylase	20	10	50.0
Aspartase	650	300	46.2
Fumarase	360	260	61.5
E. coli (aspartase)	65000[b]	30400	46.8
B. ammoniagenes (fumarase)	9670[c]	5800[d]	60.0
S. phaeochromogenes (glucose isomerase)	7910	4280	54.1

[a] Enzyme: μmol h^{-1} (mg of protein)$^{-1}$; microbial cells: μmol h^{-1} (g of cells)$^{-1}$.
[b] After ultrasonic treatment.
[c] In the presence of bile extract.
[d] After treatment with bile extract.

Microorganism cells are immobilised in κ- and ι-carrageenans, κ-carrageenan being used most often.[13,14,17,18] In studies on the immobilisation of cells in the USSR, carrageenans are little used and Soviet carrageenans are hardly used at all.[43–47] This is incomprehensible, since this method is carried out under mild conditions, simple, and is susceptible to the development of an appropriate technology. The immobilisation of cells in carrageenans consists of virtually two stages: the preparation of a suspension of cells in the carrageenan solution and gel formation. If the stability of the gel is inadequate, the gel has to be stabilised.

It is noteworthy that microbial cells are mixed with the carrageenan solution at 40 °C, the cells of different microorganisms being stable in carrageenan at this temperature; the mixture formed is readily converted into a gel. The gel does not break down during the enzymic reaction, withstands variations in pH, and the size of the pores within the given gel is sufficiently small for enzymes and cells not to leave the gel matrix, while low-molecular-weight substrates and products readily pass through it. Both enzymes and whole cells with a high degree of retention of the initial activity (40–60%) can be immobilised by this method (Table 1).[27,48]

The possibility of immobilising various microorganisms in carrageenan has been demonstrated:[27] *Achromobacter aquatilis, A. liquidum, Aspergillus oryzae, A. niger, Bacillus megatherium, B. subtilis, B. succinium, Brevibacterium ammoniagenes, B. flavum, Corynebacterium glutamicum, Erwinia herbicola, Escherichia coli, Gluconobacter melanogenus, Lactobacillus bulgaris, Micrococcus ureae, Penicillium vinaceum, Proteus vulgaris, Pseudomonas aeruginosa, P. dacunhae, P. putidum, Sarcina lutea, Streptomyces griseus, S. phaeochoromogenes, Serratia marcescens.*

Table 2. Influence of the conditions in the formation of the κ-carrageenan gel on the strength of the latter.[48]

Gel formation conditions	Strength of gel
Cooling to 10 °C	+
Treatment with NH_4Cl	+++
Treatment with metal salts: LiCl, NaCl;	–
KCl, RbCl, CsCl;	++++
$MgCl_2$, $SrCl_2$, $MnCl_2$, $FeCl_2$, $BaCl_2$	
$CoCl_2$, $NiCl_2$, $CuCl_2$, $ZnCl_2$, $Pb(CH_3COO)_2$	++
Treatment with amines:	
Methylenediamine, ethylenediamine, p-phenylenediamine, δ-hydroxy-L-lysine, L-lysine hydroxamate, L-histidine hydroxamate *S*-2-aminoethyl-L-cysteine;	+++
hexamethylenediamine, octamethylenediamine, 4-guanidinobutylamine, histidine, DL-histidine hydrazide;	++++
L-ornithine, L-lysine, L-tryptophan hydroxamate	++
Treatment with organic solvents miscible with water: methanol, ethanol, acetone	++

Note. Gel strength: (+), load from 100 to 200 g cm^{-2}; (++), 200 to 500 g cm^{-2}; (+++), 500–1000 g cm^{-2}; (++++), 1000 to 1500 g cm^{-2}; (–), no gel formation.

In order to obtain more stable gels with incorporated *S. phaeochromogenes* cells, a study was made of the influence of the conditions for the formation of the carrageenan gel (Table 2) and in addition also of the influence of the anions of the potassium salts: CO_3^{2-}, SO_4^{2-}, $S_2O_8^{2-}$, PO_4^{3-}, $P_2O_7^{4-}$, $Al_2(SO_4)_4^{2-}$, $Fe(CN)_6^{3-}$, CH_3COO^-, and $^-OOC-COO^-$. Significant differences between the stabilities of

the gels were not found when different anions were used, whereas it follows from the data in Table 2 that more stable gels are formed on contact with KCl, RbCl, CsCl, hexamethylenediamine, octamethylenediamine, agmatine, histamine, and DL-histidine hydrazide. Unfortunately these data do not make it possible to understand the reason for such stabilisation of the gel, nor do they reflect the influence of the gel formation conditions on the retention of the initial activity of the microorganism cells. However, there is no doubt that the stability of the carrageenan gel can be regulated, which encouraged investigators in the search for a more technological solution for specific processes with participation of microorganism cells immobilised in carrageenan.

Thus treatment with stabilising reagents (hexamethylenediamine and glutaraldehyde) made it possible to increase the operational stability (half life) of *E. coli* cells (aspartase activity) in carrageenan from 50 days for untreated cells to 686 days,[48] in the case of *S. phaeochromogenes* (glucose isomerase activity) the increase was from 150 to 260 days, while treatment with gelatine and glutaraldehyde resulted in an increase to 532 days.[49]

Treatment of κ-carrageenan with amines, proposed by Takata et al.,[50] increased the operational stability of immobilised *Brevibacterium flavum* cells without reducing the initial activity.

Interesting results were obtained following the addition of polycationic polymers: the fumarase activity of *B. flavum* cells immobilised in κ-carrageenan in the presence of 0.15% of polyethyleneimine was distinguished by an increased stability to changes in external factors and temperature.[51]

Bearing in mind that the melting points of κ-carrageenan gels with immobilised *B. flavum* cells are higher by 20% in the presence of polyethyleneimine than without it, it has been suggested that there is an interaction between κ-carrageenan, polyethyleneimine and *B. flavum*.

An increase in the porosity of the carrageenan matrix was noted after the introduction into carrageenan of tripotassium phosphate,[52] which improved the diffusion of nutrient substances into the gel and the mechanical properties of the latter.

Treatment of carrageenan with epichlorohydrin and with alkylenepolyamide when *Aspergillus niger* and *Saccharomyces cerevisiae* are incorporated also leads to positive results.[53]

A new method for the stabilisation of microorganism cells immobilised in carrageenan, consisting in the treatment of the gels with polyacrylamide, is noteworthy. For this purpose, acrylamide, *NN'*-methylenebisacrylamide, and β-dimethylaminopropionitrile are allowed to diffuse into the carrageenan grains with subsequent polarisation.[54]

It has also been suggested that the cells be subjected to an activating treatment with NaCl solutions before being incorporated in carrageenan.[55]

Reliable physicochemical evidence which would account for the results of studies on the stabilisation of the carrageenan gel and for the increased operational stability of the immobilised cells as a result of the chemical modification of the gel has not so far been obtained. As a result of the large number of criteria which must be taken into account and the unpredictable behaviour of a specific enzyme activity on immobilisation, it is at present difficult to give preference to any stabilising procedure. The attempts to resort to the ζ-potential of the cells in order to select the immobilisation conditions have also not been convincing.[56] However, it is clear that the gel of the carrageenan matrix plays an important role in the stabilisation of the incorporated microorganism cells, while dissolved carrageenan has no stabilising effect.[31]

Numerous studies have demonstrated that cells can grow in a carrageenan gel at the same rate as the free cells, while in many instances their growth is better: in the stationary growth phase, the number of live yeast cells in the gel was ten times greater compared with the native cells.[57] Similar results have been obtained for *S. carlsbergensis*[58] and *E. coli*.[14] The growth is concentrated in the surface layer of the gel, as a result of which the diffusional effects are slight, oxygen and the nutrient medium are satisfactorily consumed, and there is no growth in the centres of the grains.

Since one of the main purposes of immobilisation is to obtain the maximum possible concentration of the cells in the carrier, the possibility of cell growth in the carrageenan matrix is used to activate them and to accumulate the biomass.[59]

The results achieved in the preparation of biocatalysts from microbial cells immobilised in κ-carrageenan made it possible to use them for a whole series of processes.[13,17,48,60–66]

III. The use of microorganism cells immobilised in carrageenan for the synthesis of organic substances

Synthesis of L-aspartic acid. In 1969, the Tanabe Seiyaku Company (Japan) achieved the separation of the racemates of DL-aminoacids on an industrial scale using immobilised aminoacylase.[67] This was the first case of the use of immobilised enzymes. Later an attempt was made to obtain L-aspartic acid using immobilised aspartase.[68] However, the low productivity of this process led to the use of cells of *E. coli* incorporated in a polyacrylamide gel,[21] which made it possible to obtain pure L-aspartic acid in 95% yield. Taking into account the partial inactivation of the aspartase activity of the cells, the polyacrylamide matrix was replaced by κ-carrageenan.[21] In order to increase the operational stability, the immobilised cells were treated with gluaraldehyde and hexamethylenediamine. If the productivity of the cells immobilised in polyacrylamide gel is adopted as 100%, then that of the cells incorporated in κ-carrageenan was 1500%, which made it possible to initiate in 1978 the industrial manufacture of L-aspartic acid. It is of interest to note that, despite the fact that the strength of the Japanese κ-carrageenan is 650 g cm^{-2} while that of the Soviet preparation is 170 g cm^{-2}, after use for 400 h the activity of the gels immobilised in κ-carrageenan was 43 mmol g^{-1}, while that of the cells immobilised in the Soviet carrageenan was 54 mmol g^{-1}.[45]

Synthesis of L-alanine. Alanine is a valuable aminoacid required for use in medicine and the food industry. Cells of *B. dacunhae* immobilised in carrageenan were used for its continuous preparation. They were treated with glutaraldehyde,[59,63,66] and incubated at pH 4.75 and 30 °C for 1 h in order to suppress the alanine racemase and proteolytic activities. The half-inactivation time of such catalysts exceeded 280 days.

L-Alanine can be obtained more efficiently from ammonium fumarate by using in the same reactor *E. coli* cells immobilised in κ-carrageenan, which exhibit aspartase activity, and *P. dacunhae* cells with L-aspartate β-decarboxylase activity, also immobilised in κ-carrageenan.[62,64,69]

Synthesis of glutamic acid. Cells of *Corynebacterium glutamicum* ATCC 13058, immobilised in κ-carrageenan worked stably for 30 days in a flow type fermenter with a productivity of 1.08 g of glutamate per litre per hour.[61]

Synthesis of L-arginine. The reactor with *Serratia marcescens* cells immobilised in κ-carrageenan made it possible to obtain 10 mg ml^{-1} of L-arginine at pH 6.5 for a long time.[70] When pure oxygen was passed through the reactor, the immobilised cells grew five times more vigorously than the free cells.

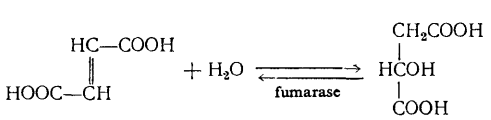
Synthesis of L-tryptophan. When *E. coli* cells with L-tryptophan synthetase activity were immobilised in polyacrylamide, alginate, and carrageenan gels, the highest activity (80% relative to the free cells) was observed for carrageenan.[71]

Synthesis of L-tyrosine. Comparison of the immobilised *Citrobacter freundii* cells with tyrosine phenol lyase activity immobilised in gelatine, agar, agar-agar, and κ-carrageenan gels indicated that the cells in κ-carrageenan were the most effective. The activity of 2–4% gels was 80% of the activity of the free cells.[72,73]

Synthesis of L-isoleucine. In order to manufacture L-isoleucine from D-threonine, a study was made of a system with growing *Serratia marcescens Ar130-1* cells immobilised in carrageenan. The reactor with the immobilised cells worked stably for 30 days with a productivity of 0.5 mg of L-isoleucine per gram of gel per hour.[14]

Synthesis of *N*-acetyl-L-methionine. *E. carotovora* cells immobilised in carrageenan cross-linked by glutaraldehyde and hexamethylenediamine catalysed the stereoselective hydrolysis of the amide of *N*-acetyl-*D*L-methionine with formation of *N*-acetyl-L-methionine and the retention of 61% of the initial activity. The optical purity was 100%.[74]

Synthesis of *L*-malic acid. In an attempt to improve the synthesis of L-malic acid, widely used in the pharmaceutical industry, from fumaric acid, the cells of *B. ammoniagenes* and *B. flavum* were immobilised in carrageenan.



If the productivity of *B. ammoniagenes* immobilised in polyacrylamide gel was adopted as 100%, then that of *B. flavum* incorporated in carrageenan was 897%.[48,75–77] It has been shown in a number of studies[51,78,79] that the thermal and operational stability of *B. flavum* cells increases sharply when they are immobilised in κ-carrageenan in the presence of polyethyleneimine and Chinese gallotannin: in the former case, the half-inactivation time for the biocatalyst increased by a factor greater than two,[79] while in the latter the increase was by a factor of 3.[78]

Manufacture of antibiotics. The cells of *Penicillium chrysogenum* immobilised in κ-carrageenan have been used to obtain penicillin *G*.[80] The cells of *Tolypocladium inflatum* synthesised cyclosporin *A*, which possesses immunodepressant properties and is used in organ transplantation.[81] The cells of *Penicillium urticae* have been used for the semicontinuous and continuous manufacture of the antibiotic patulin from glucose,[82–84] while the cells of *Xanthomonas citri*, immobilised in κ-carrageenan and treated with hexamethylenediamine and glutaraldehyde, were used to synthesise cephalexin.[85] The existence of two phases separated in time was established in the synthesis of penicillin *G* and cyclosporin *A*: cell growth (on incubation in a nutrient medium) and the formation of antibiotics.

The manufacture of ethanol. In view of the growth of the price of petroleum, there has been a growth of interest recently in the study of alcoholic fermentation by yeast immobilised in κ-carrageenan.[86–89] Problems concerning the increase in the number of cells in the gel and in the concentration of glucose and the stabilisation of gel grains with the immobilised cells have been investigated in detail.[57,58,90–92] Yeast cells immobilised in carrageenan grow more effectively than the free cells,[93] the productivity of immobilised growing cells of *Saccharomyces carlsbergensis* is three times higher than that of homogeneous cells,[58] and continuous formation of ethanol from molasses (more than 110 mg ml^{-1}) has been observed over a period of six months. The gel particles used for the manufacture of ethanol were strengthened with polyacrylamide,[54,94] by alkylenepolyamide,[53] and also by introducing tripotassium phosphate into carrageenan.[52] Metabolic inhibitors have also been introduced: dinitrophenol, azide, and arsenate.[95] Studies in which more productive microorganisms are sought are being continued.[8] When sugars were fermented by *Bacillus butacone* immobilised in carrageenan, a solution containing a butanol : acetone : ethanol = 8 : 1 : 1 mixture, which is promising for the manufacture of diesel fuel, was obtained.[96]

Synthesis of sugars. The continuous preparation of D-fructose from D-glucose has been achieved using *Streptomyces phaeochromogenes* cells immobilised in κ-carrageenan.[15] The glucose isomerase activity and the stability of the cells immobilised in carrageenan approached those for cells immobilised in polyacrylamide gel, their half-life in a column reactor amounting to 289 days.[31]

The cells of *Acetobacter* suboxydans, immobilised in a κ-carrageenan gel, produced L-sorbose in an amount of 0.2 mg per ml of the gel per hour in a stable process over a period of 15 days.[14]

The *Gluconobacter suboxydans* cells with a high alcohol dehydrogenase activity were immobilised by incorporation in a κ-carrageenan gel and were used for the biotransformation of D-arabitol into D-xylulose in 80% yield. They were activated by preliminary incubation in a nutrient medium containing D-glucose.[97]

Synthesis of acetic acid. The cells of *Acetobacter aceti* immobilised in κ-carrageenan manufactured acetic acid in a stable process for a long time at a rate of 22.5 mg per ml of the gel per hour.[98,99] *Clostridium thermoaceticum* immobilised in κ-carrageenan has been used for the same purpose.[100]

Synthesis of citric acid. *Aspergillus niger* cells immobilised in 2% carrageenan in the presence of 1% NaCl and 1% ethylenediaminetetraacetic acid, with subsequent dispersion in a mineral oil at 40 °C and treatment with polyethyleneimine or alkylenepolyamide, were used. The yield of citric acid was 5.2–5.3 g per 20 ml of the reaction mixture in 31 days.[53,101]

Synthesis of steroids and alkaloids. Steroids were one of the first substrates transformed with the aid of immobilised cells.[102] There are few instances of the employment of carrageenan for the

immobilisation of microorganisms transforming steroids and alkaloids:[103,104] these include the incorporation of *Arthrobacter globiformis* cells, which possess 3-ketosteroid Δ'-dehydrogenase activity, and the cells of *Claviceps purpurea*, which effect the biosynthesis of pharmaceutically important alkaloids: xanthoclavine, agroclavine, elymoclavine, and ergometrine. Comparison of the results with data for immobilisation in polyacrylamide gel and calcium alginate enabled the authors to conclude that they are preferable to carrageenan in the synthesis of steroids.[1]

Synthesis of vitamin B_{12}. Comparative study of the synthesis of vitamin B_{12} by cells of *Propionibacterium shermanii, P. freundenreichii subsp. shermanii ATCC 13673, Propionibacterium sp. ar I AKU 1251,* and *N_3 AKU 1253,* and *Propionibacterium sp. IAM 1714* immobilised in κ-carrageenan, calcium alginate, agar, and urethane photopolymers indicated that the cells immobilised in the urethane photopolymer have an advantage.[105]

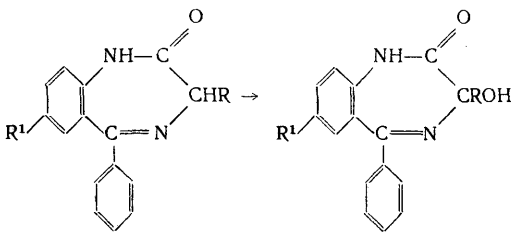
Synthesis of enzymes. The advantages of the manufacture of enzymes using immobilised cells consist in the possibility of maintaining a high concentration of cells and their rapid separation. However, the rate of diffusion of nutrients to the polymer matrix, especially of oxygen in aerobic cultures, significantly limits the growth and metabolic activity of the cells in the gel. When *Bacillus amyloliquefaciens* cells were incorporated in carrageenan, the respiratory activity and the rate of growth were suppressed to 1/2 and 1/6 respectively, but the formation of *L*-amylase was virtually similar to that by free cells.[106]

Synthesis of dihydroxyacetone. Dihydroxyacetone, which is used in the cosmetic and food industry, was synthesised from glycerol by *Gluconobacter melangenes* cells immoblised in κ-carrageenan.[107]

Synthesis of butane-2,3-diol. The cells of *Enterobacter aerogenes* immobilised in κ-carrageenan manufactured butane-2,3-diol stably over a period of ten days. On activation by incubation in a nutrient medium, the cells multiplied by a factor of 200.[108]

Reduction of nitro-compounds. The nitroreductase activity of *E. coli* cells immobilised in phyllophorin from the broad-jointed form of phyllophora and in carrageenan from spherical phyllophora is higher than that of cells immobilised in κ-carrageenan. The maximum retention of nitroreductase activity is then observed for the cells: carrageenan gel = 1:10 weight ratio. The study of the granulation conditions showed that the optimum granulation is in toluene at 0–4 °C. The gel grains with immobilised cells having a high nitroreductase activity are strengthened in 0.3–0.4 M KCl solutions. The study of the modification of the immobilised preparations by amines (ammonia, hexamethylenediamine, hydrazine hydrate, diethylamine, and triethylamine), glutaraldehyde, and titanium tetrachloride showed that the nitroreductase activity of the preparations treated with titanium tetrachloride is at the level of the unmodified preparations, while on treatment with diethylamine and triethylamine, it increases to 122 and 145% respectively. The reduction of nitro-derivatives of benzo-2,1,3-thiadiazoles and 1,4-benzodiazepin-2-ones as well as substituted nitrobenzenes with formation of the corresponding amines under column conditions has been achieved with the aid of the biocatalysts obtained.[109,110]

Synthesis of 3-hydroxy-1,2-dihydro-3*H*-1,4-benzodiazepin-2-ones. It has been shown[111] that the transformation of 1,2-dihydro-3*H*-1,4-benzodiazepin-2-ones by actinomycetes immobilised in carrageenan results in the formation, in 65% yield relative to the activity of the free cells, of optically active (+)3-hydroxy-derivatives having a more pronounced activity in their antagonism to corazole and in potentiating hexenal sleep compared with the racemate.



The achievement of this process makes it possible to obtain the corresponding 3-hydroxy-derivatives in one stage instead of the six stages required in the chemical method of synthesis.

Purification of waste water. The cells of *Clostridium pasteurianum* and *Alcaligenes eutropis*, catalysing the reaction HT + $H_2O \rightleftarrows H_2$ + HTO, were immobilised in calcium alginate and κ-carrageenan in order to remove tritium from water contaminated by radioactive impurities (for example, from the cooling systems of atomic power stations).[112] In terms of its catalytic activity, 10 g of cells immobilised in carrageenan is equivalent to 1 g of a platinum catalyst, but, in contrast to the latter, the former catalysts are cheap, can be readily obtained in a large amount, and are active in the liquid phase.

The reduction of the acidity of materials for the production of wine. It has been shown that the cells of *Leuconostoc oenos* immobilised in carrageenan effect the 100% transformation of L-malic acid into L-lactic acid in the treatment designed to reduce the acidity of materials for wine making. Thus, summarising the data presented above, one may conclude that microorganism cells immobilised in carrageenan are likely to be useful in the synthesis of organic compounds.

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In conclusion, the authors express the hope that this review will serve as a starting point for further study of the possibilities of using immobilised microorganism cells in organic synthesis, which will help create new biocatalysts for practical employment. Here attention should be concentrated on the study of Soviet produced carrageenan and its applicability in the immobilisation of cells. Evidently, more detailed investigation of the immobilisation and of the mechanism of gel formation will permit a deeper understanding of the nature of the intermolecular interactions in polysaccharide complexes and of the causes of the increased stability of microorganism cells immobilised in carrageenan and will help to develop more effective methods of synthesis of organic substances for the needs of industry, agriculture, and medicine.

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