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Fiber Fractions from Processing of Barley in Production and Conservation of a Biologic Control Agent

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Abstract

Carriers are frequently used to overcome problems associated with microbial survival in soil after inoculation. Moreover, the use of carriers can prolong the shelf lives and lessen dusting of both biofungicides and biologic fertilizers. This study investigated the suitability of barley-based fiber fractions as growth media and immobilization matrices in the cultivation of a *Streptomyces griseoviridis* biologic control agent, as well as for the conservation of obtained biomass in dehydrated hydrogel capsules. The second main ingredient in all the examined carrier matrices was alginate. The aim was to find a hydrogel formulation suited for a production process in which all individual steps, including cultivation of the organism; downstream processing; and formulation, storage, and application of the product (i.e., biologic control agent), are carried out in the hydrogel matrix. Of the tested fractions, brewer's spent grain was the best choice, when considering the price vs the nutrient contents as well as the storage time and ease of processing of the crude and the finished products. It seems that cereal fibers can be replenished with cereal fractions less rich in fiber but having a higher content of utilizable nutrients and, hence, better suited for the production of biomass. A high content of water-insoluble fiber favorably influenced the appearance as well as the applicability of the products.

Index Entries: Biofungicide; *Streptomyces* sp.; hydrocolloids; alginate capsules.

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Introduction

Immobilization and subsequent cultivation of microorganisms into hydrated hydrogel matrices are well-established techniques in the production of biologic control agents. Immobilization to hydrogel carriers can prolong the storage time of the product *(1–3)* and reduce the possibility of contamination of inoculum during storage, transport, and application *(4)*. The possibility of off-site drift (dusting and rinsing) during application may be reduced *(5)*, and the hydrogel may provide protection from biotic and abiotic environmental stresses leading to increased microbial survival in the active site *(5,6)*. In addition, immobilization to hydrogel carriers frequently increases the metabolic activity of encapsulated cells *(7)* and facilitates a slow, even, release of the active agent with reduced cell movement through soil from water flow–induced transport *(8,9)*.

In immobilization applications, sodium alginate is perhaps the most frequently utilized carrier, because it forms durable capsules under very mild circumstances through ionic crosslinking of the carboxyl groups on neighboring polyglucuronic acid segments by divalent cations *(10)*, thus allowing the cells to retain their viability. However, pure alginate capsules, although well suited for many applications, are too compact to allow sufficient diffusion of dissolved oxygen and outgrowth of encapsulated organisms *(9,11,12)*. Furthermore, disruption of the gel occurs by solubilization of the bound divalent cation (usually Ca^{2+}) when a medium containing calcium-chelating agents such as phosphates is used *(12)*. Improved stability of the alginate gel against phosphate is necessary, because phosphate is needed for maintaining growth of most microorganisms.

Various combinations of alginate and modifying fiber additives have been formulated, yielding improved stability toward phosphate *(12)*, improved diffusion properties *(12)*, prolonged storage times *(8)*, and protection during drying and rewetting cycles *(6,13)*. Previously, biologic control agents have been immobilized to pure alginate *(1,8)*, alginate–skim milk *(8)*, and alginate-clay *(9)*. The addition of skim milk clearly improved the viability and release properties of alginate beads *(8)*. Further, stability toward phosphate as well as the diffusion properties of alginate beads have been improved by adding a solution of Celite R-634 and pectin *(12)*, as well as intestinal mucus *(14)*.

In the present study, fiber fractions obtained from the processing of barley (Fig. 1) were examined for their applicability as additives to alginate beads in the production and conservation of a *Streptomyces griseoviridis* biologic control agent. The strain used in this study exhibits both α-amylase and cellulase activity, and, therefore, its growth is supported by both cellulose and starch-based media *(15)*. Culture media optimization criteria for producing biomass of this species include sugar or starch, inorganic or organic nitrogen, and phosphate *(15)*, while the incorporation of cereal material into the alginate network requires a high content of insoluble fiber (IF) or soluble fiber (SF) and a low content of phosphate and citrate, as well

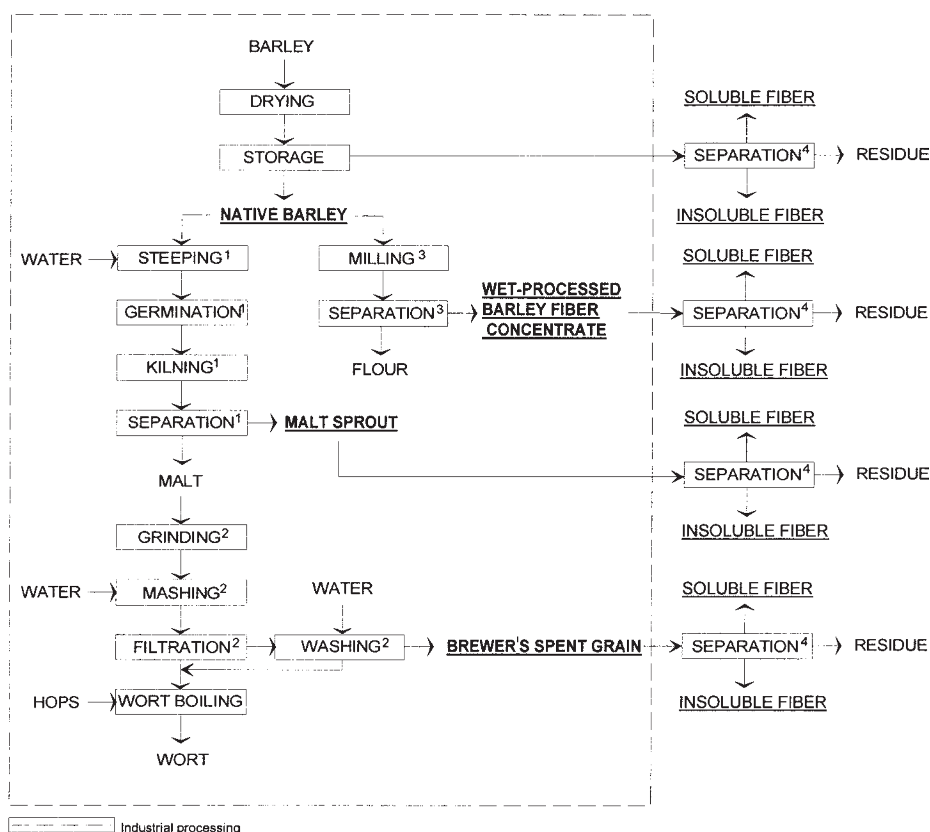

Fig. 1. Processing of malting barley. Fractions selected for the trials are underlined. The fractions are all enriched in fiber (hemicelluloses, cellulose, and pentosanes, among others) when compared to the reference material (i.e., native, unprocessed, malting barley).

as of other calcium-complexing compounds and acids *(1,12)*. The aims of the present study were to establish which part of the cereal grain is best suited for modifying alginate beads; to establish how the grain should be processed to yield a fraction which alone, or together with alginate, provides a good immobilization matrix; and to find a good growth medium and immobilization matrix for future studies.

Materials and Methods

Parallel samples were prepared and measured in each step of this investigation. Values presented represent averages of two independent replicates.

Materials

The two-rowed malting barley cultivar Kustaa, harvested in 1995, was used. Oat fiber and wet-processed barley fiber concentrate were manufac-

tured according to commercial wet-processing methods *(16)* and were supplied by Primalco, Finland. Malt sprout was supplied by the malster Lahden Polttimo, Finland, and brewer's spent grain (BSG) by the Sinebrychoff, Finland brewery (Fig. 1). They had been manufactured according to the conventional programs of the companies. In addition, barley starch (Riedel de Haën GmbH & Co. KG, Seelze, Germany), barley β-glucan (Biocon, Nagoya, Japan), and fibrous cellulose powder CF11 (Whatman plc, Kent, UK) were used.

Strains and Growth Conditions

S. griseoviridis strain K61 (DSM 7206) was supplied by Kemira Agro, Finland. The strain was maintained at –60°C in GYM medium (Difco Becton, Dickinson) containing 10% glycerol. Maintenance cultures were prepared by applying 20 mL of GYM to cultures grown on potato dextrose agar plates (Difco) (28°C for 7 d) and scraping the surface of the culture to obtain an even suspension.

The frozen inocula were first revived in liquid GYM media (28°C). On reaching the stationary growth phase (as measured with a Klett-Summerson calorimeter, filter no. 66), the culture was rotated once in liquid GYM media (28°C) prior to incubation of cereal-based media used to produce encapsulated products. All cultures were shaken at 250 rpm. Cereal-based media were inoculated with 1% inocula in the early stationary phase and incubated for 48 h at 28°C prior to encapsulation.

Encapsulation Procedure

Fiber fractions were suspended in 50 m*M* Na_2CO_3-$NaHCO_3$ buffer (pH 9.2) to give a concentration of about 0.75% (w:v) IF (7.5 mg of IF/mL of buffer). At this stage, if the medium was not used for production of microbial biomass, 0.5% (w:v) of sodium alginate (BDH, Poole, England) was added. The slurry was homogenized using an Ultra-Turrax® T 25 homogenizer (Janke & Kunkel GMBH & Co. KG, Staufen, Germany), after which it was incubated for 1 h at 37°C on a rotary shaker operated at 350 rpm (Gallenkamp orbital shaker/incubator, model INR 200 010V). In the media used to produce microbial biomass, alginate was not added simultaneously with the fiber. In these media 0.5% (w/v) of alginate was added at the end of microbial growth, just prior to incubation at 37°C and subsequent encapsulation.

The hydrated, swollen suspension was pumped dropwise with the aid of a mechanical pump (Type MV-CA4; Ismatec, Zürich, Switzerland) and nozzle about 1.5 mm in diameter into a gently stirred, sterilized 50 m*M* $CaCl_2$ solution at 22°C. Beads thus formed were stabilized in the solution for 20 min, before filtering through a mesh to remove the water and subsequent freezing (–20°C) and lyophilization (Christ Alpha 2-4; Adolf Kühner AG, Birsfelden, Switzerland).

Estimation of Biomass

Viable counts were calculated from dilution plates containing GYM agar. Samples were suspended in sterilized salt solution (9 g of NaCl/1000 mL of deionized water) and homogenized with an Ultra-Turrax T 25 homogenizer prior to further dilution and plating.

Separation of Cereal Fractions into SF and IF

The crude samples were divided into starch, SF, and IF fractions, based on solubility characteristics in aqueous suspensions *(17,18)*. A 10-g sample was first extracted for 30 min in an agitated 96% ethanol solution containing 0.9 *M* KOH. Following extraction, the sample was filtrated (Whatman No. 3 paper) under suction to preserve the retentate, which was washed thrice with pure extraction solvent. The filtrate containing denaturated and solubilized proteins and lipids along with low molecular weight carbohydrates was discarded. Next, the sample was treated under agitation at 50°C with a 0.3 *M* KOH solution, to solubilize starch and SF. The treatment was repeated twice. After each treatment, the solubilization solvent was centrifuged (5800*g* for 10 min at 50°C) to separate and retain the solubilization solvent as well as the insoluble fraction, containing mostly IF. The insoluble fraction was extracted thrice with dilute sulfuric acid (1.25%) at 75°C under agitation before oven-drying (105°C) and subsequent gravimetric analyses. The fiber was recovered through centrifugation (5800*g* for 5 min) after extraction treatments. Ethanol (90%) containing 5 mL/L of glacial acetic acid was added at a ratio of 75:10 (v:v) to the combined supernatants from the starch solubilization treatment, to precipitate soluble carbohydrates. The suspension was left overnight at 22°C. After precipitation, the precipitate was recovered by centrifugation (5800*g* for 5 min) and washed thrice with acidic ethanol before oven-drying (105°C) and subsequent gravimetric analyses. The amount of starch and fiber in the samples was estimated by measuring the loss in weight on ignition (30 min at 600°C). The residue consisted of inorganic salts and ashes.

Content of Crude Fat, Reducing Sugar, Soluble Nitrogen, Total Carbon, Nitrogen, and Phosphorus

The fat content was analyzed from dried samples (5–10 g of dry wt) using a Soxhlet extraction apparatus and dichloromethane as extraction solvent. The content of soluble reducing sugars in the growth media was analyzed using the dinitrosalicylate method of Fischer and Stein *(19)*, while the peptide-bond specific Biuret method of Itzhaki and Gill *(20)* was used to estimate total soluble protein content. Inorganic nitrogen content (NO_3 and NH_4 nitrogen) was below 0.01% (0.1 g/kg) in all native fractions; thus, it was assumed that available nitrogen was present either as soluble or insoluble protein and free amino acids. Free amino acids and insoluble protein were not measured, however.

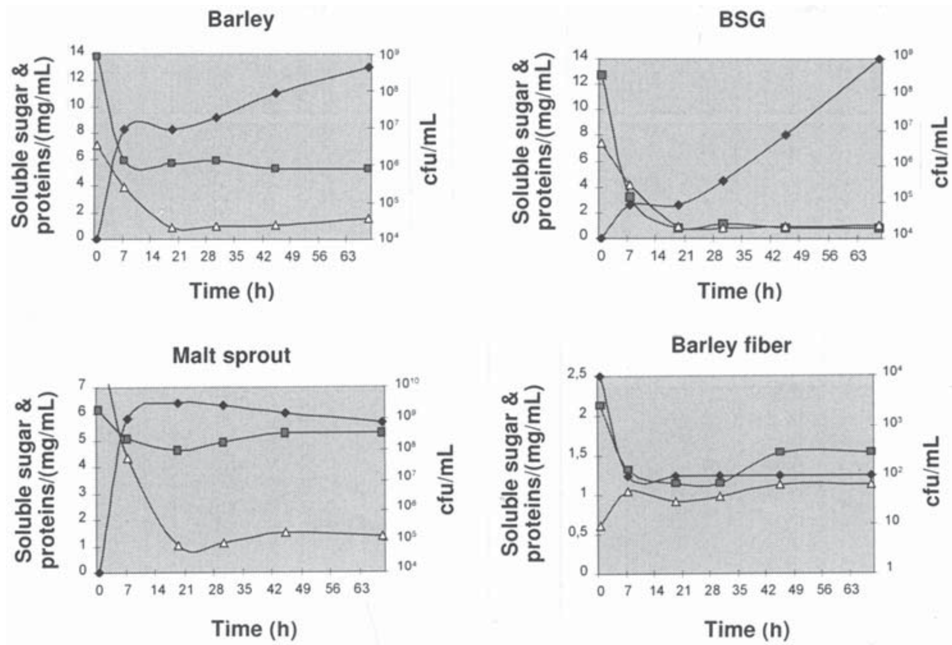

Fig. 2. Biomass development of *S. griseoviridis* and the content of soluble sugar and protein during cultivation. At the beginning of cultivation, all media contained mixed cultures, but subsequently, the antagonistic *S. griseoviridis* strain displaced contaminating microbes and within 70 h of cultivation the viable count of contaminants was below 10^{-4} cfu/cm^3 in all media. (—■—) Proteins; (—△—) reducing sugar; (—◆—) viable count.

Total carbon, nitrogen, and phosphorus, as well as inorganic nitrogen and phosphorus were analyzed by the research center of Kemira Agro, with standard methodology used in the analysis of inorganic fertilizers.

Results

Biomass Production

At first, the biomass of *S. griseoviridis* developed steepest in the malt sprout–based medium (Fig. 2). With malt sprout, the required biomass (10^9–10^{10} cfu/cm^3) was reached in <20 h. However, within 70 h from inoculation, the colony-forming unit content of *S. griseoviridis* in the barley and BSG media equalled that of the malt sprout–based medium. The wet-processed barley fiber concentrate did not support growth of the *S. griseoviridis*, not even directly following inoculation (Table 1, Fig. 2).

Composition of Cereal Fractions Prior to and During Cultivation

Malt sprout, while rich in soluble sugars, contained less soluble protein than BSG. Clearly, however, barley, malt sprout, and BSG contained enough of either soluble or insoluble nutrients to support sufficient growth,

Table 1
Composition of Cereal Fractions

Fraction	Moisture content (%)[a]	Fat (%)[a]	IF (%)[a]	IF (%)[a,b]	SF (%)[a]	Reducing sugars (mg/g dry wt)	Soluble protein (mg/g dry wt)	Total carbon[c]/ total nitrogen
Native barley	12.8	2.1	7	12	58	11.6	82.8	—
Malt sprout	4.4	2.4	13	31	26	36.3	102.0	36.1/3.3
BSG	4.2	5.3	24	42	17	21.1	209.0	41.3/2.7
Wet-processed barley fiber concentrate	7.0	3.9	17	24	33	11.0	35.9	38.0/2.0
Oat fiber	11.0	2.6	24	—	16	—	—	37.4/2.5

[a]Percentage per dry wt.
[b]IF content of the cereal fractions expressed as percentage of dry wt after 70 h of cultivation. Dry matter includes microbial biomass.
[c]Expressed as mol/kg/mol/kg.

whereas the wet-processed barley fiber concentrate did not (Fig. 2, Table 1). Soluble sugars and nitrogen had been used up within the first 20 h of cultivation in the native barley as well as the malt sprout and BSG media (Fig. 2). After this, insoluble nutrients sustained continued growth. The depletion of soluble nutrients during cultivation led to an increase in the content of IF (Table 1).

The content of crude, IF was highest in BSG (Table 1), which had been washed free of soluble starch and fiber with hot water during the mashing process, whereas the wet-processed barley fiber concentrate as well as the oat fiber had been subjected only to cold water treatments. Correspondingly, the SF content in the wet-processed barley fiber concentrate was higher than in BSG.

Encapsulation of Hydrogel Matrices to Immobilize Biomass

The IF fraction of barley was the only tested material other than alginate that had the potential to gel under the influence of calcium ions (Table 2). Gelling on immersion in calcium solution, though weaker than when using alginate, was clearly noticeable only with the IF fractions of native barley and wet-processed barley fiber. BSG, on the other hand, even though it consists of mainly barley fiber, had no capacity to gel without the addition of alginate. Comparison of the encapsulation properties of the cereal fractions on addition of alginate and enrichment of *S. griseoviridis* did not support the use of native barley (Table 2). Rather, the BSG-based and the wet-processed barley fiber concentrate–based products formed the most solid beads.

Viability and Applicability of Different Products

The viability of the *S. griseoviridis* control agent should exceed 10^8 cfu/g of dry wt. This requirement was met with all other media, but not with the wet-processed barley fiber concentrate (Table 3). Even though freeze-drying clearly diminished the viability of hydrogel beads, values still remained above this limit. Viability of dried beads remained stable for 12 mo of storage (Table 3).

The BSG-based product had the highest viability and BSG was best also when considering the appearance and applicability of the finished products. Only BSG-based and wet-processed barley fiber concentrate–based beads were reversibly hydratable. The barley and malt sprout–based products did not gel satisfactorily on immersion in calcium solution. The wet-processed barley fiber concentrate, even though forming even, reversibly hydratable capsules, failed to produce sufficient biomass.

Discussion

The encapsulating trials showed that the capsules that formed on immersion in calcium solution were held together primarily by an alginate-calcium network. The inherent weak gelling properties of the barley IF

Table 2
Encapsulation Properties of Native and Separated Fiber Fractions

	Barley	Malt sprout	BSG	WBFC[a]	Oat fiber	Barley β-glucanase	Cellulose	Barley starch	Sodium alginate
Native, untreated fraction	3.4%[d]	6.5%[d]	5.0%[d]	3.4%[d]	3.4%[d]	5.0%[d]	5.0%[d]	5.0%[d]	0[d]
IF fraction[b]	0[d]	5.0%[d]	3.4%[d]	0[d]	1.7%[d]	5.0%[d]	5.0%[d]	—	0[d]
SF fraction[b]	5.0%[d]	5.0%[d]	5.0%[d]	5.0%[d]	5.0%[d]	5.0%[d]	5.0%[d]	5.0%[d]	—
Native fraction after cultivation, i.e. enrichment of microbial biomass[c]	+[e]	–[e]	+++[e]	++[e]	+++[e]	nt[f]	nt[f]	nt[f]	nt[f]

[a]Wet-processed barley fiber concentrate.
[b]Fractionated according to the described method.
[c]8.5% (w:w) sodium alginate added per g of dry fiber
[d]Amount of alginate required for encapsulation on immersion in $CaCl_2$ solution expressed as (g of alginate/g of fiber dry wt).
[e]Visual evaluation of encapsulation properties. –, no visible encapsulation; +, formation of weak capsules that dissolve on storage; ++, formation of more durable capsules; +++, formation of durable, reversibly hydratable capsules.
[f]Not included in trials.

Table 3
Effect of Freeze-Drying and Storage
on Viable Units of Cereal Fractions (cfu/g dry wt)

Fraction	At time of inoculation	At end of cultivation	After freeze-drying	Freeze-dried product after 12 mo of storage at –20°C
Native barley	6.4×10^7	6.3×10^9	9.0×10^8	4.0×10^7
Malt sprout	3.5×10^6	5.6×10^{10}	5.7×10^8	1.1×10^8
BSG	4.2×10^5	4.6×10^{10}	3.7×10^8	1.6×10^9
WBFC[a]	4.5×10^5	$<10^4$	$<10^4$	$<10^4$
Commercial nonencapsulated agent[b]	10^7	$10^9…10^{10}$	$>10^8$	$>10^8$

[a]Wet-processed barley fiber concentrate.
[b]Viability of the product presently on the market.

fraction, however, were diminished by thermal or enzymatic treatment during the malting and mashing routines, since the IF fraction of BSG did not gel on immersion in the salt solution. Cellulose, β-glucan, or starch did not form capsules. It was therefore to be expected that malt sprout, the major fiber fraction consisting of cellulose, did not gel and, in fact, seemed rather to inhibit the gelling of alginate.

However, alginate alone cannot be used in a production process such as the one described here, because it lacks nutrients necessary for biomass production, it is susceptible to disruption by phosphate, and cell survival of aerobic organisms is limited in a matrix of pure alginate *(9,11,12)*. Cereal fiber fractions offer a promising alternative for both replenishing alginate with nutrients and modifying the alginate matrix to yield reversibly hydratable beads well suited for production of the biologic control agent cultivated in the present study. It appears that a high content of IF is required in order for the products to be firm and reversibly hydratable, but the present study shows clearly that even barley fractions rich in IF have to be replenished with alginate to yield suitable immobilization and cultivation matrices.

A successful production process for *S. griseoviris* biologic control agent requires a viable unit content in the range of $10^{-9}...10^{-10}$ cfu/cm^3 prior to encapsulation. At the same time, the content of contaminating microbes should not exceed 10^{-5} cfu/cm^3. BSG, malt sprout, and barley supported nearly equal amounts of viable biomass of the *Streptomyces* sp., but on freeze-drying, the BSG-based product sustained its viability better than the other fractions. Because BSG was best also when considering the appearance and applicability (i.e., the rewettability and consistence) of the finished product, it was the medium of choice. BSG is a good alternative also when taking into account the price of the finished product, because it is a

byproduct of a bulk process and is presently sold to fodder producers roughly at the price of delivery costs.

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