

Interactions of fungi from fermented sausage with regenerated cellulose casings

Hassan K. Sreenath · Thomas W. Jeffries

Received: 4 November 2010/Accepted: 17 March 2011 /Published online: 5 April 2011
© Society for Industrial Microbiology 2011

Abstract This research examined cellulolytic effects of fungi and other microbes present in cured sausages on the strength and stability of regenerated cellulose casings (RCC) used in the sausage industry. Occasionally during the curing process, RCC would split or fail, thereby leading to loss of product. The fungus *Penicillium sp.* BT-F-1, which was isolated from fermented sausages, and other fungi, which were introduced to enable the curing process, produced small amounts of cellulases on RCC in both liquid and solid cultivations. During continued incubation for 15–60 days in solid substrate cultivation (SSC) on RCC support, the fungus *Penicillium sp.* isolate BT-F-1 degraded the casings' dry weights by 15–50% and decreased their tensile strengths by ~75%. Similarly commercial cellulase(s) resulted in 20–50% degradation of RCC in 48 h. During incubation with *Penicillium sp.* BT-F-1, the surface structure of RCC collapsed, resulting in loss of strength and stability of casings. The matrix of industrial RCC comprised 88–93% glucose polymer residues with 0.84% xylan impurities. Premature casing failure appeared to result from operating conditions in the manufacturing process that allowed xylan to build up in the extrusion bath. The sausage fungus *Penicillium sp.* BT-F-1 produced

xylanases to break down soft xylan pockets prior to slow cellulosic dissolution of RCC.

Keywords Regenerated cellulose casings (RCC) · Cellulases · Degradation · Fungal interactions · Solid substrate cultivation (SSC)

Introduction

Regenerated cellulose, made from pure wood pulp by the viscose process [16], is widely used in meat packaging and other applications [11, 16]. Annual production of cellulose casings in the USA alone exceeds 14 million kilograms and is a billion-dollar industry there and in Europe [8, 11, 16, 27]. Regenerated cellulosic casing (RCC) is the preferred packaging material for the cooked sausage industry as it is easy to handle, hygienic, and cost effective compared with natural animal casing [3, 17, 21, 24]. RCC can also be used in producing fermented or cured sausages due to its strength and stability and its ability to withstand smoking and curing. Under most conditions of use, RCC is not subject to biodegradation. Occasionally, however, RCCs fail during curing and drying, and the loss of product can be significant.

Fermented dried sausages are popular in Italy, France, and the USA. During their manufacture, the ground, spiced meat is packed into an RCC, twisted to seal the ends, cooked, and hung for 2–4 months or more of curing depending on product size (diameter) and type. During this time, the surface of the sausage is sprayed with traditional microbial strains that enable the development of flavor and texture [2, 5, 9, 14, 18, 22, 23, 26, 28]. The microbial inoculum can be either a pure culture or a consortium of organisms.

H. K. Sreenath
Department of Biological Systems Engineering,
University of Wisconsin-Madison,
460 Henry Mall, Madison, WI 53706, USA

T. W. Jeffries
Forest Products Laboratory, 1 Gifford Pinchot Drive, Madison,
WI 53705, USA

H. K. Sreenath (✉)
Probiotic Fermentation Development, Danisco USA Inc.,
3329 Agriculture Drive, Madison, WI 53716, USA
e-mail: hassan.sreenath@danisco.com

RCC membrane curing facilitates diffusion of oxygen, moisture, and other nutrients such as sugars, amino acids, ions, etc. for the growth of external microbes to produce natural specialty flavored fermented sausages. During curing, casings occasionally break before the process is complete, leading to loss of product. The composition of RCC and microbes employed may be responsible for inconsistent performance and economic loss.

In examining the casings, we expected the composition to consist solely of glucan residues because it is derived from highly purified sulfite wood pulp, which is used to provide uniform strength and stability in various packaging applications [3, 8, 16, 21]. However, we found the presence of impurities such as xylan, mannan, and negligible lignin along with bulk glucan residues in commercial RCC (Table 1). Essentially no information existed on changes in strength, stability, and morphology of RCC during fermented-sausage production; however, we suspected that the loss of casing strength could result from microbial xylanases and cellulases.

This work was conducted to determine the reasons for RCC failure during sausage curing. Our approach was to isolate and screen cellulolytic microbes from fermented sausages and identify conditions responsible for changes in strength and morphology of RCC during microbial interactions. Primary failure appeared to result from low levels of cellulases or xylanases attacking the regenerated cellulose or from residual, reprecipitated xylan components.

Materials and methods

Isolation of microbes from sausages

In this study, we examined four brands of fermented sausages. Two were imported from Italy (Italy1 and Italy2), and two were of domestic US manufacture (US1 and US2). These were made by surface inoculation of ground sausage either using traditional microbial consortia or strains of *Penicillium nalgiovensis* to facilitate development of flavor and texture. Sliced and small flakes of surface samples from these four brands of fermented sausages were

streaked on to agar plates of bacterial heart infusion agar, tryptic soy agar, tryptone glucose agar, and potato dextrose agar. All plates were incubated at 25°C for 3–4 days. All isolated pure culture strains of fungi, yeast, and bacteria were preserved in glycerol stocks and frozen at –80°C. The traditional industrial cultures of sausage inoculant strains *P. nalgiovensis* PNT-1 and NG 14 were supplied by Viskase Corporation (Chicago, IL, USA). The transparent casing (TC), fibrous casing (FC), and pulp board (PB) used in this work were also provided by Viskase.

Screening of sausage microbes for cellulases on liquid medium

Fifty milliliters of fermentation medium containing 10 g/L carbon source [carboxymethylcellulose(CMC) 7LF/glucose/sorbitol/cellobiose/lactose/wheat bran] in the presence or absence of sorbose, 1.0 g/L asparagine, 1.0 g/L corn-steep liquor (CSL), and 10 g/L of 10× mineral salts stock solution was separately added prior to inoculation with 2 g/L cells of various isolated microbial isolates and other sausage inoculants strain of *P. nalgiovensis* PNT-1 [10]. The 10× mineral salts stock solution consisted of 15 g/L KH_2PO_4 , 5 g/L $(\text{NH}_4)_2\text{SO}_4$, 0.6 g/L MgSO_4 , 0.6 g/L CaCl_2 , 0.005 g/L $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.0016 g/L MnSO_4 , H_2O , 0.0014 g/L $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ and 0.0037 g/L $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$. For fungal-culture growth, the liquid medium pH was adjusted to 5, whereas for the bacteria and yeast, the pH was adjusted to 7.0 with NaOH and buffered with 2.0 g/L K_2HPO_4 . Shake-flask fermentation was conducted at 27°C at 100 rpm for 3–5 days.

For other screening experiments, 10 g/L CMC 7LF was substituted with various plant fibers in liquid medium such as locust-bean gum, wheat bran, rice bran, corn bran, oat bran, and flax flour for cellulase production by our laboratory-isolated fungal strain of *Penicillium sp.* isolate BT-F-1 (Table 2). Shake-flask fermentation was conducted at pH 5, 27°C at 100 rpm for 3–5 days.

Determination of enzyme activity

Filter-paper cellulase (FPase) activity of commercial cellulase Multifect B (Genencor, Palo Alto, CA, USA) and

Table 1 Chemical composition of regenerated cellulose casings (RCC)

Sample	Chemical components(%)						Total carbohydrates
	Lignin	Glucose	Xylose	Mannose	Fucose	Arabinose	
Pulp board	0.6	92.1	2.5	1.5	ND	0	96.7
FC	0.2	88.4	4.0	0.5	0.4	0.1	93.6
TC	0.4	93.5	0.8	0.7	0.4	0	95.8

Analyzed after complete hydrolysis with H_2SO_4 prior to sugar analysis with high-performance liquid chromatography (HPLC) [6, 25]

TC transparent casing, FC fibrous casing, ND not determined

Table 2 Profile of isolated microbes from various fermented sausages and their cellulolytic fermentation

Sausage type	Bacteria ^a			Fungi ^b			Yeasts ^c		
	Number of isolates	Cellulase (IU/ml)		Number of isolates	Cellulase (IU/ml)		Number of isolates	Cellulase (IU/ml)	
		FPase	CMCase		FPase	CMCase		FPase	CMCase
Italy1	4	0.02	0	3	0.04	0.06	0	0	0
US1	0	0	0	1	0.02	0.03	0	0	0
Italy2	5	0.01	0.04	3	0.01	0.03	6	0	0
US2	0	0	0	1	0.04	0.04	1	0.04	0

Fermentation conditions: 50 ml of carboxymethylcellulose (CMC) medium consisting of 1% CMC, 0.1% asparagine, 0.1% corn-steep liquor (CSL), and mineral salts [10] was inoculated with 2 g/L cells of various isolated microbial isolates in shake flasks at 100 rpm for 72 h at 27°C. For fungal-culture growth, the media pH was adjusted to 5–5.5, whereas for bacteria and yeast, the pH was adjusted to 7.0. The culture supernatant obtained after centrifugation was employed for determining filter-paper cellulase (FPase) and carboxymethylcellulase (CMCase) activity

^a For isolation of bacteria: tryptic soy agar and bacterial heart infusion agar with 0.04% (v/v) Benomyl solution (as antifungal agent) was used

^b For isolation of fungi: tryptone glucose agar and potato dextrose agar with 0.01% (w/v) streptomycin sulfate (as antibacterial agent) was used

^c For isolation of yeasts: tryptone glucose agar and potato dextrose agar with 0.04% (v/v) Benomyl solution (as antifungal agent) and 0.01% (w/v) streptomycin sulfate (as antibacterial agent) were used

in-house-produced solid substrate cultivation (SSC) cellulases were measured as described by Mandels et al. [13, 21]. The protein content of the enzyme was determined using Lowry's reagent [12]. Two milliliters of reaction mixture consisted of a 50-mg strip of Whatman no. 1 filter paper in 0.05 M sodium phosphate buffer (at both pH 4.81 and pH 7.0) and 1.0 mg of enzyme protein. The reaction mixture was incubated at 50°C for 1 h, and reducing sugars were estimated by dinitrosalicylic acid (DNS) reagent [15]. One international units of FPase unit is defined as the amount of enzyme required to liberate 1 μ mol of glucose/minute under assay conditions.

CMCase activity was determined using 1.0 ml of 1% CMC 7LF in 0.05 M sodium acetate buffer in both pH 4.81 and pH 7.0 with 0.5 mg enzyme protein [21]. The reaction mixture was incubated at 50°C for 30 min, and reducing sugars were estimated by DNS reagent. One international unit of CMCase is defined as the amount of enzyme that catalyzed the formation of 1 μ mol glucose/minute under assay conditions.

Xylanase activity was assayed using 1.0 ml of 2% soluble oat-spelt xylan in 0.05 M sodium acetate buffer pH 5.0 and 0.5 mg enzyme protein [21]. The reaction mixture was incubated at 50°C for 30 min prior to reducing sugar estimation by DNS reagent. One IU of xylanase activity referred to the amount of enzyme that catalyzed the formation of 1 μ mol xylose/min under assay conditions.

Cellulase detection by radial diffusion

The culture filtrates of isolated sausage microbes were visualized for detection of cellulases by the radial diffusion technique [19]. In this method, 100- μ m-thick CMC gels

were cast using 0.2% CMC 7LF embedded in 1.0% agar suspended in 0.05 M sodium acetate buffer, pH 4.8. Zymograms were developed by incubating 5 μ l of unknown enzyme extracts on CMC gels for 2–5 min at 48°C in a humid chamber and stained with 0.1% methylene blue (in water). After drying, the diameters of clearance zones were measured. Multifect B, 0.025 IU (in 1.0- μ l drops) was used as a positive control.

Solid-state cultivation on wheat bran

Wheat bran was wetted with 60% moisture containing tap water, 5 g/L K_2HPO_4 , 10 g/L CSL, and 10 g/L of 10 $\times$ mineral salts stock solution [10]. Fifty grams of moistened wheat bran in a 500-ml conical flask was autoclaved for 1 h at 121°C. After cooling, the moistened bran was inoculated with microbial isolates and pure cultures of *P. nalgiovensis* PNT-1 then incubated at 27°C for 12 days. The moldy bran was extracted with tap water in the solid:liquid ratio of 1:3, and the extract was centrifuged at 16,000 $\times g$ at 10°C for 20 min. The supernatant solution was decanted and saved at 4°C prior to enzyme assays.

Solid-state cultivation on RCC

Solid-state cultivation of sausage isolates were separately conducted using both casing types TC and FC: 2 g casings (0.4 $\times$ 3 cm strips) in triplicate 125 ml Erlenmeyer flasks or 25-g casings (0.4 $\times$ 3-cm strips) in triplicate 500 ml Erlenmeyer flasks. The FC and TC were first suspended in a known volume of water and sterilized for 25 min at 121°C, 15 psi for 25 min. After cooling, the water in the flask was removed aseptically. The moist

casings were then supplemented with 1.2 ml (for 125-ml flasks) or 15 ml (for 500-ml flasks) of sterile nutrient solution consisting of 10 g/L CSL, 10 g/L of 10× mineral salts stock solution, and tap water mixed at 1:1:4. The final moisture content of the casing SSC medium was about 60%. Casing SSC medium was then inoculated with 5% fungal inoculum, mixed well, and cultivated at 27°C for 2 weeks to 2 months with static incubation. Similarly, the control contained only SSC casing medium with no fungal cultures. After incubation, the moldy casing was extracted with tap water in the solid:liquid ratio of 1:3 and the extract was centrifuged at 16,000×g at 10°C for 20 min. The supernatant solution was decanted and saved at 4°C prior to enzyme assays. The eluted casings were washed four times with water and dried carefully between glass and paper towels prior to strength test and dry weight determinations.

Dry weight determination

RCC samples before and after fungal growth or before and after cellulase treatment were washed with several volumes of water to remove cells or enzymes then dried between paper towels that were sandwiched between glass plates. This was done to keep the samples flat for tensile strength tests. This sandwich predrying was gentle and prevented curling or breaking up of RCC due to its fragile nature after treatment. The treated and flattened RCC samples were dried overnight at 65°C, followed by cooling to 23–25°C to constant dry weight in a dessicator, and then dry weights and tensile strengths were determined.

Viscosity determination

Viscosity was measured using an Ostwald Viscometer at 25°C. Substrate consisted of 1% CMC 7LF (Hercules Corporation, Wilmington, DE, USA) prepared in 50 mM potassium phosphate buffer at 50°C with or without SSC enzyme extracts [19]. Viscosity is expressed in Centipoises (cps).

Tensile strength of RCC

Both TC and FC were cut into 1 × 8-cm strips or 2 × 8-cm strips in the cross-extrusion and with long-extrusion directions, respectively. After allowing the casing strips to equilibrate to the test laboratory conditions (50% moisture, 22°C), the tensile strength of the casings was checked using zero span (Pulmac Instruments Ltd., Montreal, Canada). The percent deviation in zero-span tensile strength was calculated. Similarly, the zero-span tensile strength of microbial infected casings was also checked.

Scanning electron microscopy (SEM) of fungal interaction on casing membranes

Strips of both TC and FC, 1 × 1 cm, 2 g in 125 ml Erlenmeyer flasks were sterilized in 30 ml water and later the water was aseptically drained. The RCC strips were moistened with 0.2 ml of nutrient solution consisting of 10 g/L CSL, 10 g/L of 10 × mineral salts stock solution, and tap water. After inoculating with fungal cultures of *Penicillium* sp BT-F-1 and *P. nalgiovensis* PNT-1, the flasks were incubated at 27°C for extended periods.

Samples of moldy casing membranes were aseptically taken at 4, 12, and 28 days and prepared for observation in scanning electron microscopy (SEM). The treated RCC membrane 1 × 1-cm strips were carefully washed several times with water to remove surface fungal cells or enzymes and gently dried between glass and paper towels followed by air drying at 25°C. Suitable controls were prepared using TC and FC strips. The strips were placed on double-sided silver conductive Scotch adhesive tape mounted on specimen stubs and coated with a thin layer of gold in vacuum. Observations and photographs were taken using an SEM (JSM-840, JEOL, USA).

Application of commercial cellulase(s) on RCC

Strips of both PB and FC, 1 × 1 cm, 1 g in 125 ml Erlenmeyer flasks were suspended in 10 ml 0.05 M citrate buffer, pH 4.86. Commercial cellulase (Genencor, Palo Alto, CA, USA) was topically applied at 1% level to PB and FC, and the samples were incubated at 50°C for various periods in a shaking water bath at 100 rpm. For other experiments, various levels of Multifect B cellulase were applied on PB and FC and incubated at 50°C for various periods in shaking water bath at 100 rpm. The reactions were stopped by heat inactivation with steam for 10 min. After cooling, the residue was separated from clear supernatant by filtration using Whatman filter paper no. 1, followed by reducing sugar analysis using dinitrosalicylic acid reagent [15]. The sugars in the clear supernatant were also analyzed by high-performance liquid chromatography (HPLC) analysis. The residue was washed three times with distilled water, and dry weight was determined by drying the material overnight at 65°C, followed by cooling to 23–25°C. Standard error (S_e) was calculated from data obtained, and values are presented in Figs. 1–4 on the right side of each line of the graph.

HPLC analyses

RCC was completely hydrolyzed using concentrated H_2SO_4 at 100°C for 1 h prior to monomeric sugar analysis [6]. Sugars of RCC and PB were analyzed by HPLC

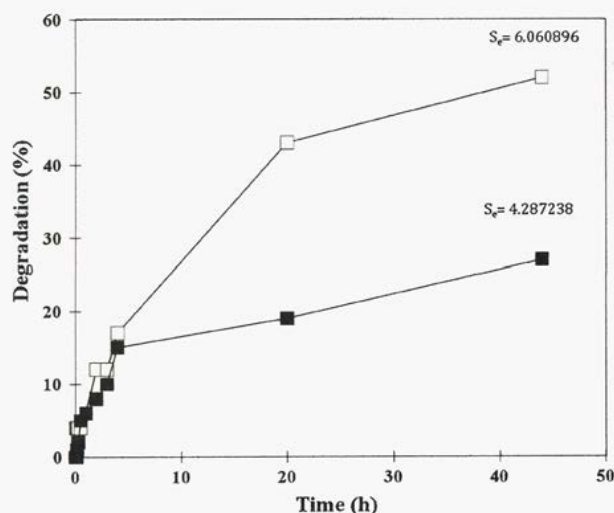


Fig. 1 Effect of extended incubation time on degradation of fibrous casing (FC) and pulp board (PB) using commercial cellulase (0.25 IU/ml) (FC open square, PB filled square)

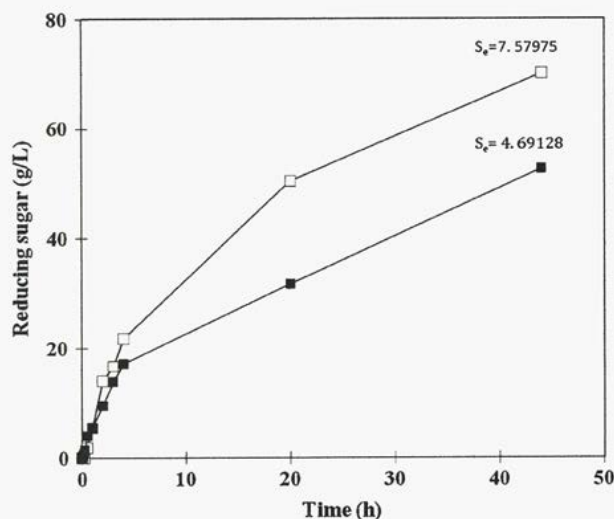


Fig. 2 Effect of incubation time on reducing sugar production during cellulolytic (0.25 IU/ml) degradation of fibrous casing (FC) and pulp board (PB) (FC open square; PB filled square)

(Dionex, Sunnyvale, CA, USA) using pulsed amperometric detection column (Carbopac PA1, 4 × 250 mm) (Dionex) with ED50 electrochemical detector [25]. The mobile phase of the column is pretreated by the reverse-gradient method consisting of elution with water for 11 min, followed by a 1-min ramp to 1 M $\text{Na}^+\text{CH}_3\text{COO}^-$ in 1 M NaOH. The 1-M level was maintained for 5 min, and then returned to the original water eluent in a 1-min gradient using GS-50 gradient pump at a flow rate of 1.1 ml/min at 18°C. These conditions were employed to elute sugars, condition the column, and re-equilibrate the column, respectively.

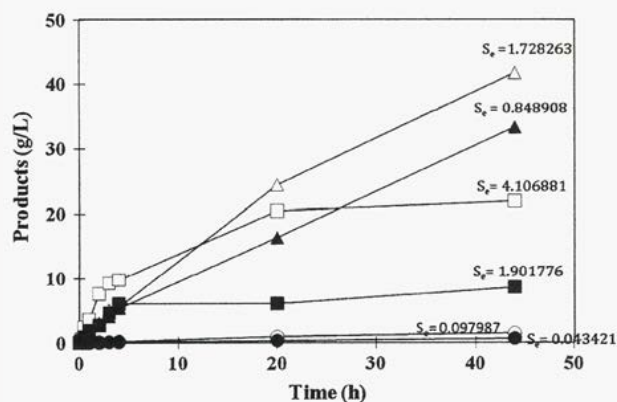


Fig. 3 Effect of extended incubation time on cellulolytic (0.25 IU/ml) degradation of fibrous casing (FC) and pulp board (PB) (FC cellobiose open square; FC glucose open triangle; FC xylose open circle; PB cellobiose filled square; PB glucose filled triangle; PB xylose filled circle)

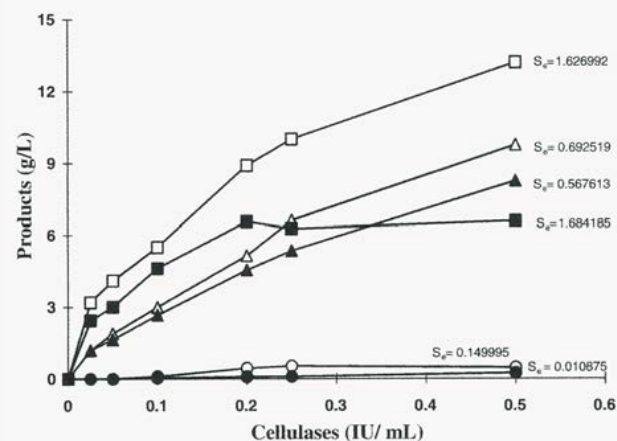


Fig. 4 Effect of various cellulase concentrations on degradation of fibrous casing (FC) and pulp board (PB) (FC cellobiose open square; FC glucose open triangle; FC xylose open circle; PB cellobiose filled square; PB glucose filled triangle; PB xylose filled circle)

Cellobiose, glucose, and xylose liberated during cellulolytic degradation of RCC were separated by HPLC analysis using ION-300 column (300 × 7.8 mm) (Inter-action chromatography, San Jose, CA, USA) with refractive index detector [20]. The mobile phase was 2.5 mM H_2SO_4 with a flow rate of 0.4 ml/min at 60–65°C, and 4,130–6,200 kPa.

Results and discussion

Isolation of sausage microbes and their cellulolytic profile in liquid media

We isolated several strains of bacteria, yeasts, and fungi from both domestic (US1 and US2) and imported (Italy1

and Italy2) fermented sausages made with traditional inocula (Table 2). Prior to receipt, these sausages had been stored under normal commercial (but unknown) conditions during distribution and marketing, so we cannot determine whether the differences in microbial populations were introduced by the manufacturing process or subsequently during shipping and handling. The isolated sausage fungal cultures grew better than yeast and bacterial cultures on liquid CMC media but produced little or no detectable cellulases. The sausage microbes failed to produce cellulase that could be detected by radial diffusion tests on CMC plates [19]. These microbes, when screened on glucose, sorbitol, cellobiose, and lactose in the presence or absence of sorbose, exhibited very good growth but had faint cellulase activity (data not shown). A low level of cellulase activity was induced in many fungal cultures but few bacteria and yeasts when cultivated on liquid medium with 2% wheat bran (Table 2). Sorbose slightly enhanced cellulase induction in wheat-bran-liquid medium. The culture fluids with cellulase activity, when incubated with a fibrous casing disc (0.4 in. diameter) at 50°C, failed to show reducing sugar release in 48 h compared with a commercial cellulase.

Cellulase production in liquid media with various plant fibers

The fungus BT-F-1, isolated from Italy1 fermented sausage was identified as *Penicillium sp.* (Analytical services, Inc. Williston, VT, USA). This fungus, which was prevalent in the sausage samples, was able grow well on various plant fibers such as locust-bean gum, corn bran, oat bran, rice bran, wheat bran, flax, and CMC in liquid fermentation, producing low cellulase activity. Table 3 also shows that the *Penicillium sp.* isolate BT-F-1 cultivation on rice bran and oat bran gave mild CMCase activity without filter-paperyase (FPase) activity. Cultivation of this fungal isolate on wheat bran produced the best CMCase and FPase activity at both pH 4.8 and 7.0 (Table 3).

Cellulase production on wheat bran by SSC

Industrial sausage fungal cultures such as *P. nalgiovensis* strain PNT-1 and NG-14 as well as *Penicillium sp.* isolate BT-F-1 produced higher cellulase activities when cultivated on wheat bran by SSC for 12 days (Table 4). Eberhardt et al. [7] also reported that wheat bran could induce cellulase formation during the sausage fermentation process. The BT-F-1 culture isolate had slightly higher CMCase activity than culture *P. nalgiovensis* strain PNT-1, but FPase activity was similar at pH 4.8. However, the culture *P. nalgiovensis* strain NG-14 showed lower activity of CMCase. The cellulase produced from both sausage

Table 3 Effects of various plant fibers in liquid fermentation media for cellulase production in sausage fungus *Penicillium sp.* isolate BT-F-1

Substrate(1%)	CMCase (IU/ml)		FPase (IU/ml)	
	pH 4.81	pH 7.0	pH 4.81	pH 7.0
Locust-bean gum	0.05	0.01	0	0.03
Wheat bran	0.23	0.14	0.03	0.08
Rice bran	0.14	0.07	0	0
Corn bran	0.09	0.07	0.01	0.04
Oat bran	0.13	0.14	0	3
Flax	0.11	0.08	0.02	0.02
CMC	0	0.02	0	0.03

Fermentation conditions: 50 ml of medium consisting 1% plant fibers, 0.1% asparagine, 0.1% CSL, and mineral salts [10] was inoculated with 2 g/L cells of *Penicillium sp.* isolate BT-F-1 in shake flasks at pH 5.0, 27°C at 100 rpm for 96 h. The culture supernatant obtained after centrifugation was employed for determining FPase and CMCase activity

CMCase carboxymethylcellulase, FPase filter-paper cellulase, CSL corn-steep liquor

fungi *Penicillium sp.* isolate BT-F-1 and traditional sausage fungi PNT-1 was able to reduce CMC viscosity. The drop in CMC viscosity was almost 50% (from 5 to 2.5 cps) in 5 h due to cellulase activity of *Penicillium sp.* isolate BT-F-1 compared with *P. nalgiovensis* PNT-1, which exhibited 40% drop in viscosity.

Chemical composition of RCC

The original PB used in the manufacture of RCC consisted of 92% glucan, 1.5% mannan, and 2.5% xylan. The TC contained 93.5% glucan, 0.8% xylan, and 0.7% mannan, whereas the FC contained 88% glucan, 4% xylan, and 0.8% mannan (Table 1). Hence, FC and TC samples as received contained significant xylan impurities.

SSC on RCC

In liquid culture, both FC and TC supported good growth of *Penicillium sp.* isolate BT-F-1 but did not produce detectable cellulase activity. The fungal growth network on RCC started after 3 days of inoculation during SSC. Consequently, the fungal growth was extensive and completely covered most of the sausage casings within 7–10 days of incubation [21]. The reducing sugar eluted from FC was 0.1–0.6 mg/ml during SSC growth. However, no reducing sugars were eluted from TC during SSC growth. The amount of xylanase detected in FC and TC by sausage fungi in the first 2 weeks of SSC growth was 1.04 and 0.11 IU/ml, respectively. However, xylanase activity disappeared in extended growth and incubation of the fungus.

Table 4 Solid substrate cultivation (SSC) on wheat bran for cellulase production by some sausage fungal strains

Culture	CMCase (IU/ml)		FPase (IU/ml)	
	pH 4.81	pH 7.0	pH 4.81	pH 7.0
<i>Penicillium sp.</i> (isolate BT-F-1)	1.00	1.00	0.42	0.30
<i>Penicillium nalgiovensis</i> (isolate NG-14)	0.70	0.24	0.40	0.48
<i>Penicillium nalgiovensis</i> (isolate PNT-1)	0.76	0.94	0.42	0.39

The moldy bran, 15 g from day 12, was eluted with 45 ml tap water at 25°C, centrifuged at 5,000 rpm for 20 min, and the cell-free clear supernatant was used as a source of enzyme

CMCase carboxymethylcellulase, FPase filter-paper cellulase

No cellulases were detected in the first 2 weeks. After 2 months of SSC, the enzyme extract of both TC and FC had faint CMCase activity at pH 4.8 and 7.0 (Table 5).

Strength and dry weight reduction of RCC after fungal treatment

Sausage fungi reduced tensile strength of sausage casings after 2 months of SSC. The strength reduction was 60–70% in FC by *Penicillium* strains of PNT-1 and isolate BT-F-1 (Table 6). The strength reduction in TC was 30–40%. It was found that the TC membrane was twice as strong as the FC membrane. The dry weight reduction of sausage casings after 2 months of SSC was significantly lower than the loss of tensile strength on comparative percentage bases. FC and TC lost 30% and 20% weight, respectively, after SSC fungal growth (Table 6). The weight reduction progressively enhanced in FC during 2 months of incubation; however, in TC, the weight decrease occurred only between months 1 and 2. In either case, the dried casing obtained after fungal growth was easily broken with little force.

Electron microscopy (EM) of fungal-treated RCC

Control FC showed smooth, highly interwoven cellulose fibrils approximately 25–30 µm in diameter (Plate 1). As the fungal growth proceeded, in FC, the walls of interwoven fibrils were degraded progressively, loosening the surface structure due to low fungal enzyme secretion (Plate 2). Hence, further incubation resulted in strength loss of RCC due to fungal enzyme interactions. The TC

exhibited rough, long slits of cellulose fibrils of diameter 0.25 µm (Plate 3). The microfibril slits of cellulose were heavily disorganized due to fungal growth. This led to cracks and crevices, which we hypothesize was related to strength loss and to collapse of structure (Plate 4). The structural damage to TC membranes was more prevalent with the PNT-1 fungus than with BT-F-1, leading to strength loss of RCC due to enzymatic interactions.

EM was valuable in understanding the mechanisms of degradation. Significant dissolution was observed in the morphology of these casing membranes when grown with sausage fungal strains of *Penicillium sp.* isolate BT-F-1 and PNT-1. Following extended incubation, the fungal-inoculated casings exhibited disorganized and delicate surface structure, resulting in loss of weight and tensile strength. In the case of FC, due to fungal attack, the surface-interwoven cellulose structure was digested, leading to heavy damage with many crevices. The heavy lesions on the cellulosic fibrils paved the way for hyphal contact that increased structural disorganization (Plate 2). In TC, the fungal contact initiated digestion of several “blebs” in an orderly fashion. These correlated with holes in the slit structure. The holes increased in volume during hyphal contact, which thereby led to morphological collapse of the slit structure (Plate 5).

The chemical nature of the blebs was not determined but is hypothesized to consist of reprecipitated xylan. In the manufacture of RCC, sulfite pulp is solubilized in a strong solution of sodium hydroxide, which frees the pulp from residual hemicelluloses and converts it into alkali cellulose. This also allows the cellulose to be further solubilized by CS₂. The viscose solution of cellulose is filtered and then

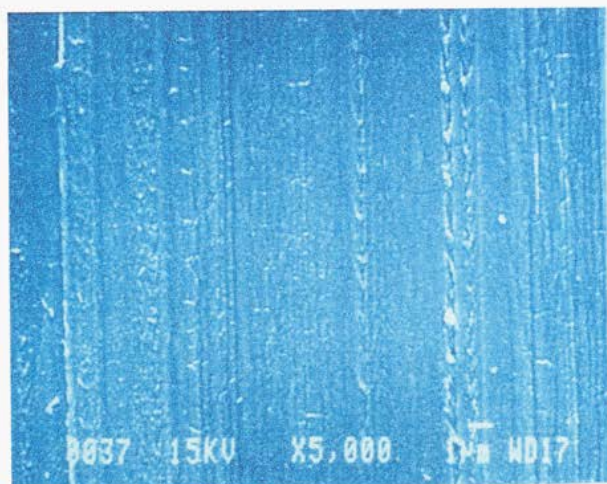
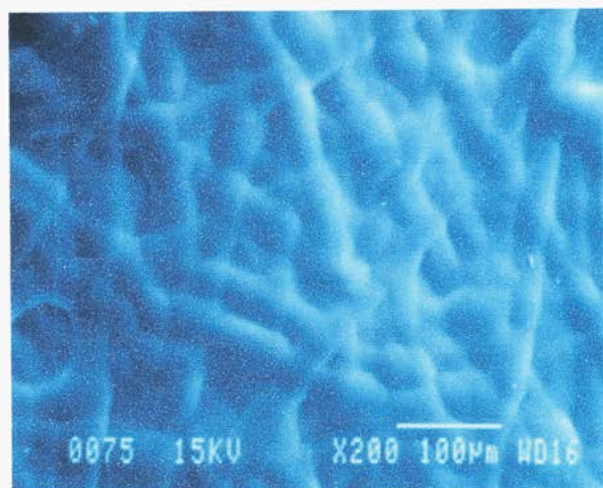
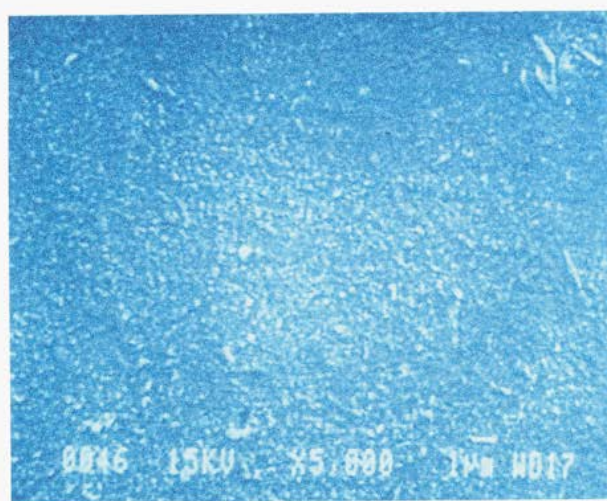
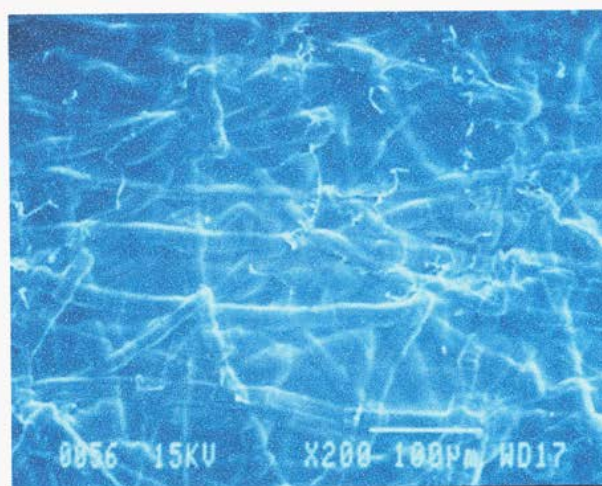
Table 5 Solid substrate cultivation (SSC) on regenerated cellulose casings (RCC) for cellulase production by some sausage fungal strains

Sample	<i>Penicillium sp.</i> (BT-F-1) CMCase (IU/ml)		<i>Penicillium nalgiovensis</i> (PNT-1) CMCase (IU/ml)	
	pH 4.81	pH 7.0	pH 4.81	pH 7.0
Fibrous casing (FC)	0.04	0.02	0.03	0.01
Transparent casing (TC)	0.01	0.05	0.06	0.06

Two-month-old moldy casing, 15 g, eluted with 45 ml tap water at 25°C, centrifuged at 5,000 rpm for 20 min, and the clear cell-free supernatant was used as a source of enzyme

Table 6 Effect of fungal solid substrate cultivation (SSC) on dry weight and tensile strength of regenerated cellulose casings (RCC)

Treatment	Dry weight ^a (g)	Reduction (%)	Tensile strength ^b (psi)	Reduction (%)
Fibrous casing (FC)				
Control	1.58 ± 0.2	0	28.2 ± 5.5	0
<i>Penicillium</i> sp Isolate BT-F-1	1.12 ± 0.1	29.1	11.5 ± 4.2	59.2
<i>P. nalgiovensis</i> PNT-1	1.12 ± 0.1	29.1	8.6 ± 2.8	59.5
Transparent casing (TC)				
Control	1.35 ± 0.15	0	60.0 ± 4.1	0
<i>Penicillium</i> sp Isolate BT-F-1	1.07 ± 0.1	20.7	42.2 ± 7.8	29.7
<i>P. nalgiovensis</i> PNT-1	1.15 ± 0.1	14.8	35.3 ± 14.6	40.7

^a Dried at 65°C over night^b Both casing strips FC and TC, 1 × 8-cm strips/2 × 8-cm strips in the cross-extrusion and with long-extrusion directions were measured by zero span (Pulmac instruments) for tensile strength at 50% moisture at 22°C**Plate 1** Ultrastructure of fibrous casing (FC)**Plate 3** Ultrastructure of transparent casing (TC)**Plate 2** Destabilized ultrastructure of fibrous casing (FC) due to *Penicillium* sp. isolate BT-F-1 fungal interactions**Plate 4** Collapsed ultrastructure of transparent casing (TC) due to *Penicillium* sp. isolate BT-F-1 fungal interactions

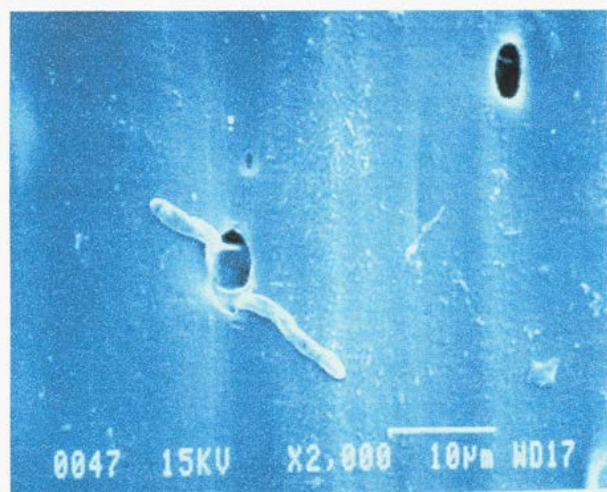


Plate 5 Fungal hyphae attacking through xylan bleb of transparent casing (TC) leading to cellulose dissolution

spun or extruded into an acid bath. This produces regenerated fibers in the spinning process or sheets of casing material in the extrusion process [8, 16]. Under optimal conditions, the residual xylan passes into the acid solution, and the regenerated cellulose forms an antiparallel crystal structure. In commercial operations, the acid bath solution is recycled, thereby allowing accumulation of residual xylan, which is normally not allowed to exceed established process limits. We hypothesize that this noncellulosic component of the reprecipitated pulp is what forms the blebs in the extruded casing and that the parallel lines observed in the EM pictures were formed by rough surfaces on the edge of the extruder.

Effect of topical addition of commercial cellulases on RCC

Regenerated casings were more susceptible to degradation than were the PB (Fig. 1) from which the casing material was manufactured. The percent dry weight decrease of regenerated cellulose recorded in this study was termed degradation. Regenerated cellulose FC degradation with a commercial cellulase was almost twice as high in 48 h compared with the starting material PB. Similarly, the reducing sugar release was 1.5 times higher in FC compared with PB during cellulolytic degradation (Fig. 2). Cellulolytic degradation of FC in the first 4 h initially produced more cellobiose than glucose (Fig. 3). Increasing cellulolytic activity gave higher amounts of cellobiose and glucose in FC compared with PB (Fig. 4). The higher rates of FC degradation due to added cellulases could be attributed to the structure of regenerated cellulose itself [11, 17]. Digestibility of celluloses by cellulases has been

directly related to amorphous structure of the cellulose and degree of swelling under aqueous conditions [1]. Unlike native cellulose, which consists of parallel β [1, 4] glucan chains that form a crystallite, regenerated cellulose consists of antiparallel β [1, 4] glucan chains [16]. Further study is necessary to determine whether the greater degradation of FC was due to differences in amorphous content, crystalline form, or other factors, such as available surface area. Also, the regenerated celluloses are reported to be more recalcitrant than native celluloses to biodegradation [4, 29].

Xylose was also produced during casing degradation. Cellobiose production was much higher with regenerated cellulose. In long-term assays, glucose production predominates (Fig. 3). Thus RCC degradation clearly suggested the presence of small amounts of xylose polymer with major glucose polymer in the casing matrix [21].

Facile xylan degradation in casing matrix could be the starting point of strength reduction of RCC during fungal sausage fermentation. Xylanase was initially observed during fungal growth leading to a low profile of cellulase production, resulting in structural collapse of the casings on prolonged fungal interactions. Hence, xylan-free cellulose casings were recommended for better performance during fermented sausage production.

Conclusions

Regenerated cellulose is susceptible to degradation due to fungal interactions. The greater RCC degradation is likely due to a combination of factors that may include cellulose form, degree of amorphous content, and surface area. Fungi in fermented sausages produced very low levels of cellulases and xylanases, which upon extended incubation, initially degraded hemicellulosic RCC components, followed by destabilization of the cellulose matrix, thereby resulting in strength loss and RCC failure.

Acknowledgments Viskase Corporation, Chicago, IL, USA, funded this work under a confidentiality agreement that allowed for publication of the findings 3 years after completion of the study. The authors are solely responsible for observations and conclusions reported herein. The authors are thankful to Mr. Brian D. Rosin for his excellent technical assistance to conduct this work. The authors are thankful to Mr. Thomas A. Kuster, FPL, Madison, WI, USA, for his technical work with SEM.

References

1. Akhishima Y, Isogai A, Kuga S, Onabe F, Usada M (1992) Kinetic studies on enzymatic hydrolysis of celluloses for evaluation of amorphous structures. *Carbohydr Polym* 19:11–15
2. Bruna JM, Fernández M, Ordóñez JA, Hoz L (2002) Enhancement of the flavour development of dry fermented sausages by

- using a protease (Pronase E) and a cell-free extract of *Penicillium camemberti*. J Sci Food Agri 82:526–533
3. Cumba HJ, Bellmer D (2005) Production of value-added products from meat processing cellulosic waste. Proceedings of American society of agricultural engineers annual international meeting, Tampa, Florida, July 17–20, Paper number 057032, pp 1–16
 4. Dadi AP, Schall CA, Varanasi S (2007) Mitigation of cellulose recalcitrance to enzymatic hydrolysis pretreatment. Appl Biochem Biotechnol 137:407–421
 5. Dainty R, Blom H (1995) Flavour chemistry of fermented sausages. In: Campbell-Platt G, Cook PE (eds) Fermented meats. Blackie Academic & Professional, London, pp 176–193
 6. Davis MW (1998) A rapid modified method for compositional carbohydrate analysis of lignocellulosics by high pH anion-exchange chromatography with pulsed amperometric detection (HPAEC/PAD). J Wood Chem Technol 18:235–252
 7. Eberhardt A, Galensa R, Henning W (1997) Detection of cellulose in sausages using a coupling of enzyme reactors to HPLC. Deutsche Lebensm Rundschau 93:80–84
 8. Gentry JL, Hussein HS, Berger LL, Fahey GC Jr (1996) Spent cellulose casings as potential feed ingredients for ruminants. J Anim Sci 74:663–671
 9. Gillmann L, Lebeault JM, Cochet N (1994) Influence of the casing on the microflora of compost colonized by *Agaricus bisporus*. Acta Biotechnol 14:275–282
 10. Ilmen M, Saloheimo M, Onnela ML, Penttilä ME (1997) Regulation of cellulase gene expression in the filamentous fungus *Trichoderma reesei*. Appl Environ Microbiol 63:1298–1306
 11. Lacoste-Bourgeacq JF, Fawaz M (2000) Method of removing cellulose casings from sausages. US Patent 6,132,779
 12. Lowry OH, Rosenbrough NJ, Farr AL, Randall RJ (1951) Protein measurements with the Folin phenol reagent. J Biol Chem 193:265–272
 13. Mandels M, Andreotti R, Roche R (1976) Measurement of saccharifying cellulase. Biotechnol Bioeng Symp 6:21–33
 14. Marc B (2003) Process for manufacturing a fermented food product using cell extracts. US Patent 6649199
 15. Miller GL (1959) Use of dinitrosalicylic acid reagent for determination of reducing sugar. Anal Chem 31:426–428
 16. Nicholson MD (1991) Flexible nonwoven composites for food packaging. TAPPI J 74:227–231
 17. Sanders DA, Belyea RL, Taylor TA (2000) Degradation of spent casings with commercial cellulases. Bioresour Technol 71:125–131
 18. Selgas MD, Ros J, Garcia ML (2003) The effect of selected yeast strains on the sensory properties of dry fermented sausages. Eur Food Res Technol 217:475–480
 19. Sreenath HK (1993) Hydrolysis of carboxymethyl celluloses by cellulases. Lebensm Wiss Technol 26:224–228
 20. Sreenath HK, Moldes AB, Koegel RG, Straub RJ (2001) Lactic acid production from agricultural residues. Biotech Lett 23:179–184
 21. Sreenath HK, Koegel RG (2008) Bioconversion of spent cellulose sausage casings. Enzyme Microbial Technol 43:226–232
 22. Sugimoto C (2004) Die Salami in der reifepfung. Coop Zeitung 24:54–57
 23. Sunsen LO, Dorigoni V, Zanardi E, Stahnke L (2001) Volatile compounds released during ripening in Italian dried sausage. Meat Sci 58:93–97
 24. Van Soest PJ (1973) The uniformity and nutritive availability of cellulose. Fed Proc 32:1804–1808
 25. Verhaar LATH, Kuster BFM (1981) Improved column efficiency in chromatographic analysis of sugars on cat-ion exchange resins by use of water-triethylamine eluents. J Chromatogr 210:279–290
 26. Verluyten J, Leroy F, Vuyst L (2004) Effects of different spices used in production of fermented sausages on growth and curvacin A production by *Lactobacillus curvatus* LTH 1174. Appl Environ Microbiol 70:4807–4813
 27. Viikari L, Mustranta A, Ojamo O, Itavaara M, Johansson T (1998) Method of dissolution of sausage skins and other cellulosic substances by means of enzyme solution. US Patent 5,814,515
 28. Wood RD, Hose H, Zurbruggen BD (2000) Production of an aromatizing agent. US patent 6020009
 29. Zhao H, Jones CL, Baker GA, Xia S, Olubajo O, Person VN (2009) Regenerating cellulose from ionic liquids for an accelerated enzymatic hydrolysis. J Biotechnol 139:47–54