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Faculdade de Engenharia de Alimentos

Departamento de Ciência de Alimentos

"ENCAPSULAMENTO DE *BEIJERINCKIA SP* UTILIZANDO SPRAY-DRIER"

PARECER

Este exemplar corresponde à redação final da tese defendida por **Yolanda Eugenia Álamo Gabrine Boza** aprovada pela Comissão Julgadora em 11 de março de 2003.

Campinas, 11 de março de 2003.

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RESUMO

A escolha adequada do material de parede é um dos fatores determin antes no grau de viabilidade e na manutenção da atividade fermentativa do inóculo encapsulado. Maltodextrina, glicose, amido modificado e goma arábica foram testadas como encapsulantes. *Beijerinckia* encapsulada em maltodextrina, reidratada e inoculada em m eio de fermentação previamente esterilizado, após um período de estocagem de dois meses, apresentou maior estabilidade da atividade fermentativa. A sobrevivencia e a atividade fermentativa das células de *Beijerinckia* encapsulada em maltodextrina foram tes tadas sob várias condições do spray-drier e durante posterior período de estocagem. A viabilidade bacteriana para todas condições testadas apresentou declinio pórem quando se utilizou menores temperatura de saída no spray –drier e teor de sólidos na suspen são obteve-se maior grau de sobrevivência. Para verificar a influência da encapsulação em maltodextrina sobre a produção de biopolímero comparou -se a atividade fermentativa das células de *Beijerinckia* encapsulada e não encapsulada. A partir das caracterist icas reológicas e químicas dos biopolímeros produzidos e da cinética fermentativa dos inóculos concluiu -se que encapsulaçao de *Beijerinckia* influe no crescimento celular e na produção de biopolímero.

SUMMARY

An adequate choice of the wall (coating) m aterial is one of the factors, which will determine the degree of cell survival and the maintenance of fermentative activity in the encapsulated inoculum. Malt dextrin, dehydrated glucose syrups, modified starch and acacia (gum Arabic) were used as wall ma terials. The results showed that spray dried *Beijerinckia* encapsulated in malt dextrin, stored for two months and inoculated into sterile must after re -hydration, presented the greatest stability related to fermentative activity and percent viability durin g spray drying and storage period. The survival and fermentative activity of *Beijerinckia sp* cultures encapsulated in malt dextrin by spray drying were determined under various conditions of spray drying and during the subsequent storage period of the products. The survival of *Beijerinckia sp* was greater with lower outlet air temperatures and with lower solids contents in the feed suspension during spray drying. Increasing either the spray dryer outlet air temperature or the feed solids content resulted in a decrease in the moisture content of the spray dried powders. A gradual decrease in viability was observed during storage for all treatments, although there was a relatively greater retention of fermenting activity in the inoculae obtained with lower ou tlet air temperatures and lower solids contents in the feed suspension. Was to examine the effects of encapsulation in malt dextrin on the production of biopolymer, comparing encapsulated and non -encapsulated *Beijerinckia* cells. From the characterization o f the biopolymers and study of the

fermentation kinetics, it was shown that the encapsulation of *Beijerinckia* mainly influenced cell growth and biopolymer production.

1. INTRODUÇÃO

As bactérias do gênero *Beijerinckia* têm sido objeto de constante estudo em todo o mundo. Estas bactérias são fixadoras de nitrogênio no solo, produtoras de polímeros cujas características físico-químicas são adequadas ao uso em associação com a lama de perfuração no fraturamento hidráulico da rocha na extração do petróleo e ainda produzem o polihidroxibutirato, matéria-prima na obtenção de plásticos biodegradáveis. Estudos recentes relativos a *Beijerinckia sp* concentram-se no uso destas bactérias como biofertilizante e ainda no tratamento de resíduos, através da oxidação de compostos aromáticos e de organo-sulfurados.

As fermentações industriais para obtenção de biopolímeros freqüentemente apresentam baixa reprodutibilidade, a qual em geral está associada a contaminações e alterações genéticas da cultura bacteriana, decorrentes de técnicas inadequadas de manutenção, de propagação e de produção dos inóculos. Os problemas associados com a reprodutibilidade podem alterar a produtividade, o rendimento e a qualidade do biopolímero. A qualidade do biopolímero é um fator particularmente importante, que é medida prontamente por suas propriedades reológicas uma vez que a aplicação de goma depende principalmente deste parâmetro (Amanullah et al., 1996).

Há uma grande variedade de técnicas disponíveis para preservação dos microrganismos e preparo de inóculos bacterianos, também chamados "starters" para a indústria de alimentos. As principais características que devem ser consideradas na escolha do método de preservação são: a manutenção da viabilidade celular durante o armazenamento; a estabilidade das características genéticas importantes das culturas; o controle eficiente de pureza, o custo com os materiais e equipamentos, o volume das culturas a ser preservadas, o fornecimento e o transporte das culturas (Baiocco, 1997).

Segundo Luria & Delbruck (1973) durante o crescimento bacteriano ocorrem mutações espontâneas e aleatórias, assim nos procedimentos de manutenção que dependem de propagação contínua a probabilidade de ocorrência de alterações nas características fisiológicas das culturas é diretamente proporcional ao número de gerações envolvidas. Este fato tem conduzido a investigação de técnicas de manutenção de cultura não propagativas como a liofilização e a secagem em spray-drier.

Os métodos de preservação de culturas, por liofilização e por secagem em spray-drier, mantém a cultura por longo período em estado de anabiose, este importante para manutenção das características da cultura. Comparando os dois métodos de preservação, a liofilização é um processo mais caro, pois a sublimação consome mais energia do que a evaporação e os custos com a implantação da planta são maiores, além disto o vácuo requerido na liofilização é difícil de se obter e manter em processo contínuo (Lievense & Riet, 1993).

Para uso industrial, onde grandes quantidades de bactérias ativas são requeridas, o processo de manutenção por uso do spray-drier é o mais econômico, sendo disponível no mercado vários tipos de equipamentos. A técnica propícia à obtenção de boa densidade de células com atividade metabólica substancialmente intacta a qual se mantém durante a distribuição e a estocagem. Os processos comerciais de desidratação de culturas usando spray-drier são usados na produção de leveduras secas para panificação e de culturas lácticas para preparação de iogurte caseiro, e na produção de queijo cheddar. Devido o produto estar desidratado, ocupa menor volume reduzindo os custos com transporte e com estocagem (Johnson & Etzel, 1993).

A liofilização quanto a desidratação em spray-drier podem ser responsáveis por considerável inativação dos microrganismos e ainda o alto custo do processo de liofilização tem levado à busca contínua de outros métodos de desidratação de culturas microbianas, sendo que o encapsulamento dos microrganismos por spray-drier surge como alternativa promissora. O processo de secagem em spray-drier embora freqüentemente seja considerado um processo de desidratação é também uma técnica de encapsulamento, quando o "material ativo" a ser desidratado está envolvido por uma matriz protetora (Shahidi & Han, 1993). Neste processo as células bacterianas são desidratadas em suspensão na presença de substâncias protetoras (ex: amido, goma, maltodextrina) (Lievense & Riet, 1993).

Este trabalho teve como objetivo avaliar a influência das condições de encapsulamento utilizando spray-drier sobre a atividade e viabilidade da *Beijerinckia sp* e estabelecer condições de processamento na produção de inóculos para uso na produção de biopolímero.

2.CAPÍTULO I - REVISÃO BIBLIOGRÁFICA

Na indústria de alimentos os biopolímeros encontram um vasto campo de aplicação como agentes espessantes, estabilizantes e gelificantes e vêm substituindo gradativamente os polissacarídeos obtidos de fontes convencionais como plantas. A preferência por biopolímeros microbianos se deve às suas propriedades funcionais, somadas às vantagens de sua produção ser independente de condições climáticas.

Apesar da potencialidade do uso de biopolímeros na indústria de alimentos como ferramenta poderosa para se criar novas texturas propiciando a possibilidade de desenvolvimento de novos e diferenciados produtos, os mercados de consumo mais importantes para polissacarídeos são as indústrias de petróleo, de mineração, têxtil, cosmética, farmacêutica e de produtos agropecuários.

A seleção de microrganismos que produzam polissacarídeos com propriedades funcionais economicamente interessantes bem como os estudos para otimizar o rendimento e a produtividade dos processos fermentativos na obtenção dos polissacarídeos é um desafio constante.

2.1 Características da *Beijerinckia* sp e de seu biopolímero

Atualmente apenas quatros espécies do gênero *Beijerinckia* foram identificadas: *Beijerinckia indica, Beijerinckia mobilis, Beijerinckia derxii e Beijerinckia fluminensis* (Embrapa, 2001). A presença de *Beijerinckia* no solo do Brasil dá-se nos seguintes estados: Pernambuco, Pará, Rio Grande do Norte, Alagoas, Bahia, Minas Gerais, Paraná, Rio de Janeiro e São Paulo. Hidrofílica, a *Beijerinckia* prefere solos com alto teor de umidade, até mesmo os encharcados, sendo que não é encontrada nos períodos de seca prolongada (Dobereiner, 1959).

As bactérias desta espécie são aeróbias, no entanto desenvolvem-se e fixam nitrogênio em meios com baixo teor de oxigênio, Gram negativas, uni ou bicelulares (Borzani, 1986). A faixa de temperatura de crescimento de *Beijerinckia sp* é de 10 a 35°C e possuem tolerância a baixos valores de pH (4,5 a 5,2), porém já foram isoladas em pH neutro (Florenzano et al., 1968).

A vegetação apresenta grande influência sobre a *Beijerinckia sp* que se desenvolve bem na rizosfera de certas plantas forrageiras, notadamente na rizosfera da cana-de-açúcar. A associação *Beijerinckia*-cana-de-açúcar é altamente benéfica, pois as taxas de fixação do nitrogênio apresentam-se muito superiores às normalmente encontradas (Borzani, 1986).

Biopolímeros são macromoléculas polissacarídicas que atuam em baixas concentrações e em ampla faixa de pH e de temperatura, sendo amplamente utilizados em diversos ramos da indústria como agente espessante, estabilizante e geleificante, devido à diversificação de seu consumo torna-se necessária à produção em maior quantidade e qualidade.

Jones et al. (1963) realizaram os primeiros estudos de fermentação para obtenção de polissacarídeos produzidos por *Beijerinckia* utilizando inóculo de *Beijerinckia indica* a 25°C em erlenmeyer de um litro contendo 300 mL de meio constituído por (% p/v): glicose, 5,0; peptona, 0,1; K_2HPO_4, 0,05; $MgSO_4.7H_2O$, 0,05; NaCl, 0,02; $CaSO_4$, 0,01; $FeCl_3.6H_2O$ traços e $CaCO_3$, 0,02. A recuperação do biopolímero foi através de precipitação com acetona após 38 dias de fermentação. O rendimento em relação à fonte de carbono foi de 2% após seis dias e 18% ao final da fermentação. Ensaios de caracterização do polímero foram realizados e verificou-se que o polímero produzido era composto por duas frações, sendo a acídica a maior

fração. A fração acídica possuía estrutura linear composta por unidades de ácido D-glucurônico, D-glicose e D-glicero-manoheptose.

O crescimento de bactérias do gênero *Beijerinckia* é exponencial quando utilizado carboidrato como fonte de carbono.Vendruscolo (1995) produziu células da cepa 7070 de *Beijerinckia sp* utilizando como fontes de carbono sacarose e glicose em concentrações de 0,1 a 5,0% (p/v), obtendo o melhor resultado com 1% de sacarose. A fase logarítmica de crescimento celular variou de 15 a 18 horas. O meio mais eficiente para produção de biopolímero era composto por (g/l) : K_2HPO_4, 2,0; KH_2PO_4, 13,6; $MgSO_4.7H_2O$, 2,7; extrato de levedura, 0,2; triptose, 2,0; sacarose, 50,0.As melhores condições de fermentação foram pH inicial de 6,5, temperatura entre 20-24°C, 200 rpm por 96 horas e volume de 200 a 300 mL em frasco de um litro. A composição química do polissacarídeo produzido por *Beijerinckia sp* foi realizada, ápos hidrólise total do biopolímero com ácido clorídrico 1N sob refluxo a 70°C por 16 horas, neutralizando posteriormente com bicarbonato de sódio, por cromatografia em papel e por cromatografia de camada delgada. A composição química encontrada foi glicose, galactose, manose, ácido galacturônico e ácido glicurônico.

Através de hidrólise, derivação química e de métodos cromatográficos e espectrométricos foi determinada a composição química do biopolímero produzido por *Beijerinckia sp* produzido em meio de fermentação contendo: sacarose , 5%; $MgSO_4$ 0,05%; K_2HPO_4 , 0,01%; KH_2PO_4 0,05% e triptose , 0,5%, a 200 rpm por 72 horas a 25 °C , sendo encontrada a seguinte composição: glicose, galactose e fucose nas proporções de 3:1: 3, respectivamente (Mariuzzo, 1996).

Uma das mais importantes indicações de que um biopolímero apresenta propriedades funcionais com viabilidade de aplicação comercial é o resultado da medida da viscosidade de suas soluções aquosas (Szczesniack, 1985). O polímero produzido pela *Beijerinckia sp* possui características pseudoplásticas e apresenta viscosidade maior do que a da goma xantana. Em meios com pH inferior a três ou superior a doze o polímero degrada-se. Essas propriedades permitem seu uso no fraturamento hidráulico da rocha na extração de petróleo, pois após o seu uso na fratura da rocha o polímero dever ser recuperado através de bombeamento da goma liquefeita.Assim tem-se neste polímero um substituto viável da goma xantana (Vendruscolo, 1995).

Estudos recentes concentram-se no uso da *Beijerinckia* em tratamentos de resíduos, para a oxidação de compostos aromáticos. A capacidade de utilizar compostos orgânicos aromáticos e sulfurosos como fonte de carbono, sob condições de anaerobiose, permitem que estas bactérias sejam usadas em tratamentos de resíduos (Blinkovsky & Dordick, 1994).Ainda o interesse pelo estudo das bactérias do gênero *Beijerinckia* deve se ao fato de ser produtora de polihidroxibutirato, matéria-prima para produção de plásticos biodegradáveis.

2.2 Encapsulamento de microrganismos

A microencapsulação é um processo de formação de partículas onde um ingrediente ativo é recoberto por uma fina camada de outro material. O material de parede ou encapsulante deve formar um filme contínuo para proteger o ingrediente. A microencapsulação é usada para conferir as seguintes características a um ingrediente: 1) liberação controlada: o encapsulante libera o núcleo sobre condições específicas tais como: mudança de pH, aplicação de calor ou mastigação; 2) proteção

contra luz, calor, água e oxidação; e 3) facilidade no manuseio e estocagem (Shahidi &Han, 1993).

Rogers (1914) realizou o primeiro estudo de obtenção de culturas bacterianas láticas por spray-drying. Posteriormente Mamaeva (1956) utilizando spray–drier obteve uma cultura desidratada constituída por *lactobacillus* e leveduras, usada na manufatura de koumiss, e suficientemente ativa para uso por um período de seis meses.

O uso de encapsulamento de microrganismos consiste numa tecnologia simples e aplicável a uma série de microrganismos em vários processos biotecnológicos. A área ambiental é uma das mais promissoras para o uso desta tecnologia, na biodegradação de contaminantes da água, do solo e ainda na fixação de nitrogênio molecular (Cassidy et al., 1996).Várias formulações de inóculos especialmente do gênero *Rhizobium* encapsulado em polímeros e desidratados em spray-drier têm sido estudados. Os polímeros têm como função proteger a cultura do estresse ambiental liberando gradualmente os microrganismos no solo à medida que se degrada (Mary et al., 1993).

A microencapsulação tem melhorado a estabilidade de culturas "starters", especialmente de bactérias lácticas, para indústria de alimentos. Jackson & Lee (1991) verificaram que as culturas de bactérias lácticas encapsuladas em alginato de sódio ou carboximetilcelulose e armazenadas a vinte e dois graus Celsius tiveram a estabilidade sensivelmente aumentada. Outros exemplos de encapsulamento de microrganismos especialmente para uso em alimentos são: a co-encapsulação de metionina e *Brevibacterium linens* usados como acentuadores do flavor do queijo cheddar e ainda o uso de microrganismos microencapsulados para reduzir o tempo de maturação do queijo azul (Shahidi & Han, 1993).

2.3 Produção das microcápsulas

A microencapsulação é a tecnologia de envolver sólidos, líquidos e gases em cápsulas microscópicas, tais estruturas possuem tamanhos de micrômetros e podem ter inúmeras formas (Bakan , 1978). O encapsulamento de produtos alimentícios, farmacêuticos e cosméticos dentre outros, tem sido realizado por diversas técnicas. Segundo Nori (1996) as técnicas para produção de microencapsulados são divididas em três grupos: métodos físicos, químicos e físico-químicos.

A seleção do método de encapsulação depende do custo, da sensibilidade do encapsulado, do tamanho de microcápsula desejado, das características físicas e químicas do ingrediente ativo e do material de parede, da aplicação das microcápsulas e do mecanismo de liberação do material ativo (Jackson & Lee, 1991).

A microencapsulação por spray-drying consiste em processo físico, no qual o contato do ingrediente ativo com o material de parede é realizado por meio mecânico. O processo envolve as seguintes etapas: a) preparação da emulsão ou dispersão do ingrediente ativo onde é adicionado o material de parede; b) homogeneização da dispersão; c) atomização da dispersão dentro de uma câmara onde circula ar quente e seco, onde ocorre a secagem do líquido pulverizado e d) separação do produto seco com o ar quente (Jackson & Lee, 1991).

A escolha do material de parede é o passo inicial na encapsulação, sendo que a composição depende primeiramente do produto a ser encapsulado e das características desejadas do produto final. Eles são basicamente materiais formadores de filmes e podem ser selecionados entre um grande número de polímeros sintéticos ou naturais (Bakan, 1978). Os materiais de parede mais usados

são gomas vegetais, gelatina, amido modificado, dextrinas, lipídios e emulsificantes, podendo ser usados combinados ou apenas um componente na constituição da estrutura externa das microcápsulas (Shahidi & Han, 1993).

A composição do material de parede é determinante nas propriedades funcionais das microcápsulas. Um bom material de cobertura deve apresentar algumas características tais como: a) baixa viscosidade; b) facilidade de manipulação durante o processo de encapsulação; c) habilidade em dispersar ou emulsificar o ingrediente ativo; d) estabilizar a emulsão produzida; e) não ser reativo com o material a ser encapsulado, pois poderia causar danos durante o processamento e na estocagem; f) reter o material ativo dentro da estrutura durante o processamento e estocagem; g) oferecer máxima proteção ao produto microencapsulado quanto à umidade, luz, calor e oxigênio; h) ser solúvel em solventes permitidos para sua finalidade e i) ser econômico (Shahidi & Han, 1993).

Existem vários mecanismos de liberação dos ingredientes encapsulados, sendo que as propriedades físicas e químicas do encapsulado (ex: solubilidade, pressão de vapor, coeficiente de partição) e do material de parede (ex: espessura, porosidade) influem na forma de liberação (Jackson & Lee, 1991). Os mecanismos de liberação que se destacam são: os processos de fratura, de dissolução e de biodegradação.

A Tabela 1 mostra alguns sistemas de parede encapsulados solúveis em água e seus prováveis mecanismos de liberação.

Tabela 1. Mecanismo de liberação para sistemas de parede solúveis em água.

Sistema de parede	Mecanismo de liberação			
	Mecânico	Térmico	Dissolução	Químico
Alginato	*		*	
Carragena	*		*	
Caseinato	*		*	
Derivados de celulose	*		*	
Quitosanas	*			
Gelatina	*		*	
Goma Gelan	*	*		
Goma arábica	*	*		
Látex	*		*	
Polietilenoglicol	*	*	*	
Óxido de polietileno	*	*	*	
Polipectato	*		*	
Álcool polivinílico	*		*	
Polivinilpirrolidina	*		*	
Amidos	*		*	
Derivados de açúcares	*	*	*	*

Fonte: Southwest Research Institute, A Capability Statement for microencapsulation, 1995.

2.4 Danos provocados pelo encapsulamento de bactérias por spray-drying

Segundo Cassidy et. al. (1996) o encapsulamento em spray-drier reduz o número de células viáveis ao redor de 1,0% em relação ao número de células viáveis inicial, contudo o percentual de viabilidade residual depende da linhagem, da composição do material de parede, do método e das condições de secagem.

Lian et. al. (2002) verificaram que o grau de sobrevivência de *Bifidobacteria* encapsulada varia com a linhagem e com o composição do material de parede.A inativação das *Bifidobacteria* encapsuladas em amido solúvel foi maior em relação ao grau de inativação observado nas *Bifidobacteria* encapsuladas em leite desnatado.

Teixeira et al. (1995) compararam a influência da liofilização e do spray-drying sobre culturas lácticas quanto à sobrevivência e extensão da fase lag e verificaram não ter diferenças significativas. Foster (1962) verificou que as curvas de crescimento das culturas bacterianas lácticas secas e reidratadas são praticamente idênticas as das culturas frescas, porém ocorre acentuadas diferenças na extensão da fase lag no ciclo vital, sendo que estas diferenças era específica para cada cultura, contudo quando reativadas produziram ácido láctico com igual produtividade e rendimento.E ainda observou que as diferenças metabólicas que apareceram após secagem se mantiveram durante a estocagem.

Segundo Teixeira et al. (1997) culturas "starters" de bactérias lácticas desidratadas por spray-drier não podem ser usadas diretamente para inocular o leite devido a extensão da fase lag das células injuriadas presentes. De acordo com Cassidy et al. (1996) a extensão da fase lag e a velocidade de crescimento podem ser afetadas pelas condições de encapsulamento.

Os efeitos biológicos do calor têm sido pesquisados sob vários aspectos, contudo os mecanismos de morte térmica e de injúria das células microbianas, ainda não foram esclarecidos. Uma suspensão de células bacterianas submetidas a estresse térmico pode conter células mortas apresentando injúria ou inativação irreversível, células danificadas ou injuriadas e células normais (Teixeira et al., 1997).

A injúria microbiana é expressa pela inabilidade destas células em produzir crescimento detectável sob condições normalmente apropriadas para sua proliferação. Os microrganismos injuriados se distinguem dos mortos ou dos que sofreram mutações, por sua habilidade em readquirir atividade fisiológica normal quando se encontram em condições favoráveis. A restauração das funções fisiológicas normais é denominada reparo ou recuperação. A extensão e severidade

da injúria subletal, os mecanismos de dano e os mecanismos de recuperação variam com: os processos térmicos, as espécies e linhagens microbianas, as condições fisiológicas do microrganismo e os métodos de recuperação celular (Lievense & Riet, 1994).

Sabe-se que fatores intrínsecos e extrínsecos associados com a bactéria e ao ambiente no qual esta será submetida ao tratamento térmico afetam sua resposta ao calor. O aquecimento subletal causa danos aos componentes celulares a nível molecular. Os danos celulares freqüentemente relatados são: aumento da hidrofobicidade da superfície celular, perda de componentes da membrana citoplasmática, inativação de enzimas celulares, degradação do rRNA e rompimento das fitas de DNA levando a mutações (Czechowicz et al., 1996).

Os danos celulares promovem uma perda substancial na viabilidade celular e alterações metábolicas, sendo que, quando o metabolismo é alterado as bactérias injuriadas pelo calor podem expressar diferentes requerimentos fisiológicos e nutricionais.

Em geral, são considerados quatro sítios principais nas células microbianas susceptíveis à injúria térmica: a) DNA; b) RNA; 3) proteínas (enzimas) e 4) membrana celular (Lievense & Riet, 1994). O dano térmico é principalmente determinado pela combinação tempo - temperatura, conteúdo e distribuição de água nas cápsulas e resistência térmica dos constituintes termolábeis.

De acordo com Paul et al. (1993) a redução de água altera profundamente as características das células por modificações físicas e estruturais assim como por degradação de moléculas através de reações químicas e enzimáticas, pois as velocidades destas reações são influenciadas pela atividade de água. Scott (1958) sugeriu reações entre agrupamentos amino ($-NH_2$) e compostos carbonilos durante a

secagem e a estocagem das células, como uma das causas de injúria protéica, enquanto Lievense & Riet (1994) afirmaram que a desidratação das células remove a água de hidratação das proteínas hidrossolúveis destabilizando sua conformação desnaturando-as.

Anteriormente Rogers (1914) atribuiu o aumento da pressão osmótica como provável mecanismo de inativação das bactérias lácticas durante a secagem, pois poderia levar a plasmólise das células, uma vez que a perda de água aumenta a concentração de solutos intracelulares e alguns compostos solúveis podem precipitar. O aumento da concentração de sais poderia causar danos nas membranas celulares e desnaturação de proteínas.

A membrana citoplasmática é em geral considerada como o principal sítio de injúria térmica, sendo que o dano à sua estrutura pode ser extensivo aos componentes intracelulares. De acordo com Crowe et al. (1990) as características das membranas dependem da organização do seu respectivo sistema fosfolipídios-água, o qual pode apresentar-se sob vários graus de organização estrutural (polimorfismo) quando hidratados. As estruturas mais freqüentemente observadas são: a bicamada lamelar, a fase líquida cristalina e a fase gel. O grau de organização estrutural do sistema água – fosfolipídio depende principalmente de sua composição, da temperatura e do pH do meio. Durante a desidratação o sistema água-fosfolipídio da membrana passa por uma fase intermediária ou de transição, a qual consiste na passagem da fase líquida cristalina para fase gel, período no qual podem ocorrer danos à membrana por dois mecanismos conforme abaixo descrito:

a) através da entrada dos fosfolipídios da bicamada na fase de transição a diferentes intervalos de tempo, o que pode danificar a bicamada, fato observado quando não há uniformidade do aquecimento, e;

b) pela separação de lipídios durante a secagem, pois as membranas biológicas são constituídas por uma mistura de fosfolipídios e cada fosfolipídio entra na fase de gel à determinada temperatura e num estado de hidratação. Este último mecanismo é considerado o mais importante na danificação das membranas durante a desidratação.

Vários estudos demonstram que ocorre um rompimento das fitas do DNA e/ou RNA durante a desidratação de microrganismos (Lievense & Riet 1994). A relação entre os danos sofridos pelo DNA e sua influência na viabilidade dos microrganismos injuriados subletalmente tem sido estudada, embora haja evidências de que o DNA seja realmente danificado e reparado após os microrganismos terem sido submetidos a processos térmicos, de congelamento e de desidratação, é ainda prematuro apresentar o DNA como a principal estrutura responsável pelo comportamento das culturas danificadas. Danos na molécula de DNA resultam em geral mutações estáveis ou inativações irreversíveis (Pierson et al., 1978; Baiocco, 1997). Segundo Lievense & Riet (1994) a condensação da cromatina durante os estágios iniciais da secagem da levedura consiste num mecanismo de proteção típico das culturas com maior viabilidade após secagem.

As reações de oxidação dos componentes celulares são também mencionadas como um dos possíveis mecanismos de inativação. Segundo Czechowicz et al. (1996) um dos primeiros eventos que ocorre durante a inativação térmica de microrganismos é a inativação de enzimas cuja função é proteger as células da ação destrutiva do oxigênio e da luz. As enzimas catalase e superóxido dismutase permitem que as células reajam com radicais, tais como: os íons superóxidos e o peróxido de hidrogênio, os quais formam-se espontaneamente na presença de oxigênio. Caso esses radicais permaneçam a morte celular pode ocorrer

devido à oxidação de lipídios e por danos na membrana celular (Kellog & Fridovich, 1975). Assim, um período de recuperação estritamente anaeróbio pode eliminar o estresse oxidativo pela ausência do oxigênio, precursor necessário para a formação destes radicais tóxicos (Knabel et al., 1990).

2.5 Aspectos dos parâmetros de secagem em spray-drier

A inativação das células bacterianas durante a secagem em spray –drier pode ser causada por dois mecanismos a inativação térmica e a desidratação térmica. Segundo Lievense & Riet (1993) durante o primeiro estágio do processo de secagem a superfície das partículas permanece úmida devido a evaporação da água e a temperatura não excede a temperatura de bulbo úmido. Assim, a inativação térmica é limitada nesta fase, pois a alta velocidade de evaporação e a temperatura resultante de bulbo úmido protegem as células durante a secagem. Na fase seguinte a superfície das partículas está seca e a temperatura aumenta paralelamente à temperatura de entrada do spray-drier, nesta fase a inativação térmica aumenta, porém devido a menor concentração de água a resistência térmica das células também aumenta.

Durante a secagem a altas temperaturas a inativação celular ocorre predominantemente por inativação térmica, sendo que a resistência térmica das células bacterianas aumenta com a redução do teor de água. No spray-drying altas temperaturas de entrada são necessárias, pois o calor de evaporação específico deve ser fornecido durante curto período de tempo para minimizar significativamente a inativação térmica das células (Lievense & Riet, 1994).

A sobrevivência das células bacterianas durante o processo de secagem no spray-drier está altamente correlacionada com a temperatura de saída

devido ao resfriamento evaporativo na primeira fase do processo de secagem. Portanto maior sobrevivência é obtida a menores temperaturas de saída. Baixas temperaturas de saída resultarão em menor inativação térmica e possivelmente também resultará em maior teor de água residual, o qual pode influenciar na sobrevivência da cultura.

Estudos da produção de iogurte em pó utilizando spray-drier demonstraram que o uso de menor temperatura de saída (60-65°C) durante o processo de secagem resulta em maior sobrevivência das culturas de *Lactobacillus delbrueckii* e de *Streptococcus thermofilius* assim como maior teor de umidade no produto, porém o iogurte apresentou textura desagradável. Iogurte com boas propriedades sensoriais e com satisfatória viabilidade das culturas foi obtido usando maior temperatura de saída (70-75°C) durante a secagem no spray-drier (Bielecka & Majkowska, 2000).

A secagem em spray-drier é uma técnica altamente promissora na produção de algumas culturas probióticas. Trindade (2001) obteve maior sobrevivência das culturas de *Lactobacillus acidophylus* e de *Bifidobacterium lactis* encapsuladas em acetato ftalato de celulose, usando menores temperaturas de entrada e de saída (75 °C e 130 °C respectivamente) durante a secagem no spray-drier.

O tamanho da partícula (cápsulas) depende de três propriedades físicas da suspensão celular: viscosidade, densidade e tensão superficial. Segundo Lievense & Riet (1994) quanto maior a concentração de sólidos na suspensão maior serão as partículas e concomitantemente maiores os tempos de secagem e de contato das partículas com o ar quente.

A pressão de atomização influe no tamanho das partículas, sendo que há um decréscimo da sobrevivência bacteriana em função da maior desidratação e inativação térmica devido o aumento do tamanho de partícula resultante da redução da pressão de atomização.

De acordo com Lievense & Riet (1994) outros fatores além das condições operacionais do spray-drier influem no grau de inativação das culturas bacterianas tais como: a) a espécie bacteriana; b) as condições de crescimento; c) a presença de aditivos protetores; d) a concentração celular e o pH; e) o gás, a velocidade e o grau de secagem; f) a reidratação; e g) técnica usada para medir o grau de inativação das culturas.

As espécies bacterianas diferem quanto à sensibilidade ao calor sendo as Gram positivas mais resistentes à secagem. Uma bactéria é danificada quando a diferença de pressão osmótica intracelular e extracelular é suficientemente alta ou baixa para retardar a taxa de crescimento ou afetar sua capacidade de reprodução. As bactérias Gram negativas possuem uma concentração de solutos intracelulares comparativamente menor e são, portanto, mais susceptíveis à plasmólise do que outros tipos de microrganismos. A capacidade das células em reter a viabilidade, quando expostas a um ambiente com maior ou menor concentração de solutos, depende da integridade da membrana celular, sendo que durante o choque osmótico podem ocorrer perdas de proteínas associadas às membranas, tais como as proteases e conseqüentemente diminuir a viabilidade das células (Beuchat, 1978).

A fase de crescimento da cultura bacteriana é importante para sua sobrevivência. Em geral as células na fase estacionária são mais resistentes à desidratação, contudo o ponto da fase estacionária em que células são mais resistentes não está bem estabelecido. Segundo Krallish et al. (1989) o grau de

sobrevivência das leveduras durante a desidratação pode ser influenciado pelos níveis de energia das células, as células na fase estacionária apresentam maior nível de ATP e esta energia acumulada pode auxiliar no processo de reidratação celular.

A resistência celular à desidratação é também influenciada pelo meio de crescimento. Assim alterações das condições de cultura para aumentar os teores de alguns ácidos graxos nas células bacterianas podem conferir à cultura maior resistência à desidratação (Gilliland, 1977).

As presenças de aditivos protetores nas suspensões celulares têm sido intensivamente estudadas, pois o grau de comprometimento das estruturas celulares depende diretamente da composição do meio. Os efeitos positivos sobre a viabilidade celular dos inóculos desidratados têm sido atribuídos as seguintes substâncias: açúcares (ex: sacarose, trealose, lactose), glicerol, sorbitol, ácidos carboxílicos, meios de cultura (ex: peptona, extrato de levedura), dextrana, aminoácidos, proteínas (ex: albumina) e leite desnatado reconstituído. (Lievense & Riet, 1994). O mecanismo de ação destes agentes protetores ainda não está bem esclarecido, mas sabe-se que a presença de sacarose e leite desnatado reconstituído altera as características de permeabilidade da membrana celular, permitindo o fluxo de solutos do meio intracelular para o meio extracelular durante a desidratação e o fluxo inverso durante a reidratação das suspensões (Baiocco, 1997).

Segundo Beauchat (1978) os peptídeos e as peptonas podem estabilizar as ligações hidrofóbicas e corrigir algumas alterações estruturais das proteínas e dos lipídios presentes nas membranas citoplasmáticas. Produtos complexos como leite desnatado reconstituído, carboidratos, glicerol, dextrana e albumina, por exemplo, são alguns dos agentes que apresentam a capacidade de proteger as bactérias Gram negativas contra as injúrias provocadas pela liofilização, reduzindo seus efeitos letais.

Os carboidratos, como a sacarose e a lactose, inibem a produção de radicais livres, os quais são freqüentemente associados à perda de viabilidade das bactérias desidratadas. Estes mesmos compostos podem formar pontes de hidrogênio com proteínas, substituindo a água removida e assim reduzindo o número de grupos polares e apolares das moléculas protéicas expostos ao meio extracelular (Champagne et al., 1991). Devido à ação estabilizante destes carboidratos através da manutenção da atividade biológica das macromoléculas celulares, eles são recomendados como agentes protetores por aumentar a taxa de recuperação das células desidratadas.

A porcentagem dos microrganismos sobreviventes aumenta com o aumento da concentração celular na suspensão a ser desidratada. Uma possível explicação para este fato é que a liberação de componentes intracelulares das células danificadas teria efeito protetor sobre as demais células, provavelmente atuando como antioxidantes ou ainda reduzindo a área interfacial entre as células vivas e o meio externo (Bozoglu et al., 1987).

A composição do gás de secagem pode influenciar no nível de sobrevivência da cultura desidratada. O nitrogênio é o gás mais indicado, pois reduz a oxidação dos componentes celulares, porém devido ao custo do uso do nitrogênio em escala industrial alguns autores recomendam o uso de antioxidantes (Lievense & Riet, 1994).

Poucos autores estudaram a influência da velocidade de secagem sobre a taxa de sobrevivência bacteriana após secagem em spray-drier. Kuts & Tutova (1983) relataram que a velocidade de secagem é um dos mais importantes fatores que influenciam na taxa de sobrevivência, porém outros autores não verificaram influência significativa da velocidade de secagem sobre a taxa de sobrevivência.

REFERÊNCIAS BIBLIOGRÁFICAS

AMANULLAH, A; SERRANO; L.C. GALINDO, E.; NIENOW, A.W. Reproducibity of pilot scale xanthan fermentations. **Biotechnology Progress**, v.12, p.466-473, 1996.

BAIOCCO, L.M. **Estudo de parâmetros para a produção de inóculos liofilizados de *Xanthomonas campestris pv. manihotis***. Campinas, 1997. 138p. Dissertação (Mestrado)- Faculdade de Engenharia de Alimentos, Universidade de Campinas.

BAKAN, J.A. Microencapsulation. In: Peterson, M.S.; Johnson, R. (eds.). **Encyclopedia of Food Science**, Westport, AVI, 1978. p.499.

BEAUCHAT, L.R. Injury and repair of Gram-negative bacteria, with special consideration of the involvement of the cytoplasmatic membrane. **Advances in Applied Microbiology**, v.23, p.219-243, 1978.

BLINKOVSKY, A. M.; DORDICK, J. S. – Enzymatic conversion of coal in no aqueous media. **Abstract Paper of American Chemical Society**, p.207 – 211, 1994.

BIELECKA, M.; MAJKOWSKA, A. Effect of spray drying temperature of yoghurt on the survival of starter cultures, moisture content and sensoric properties of yoghurt powder. Nahrung, v.44, p.257-260, 2000. In: **Food Science and Technology Abstracts**, v.11, p.1821, 2000.

BORZANI, W. Cinética do processo fermentativo. In: BORZANI, W.; LIMA, U. A.; AQUARONE, E. ED. **Engenharia Química**. São Paulo: Edgar Blucher, v. 3, p. 165 - 184, 1986.

BOZOGLU, T.F.; OZILGEN, M.; BAKIR, U. Survival kinetics of lactic acid starter cultures during and after freeze-drying. **Enzyme Microbiology and Technology**, v.19, n.9, p.531-537, 1987.

CASSIDY, M.B.; LEE, H.; TREVORS, J.T. Environmental applications of immobilized microbial cells: a review. **Journal of Industrial Microbiology**, v.16, p.79-101, 1996.

CHAMPAGNE, C.P.; GARDNER, N.; BROCHU, E.; BEAULIEU, Y. The freeze-drying of lactic acid bacteria. A review **Canadian Institute of Food Science and Technology Journal**, v.24, n.3/4, p.118-128, 1991.

CROWE, J.H.; CARPENTER, J.F.; CROWE, L.M.; ANCHORDOGUY, T.J. Are freezing and dehydration similar stress vectors? A comparison of modes of interaction of stabilizing solutes with biomolecules. **Cryobiology**, v.27, p.219-231, 1990.

CZECHOWICZ, S.M.; SANTOS, O.; ZOTTOLA, E.A. Recovery of thermally-stressed *Escherichia coli* 0157:H7 by media supplemented with pyruvate. **International Journal of Food Microbiology**, v.33, n.2/3, p.275-284, 1996.

DOBEREINER, J.Sobre a ocorrência de *Beijerinckia* em alguns estados do Brasil. **Revista Brasileira de Biologia**,v.19, n.2, p. 151-160, 1959.

EMBRAPA/CNPAB.**Biodiversidade:Perspectivas e Oportunidades Tecnológicas**.BrasíliaDisponível:http://www.bdt.org publicações/padct/bio [capturado em 13 dez.2001].

FLORENZANO, G; BALLONI, W.; MATERASSI, R. Nitrogen-fixing bacteria of the genus *Beijerinckia* in Venezuelan soils. Trans.,9. **International Congress Soil Science**, Adelaide, 9. Australia, 1968, v. 2 p.125- 128.

FOSTER, E.M. Culture preservation. **Journal of Dairy Science**, v.45, p.1290-1294, 1962.

GILLILAND, S.E. Preparation and storage of concentrated cultures of *Lactic Streptocci*. **Journal of Dairy Science**, v.60, n.5, p.805-809, 1977.

JACKSON, L.S. e LEE, K. Microencapsulation and the food industry. **Lebensmittel Wissenschaft und Technologie**, v.24, p.289-297, 1991.

JOHNSON, J.A.C. ; ETZEL, M.R. Inactivation of lactic acid bacteria during spray drying. **AICHE Symposium Series**, v.89, n.297, p.98-107, 1993.

JONES, J. K.; PERRY, M. B.; SOWA, W. The occurence of D-glycero-D-manno – heptose in extracellular polysaccharide produced by *Azotobacter indicum*. **Canadian Journal Chemistry**, v. 41, p. 2712 –2715, 1963.

KNABEL,S.J.; WALKER,H.W.:HARTMAN,P.A.;MENDONÇA, A.F. Effects of growth temperature and strictly anaerobic recovery in the survival of *Listeria monocytogenes* during pasteurization. Applied and Environmental Microbiology, v.56, n.2, p.370-376, 1990.

KELLOGG, E.W.; FRIDOVICH, I. Superoxide, hydrogen peroxide, and singlet oxygen in lipid peroxidation by xanthine oxidase system. **The Journal of Biological Chemistry**, v.250, n.22, p.8812-8816, 1975.

KRALLISH, I.L.; DAMBERGA, B.E.; BECKER, M.J. State of adenosine phosphates during dehydration of yeast. **Applied Microbiology and Biotechnology**, v.31, n.2, p.194-199, 1989.

KUTS,P.S.;TUTOVA,E.G. Fundamentals of drying of microbiological materials.**Drying Technology**, v.2., p.171-201, 1983.

LIAN,W.C.; HSIO,H.C.; CHOU,C.C. Survival of *bifidobacteria* after spray-drying. International Journal of Food Microbiology, v.74, p.79–86, 2002.In: **Food Science and Technology Abstracts**, v.6, p.840, 2002.

LIEVENSE, L.C. e RIET, K. Convective drying of bacteria. **Advances in Biochemical Engineering/Biotechnology**, v.50, p.46-63, 1993.

LIEVENSE, L.C. e RIET, K. Conective drying of bacteria – factors influencing survival. **Advances in Biochemical Engineering/Biotechnoogy**, v.51, p.72-89, 1994.

LURIA, S.E. ; DELBRÜCK, M. Mutations of bacteria from virus sensitivity to virus resistance. **Genetics**, v.28, p.491-511, 1973.

MAMAEVA, P. Dry koumiss culture. Konevodstvo, v.25, p.46, 1955. In: **Dairy Science Abstract**, v.18, p.639, 1956.

MARIUZZO, D. M. **Contribuição ao estudo da estrutura química do exopolissacarídeo PS-32 obtido por fermentação aeróbica da bactéria *Beijerinckia sp.*** Campinas, 1996. 96 p. Dissertação (Mestrado) - Faculdade de Engenharia de Alimentos, Universidade de Campinas.

MARY, P.; MOSCHETTO, N.; TAILLIEZ, R. Production and survival during storage of spray-dried *Bradyrhizobium japonicum* cell concentrates. **Journal of Applied Bacteriology**, v.74, p.340-344, 1993.

NORI, M.A. Ingredientes e aditivos microencapsulados na indústria de alimentos. **Engenharia de Alimentos**, n.9, p.33-35, 1996.

PAUL, E.; FAGES, J.; BLANC, P.; GOMA, G.; PAREILLEW, A. Survival of alginate - entrapped cells of *Azospirillium lipoferum* during dehydration and storage in relation to water proprieties. **Applied Microbiology and Biotechnology**, v.40, p.34-39, 1993.

PIERSON, M.D.; GOMEZ, R.F.; MARTIN, S.E. The involvement of nucleic acids in bacterial injury. **Advances in Applied Microbiology**, v.23, p.263-285, 1978.

ROGERS, L.A. The preparation of dried cultures. **Journal of Infectious Diseases**, v.14, p.100-123, 1914.

SCOTT, W.J. The effect of residual water on the survival of dried bacteria during storage. **The Journal of General Microbiology**, v.19, n.3, p. 624-633, 1958.

SHAHIDI, F. e HAN, X.Q. Encapsulation of food ingredients. **Critical Reviews in Food Science and Nutrition**, v.33, n.6, p.501-547, 1993.

SZCZESNIAK, A. S. Rheological basis for selecting hydrocolloids for specific applications. In: PHILLIPS, G. °; WEDLOCK, D. J.; WILLIAMS, P. A. 3 ED. **Gums and stabilisers for the food industry**. London: Elsevier, p.311 –323, 1985.

TEIXEIRA, P.; CASTRO, H.; KIRBY, R. Spray drying as a method for preparing concentrated cultures of *Lactobacillus bulgaricus*. **Journal of Applied Bacteriology**, v.78, p.456-462, 1995.

TEIXEIRA, P.; CASTRO, H.; MOHÁCSI-FARKAS, C.; KIRBY, R. Identification of sites of injury in *Lactobacillus bulgaricus* during heat stress. **Journal of Applied Microbiology**, v.83, p.219-226, 1997.

Trindade, C.S.F.F. **Encapsulação de *Lactobacillus acidophylus* (L9-05) e de *Bifidobacterium lactis* (Bb-12) e avaliação in vitro do nível de tolerância dos mesmos às secreções gastroentestinais.** Campinas, 2001. 111p. Tese (Doutorado). Faculdade de Engenharia de Alimentos, Universidade Estadual de Campinas.

VENDRUSCOLO, C.T. **Produção e caracterização do biopolímero produzido por *Beijerinckia sp.* isolada do solo cultivado com cana-de-açúcar da região de Ribeirão Preto - São Paulo - Brasil.** Campinas, 1995. 143p. Tese (Doutorado). Faculdade de Engenharia de Alimentos, Universidade Estadual de Campinas.

3. CHAPTER II - SURVIVAL OF *BEIJERINCKIA SP* MICROENCAPSULATED IN CARBOHYDRATES BY SPRAY-DRYING.

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SURVIVAL OF *BEIJERINCKIA SP* MICROENCAPSULATED IN CARBOHYDRATES BY SPRAY-DRYING.

In this study, the encapsulation of *Beijerinckia sp* cell suspension in different wall materials using the spray drying technique was performed. Malt dextrin, dehydrated glucose syrups, gum acacia and modified starch materials were tested. Cell viability assays were carried out before, after drying and during storage of the products. The surface area and characteristics of the encapsulated powders were examined using BET- adsorption of N_2 and scanning eletron microscopy (SEM) respectively. The residual moisture content and water activity of the powders were also determined. The best results were obtained with the dehydrated glucose syrup, which resulted in products with the greatest percent survival during the drying process and subsequent storage period. The products obtained with the dehydrated glucose syrup showed more uniform microcapsule surfaces at lower A_w values and residual moisture content.

Keywords: spray drying, *Beijerinckia*, encapsulation.

Introduction

Beijerinckia sp bacteria have been the focus of several studies. These bacteria are capable of N_2 fixation in the soil and of producing polymers with physical-chemistry characteristics of industrial interest, besides showing the capacity to produce polyhydroxybutyrate (PHB), which is the material currently, used to produce biodegradable plastics.

Studies on *Beijerinckia sp* are mainly directed toward the use of this bacteria as a biofertilizer and for waste treatment via the oxidation of aromatic and sulphur compounds (Kim and Zylstra1995). The extracellular polysaccharide produced by these microorganisms has widespread commercial applications as a viscosity

enhancing agent and stabilizer in a variety of industries. Reproducibility of the results is an essential prerequisite for a successful fermentation process. The lack of reproducibility in gum fermentation arises mainly because of inconsistencies in inoculum development and strain instability. The errors associated with the inoculum preparation increase with the number of culture propagation steps required for larger scale operation. Problems associated with fermentation reproducibility on an industrial scale may result in inconsistent productivity, yield and quality, all of which can translate into financial losses. Quality is particularly important in the manufacture of gums, which can most readily be measured by their rheological properties. Since the final application of the gum is mainly dependent on this parameter, it is also desirable to obtain reproducible gum rheological characteristics (Amanullah et al. 1996).

Several investigators have considered the possibility of drying large quantities of bacterial cultures, with the idea of using this as a way to replace the usual liquid bulk starter in the production of a gum. Alternative methods for culture maintenance and inoculation are freeze and spray drying for the non-propagative maintenance of cultures, since the successive transfers used in the preparation of the inoculum under propagative conditions increase the possibility of genetic instability by a factor directly proportional to the number of generations involved (Teixeira et al. 1995). For this purpose spray drying would be preferable to freeze drying because it is a more economical process, especially on a large-scale basis. It is a continuous operation in which the particles are dried within a few seconds, minimizing thermal degradation. Since the product is dry and occupies a small volume, transportation and storage costs are also reduced. Although this process proved promising, it has not been developed commercially for use with bacteria. Reasons for this are mainly low survival rates

during drying of the cultures, low stability under storage and difficulty in rehydrating the product.

Although spray drying is mostly considered as a dehydration process, it can be used as an encapsulation process, entrapping "active" materials within a protective encapsulating matrix. The encapsulating or wall materials used generally consist of starch, starch derivatives, proteins, gums or any combination of these. Wall materials, which form high-solid solutions with a viscosity that allows for good atomization and droplet formation, are suitable for microencapsulation by spray drying. The proper choice of capsule composition and drying conditions can improve survival and stability during storage of the encapsulated bacteria (Shaihidi and Han 1993). The coating material has also been referred to as the encapsulation matrix, "shell" or "wall" material, encapsulating agent or carrier in the literature.

The purpose of this study was to examine the possibility of preserving *Beijerinckia* cultures by encapsulation and spray drying for the large scale preservation of cells and to determine the effects of different coating material on cell survival during o spray drying and subsequent storage.

Materials and methods

Materials

Malt dextrin and dehydrated glucose syrups (Corn Products International, Mogi Guaçu, Brazil), modified starch (Capsul®,National Starch, São Paulo, Brazil) and gum acacia (Spray Gum®, Colloids Naturels Brasil Commercial Ltda., São Paulo, Brazil). The materials used were all commercial food grade.

Organism

Beijerinckia sp. isolated from sugar cane roots, maintained at 4°C on YM agar slants and transferred every 30 days, according to Muro and Luchi (1989).

Pre-culture and Production of Cell Concentrate

Beijerinckia was grown in shaker flasks containing YM medium. The medium was adjusted to pH 6.5, and sterilized at 121°C for 15 min. The preculture was inoculated with two loops of culture from a slant and incubated at 25°C and 200 rpm for 24 hours in a model G25 New Brunswick shaker. This pre-culture was then used to inoculate a second broth (5% v/v). The second broth contained sugar cane molasses (1.5% w/v) and autolyzed brewers yeast (2.0% w/v) (Maldonade, 1996). All fermentation broths were made using the same lot numbers of the components. The cultures were incubated at 25°C and 200 rpm for 18 h. Stationary phase cells were harvested by centrifugation (22.300xg for 15 min. at 5°C) and washed twice with sterile distilled water.

Spray Drying Experiments

A cell pellet from 4L culture was resuspended in 390 mL of a sterile solution of 10% (w/v) skim milk according to Mary et al. (1993). This medium base was aseptically supplemented with 15% (w/v) non-sterilized wall material. Bacterial suspensions (450 mL) were incubated at 25°C and 100 rpm for 30 min to allow for cell adaptation and then sprayed. In preliminary experiments, this ratio resulted in suspension with a total

viable of $\geq 10^9$ cells g^{-1}. The total solids content was 25% (w/v) based on the percent dry matter of the bacterial cell. The proportion of other ingredients in the formulation of the spray-dried cultures was determined from the results of the preliminary trials. Five different wall materials were employed: dehydrated glucose syrups, 10 and 20 dextrose equivalent (DE) malt dextrin and of 20 DE, capsul® and a 3:2 blend of 20 DE malt dextrin and acacia gum according to Reineccius (1991). A magnetic stirrer was used for the bacterial suspensions to guarantee a homogeneous feed during drying. The viscosity of all the bacterial suspensions was determined.

A LabPlant model SD-04 (Leeds, UK) spray- drier was used. The entrance and exit temperatures, liquid flow rate, air pressure and nozzle diameter were respectively: 135°C, 75°C, 10 mL/min, 5 kgf/cm^2 and 1 mm. The processing conditions were standardized from the results of the preliminary trials. Microencapsulation of bacteria with five different wall materials was performed in triplicate, maintaining the same drying conditions. The resultant spray-dried bacteria were homogenized and stored separately in 2g quantities in sealed sterile glass bottles at 4°C. Viability assays were performed on two bottles of each of the different powders.

Viability assays were carried out on the powders dried under different test conditions just before drying the feed suspension, after drying and during storage at 4°C (after 14, 30 and 60 days).

Each sample of spray-dried bacteria was tested after rehydrating the powder. The rehydration conditions were determined according to the results of the preliminary trials, to give rehydrated products with a total viable count between 10^7 and 10^8 cells per mL, a level of inoculum comparable to that obtained with the traditional loop transfer in the pre-culture method according to Kidby et al. (1977). For all microcapsules, 1g of spray-dried bacteria were rehydrated at 25°C and 100 rpm for

24h in 10 mL of a sterile solution of skim milk (10% w/v). One milliliter of the cell suspension was then subjected to serial dilutions with a sterile NaCl solution (0.85% w/v) before transferring to YM agar plates and incubated at 25°C for 36h. The total viable cell number was expressed as colony forming units per gram (CFU/g) of dry powder. Survival was defined as the ratio of viable cells before and after drying at each time, converted to the base 10-logarithm value (logNo/N). Physico-chemical analyses on the encapsulated samples included moisture, water activity and structure.

Analytical procedures

Moisture content and water activity. Moisture content was determined in a vacuum-oven at 100°C and 0.6 atm, to constant weight. Water activity was determined using an Aqualab Mod CX2 (Decagon, Pullman, Washington) at 25 °C.

Scanning electron microscopy (SEM). Encapsulated samples were fixed in stubs on carbon adhesive gold plated paper (metallizer MED 020 BALZERS, Lichtenstein, Austria). Images of the specimens were obtained at accelerating voltage of 20 Kv (Scanning Eletron Microscope DSM 900 Zeiss), and taken at a magnification of x1000.

Powder surface area. The powder surface area was measured using BET (Brunaver, Emmet, Teller) – adsorption. The instrument used was a micromeritics ASAP 20110 with nitrogen gas (Webb and Orr 1997).

Viscosity. Viscosity of the bacterial suspensions obtained under the different test formulations was determined as described by Man et al. (1999) using a Haake Rotovisco CV20 coaxial cylinder viscometer at 25°C.

Statistical analysis. To verify if the bacteria encapsulated in the different wall materials (WM) behaved in distinct manners with respect to the variable of viability (log No/N) during the different periods of time, and considering that the levels were quantitative, the analysis of variance via polynomial ortogonal regression was applied to the data obtained for this variable. Following this, a group analysis of variance was applied, uniting the 5 individual experiments in a single analysis. The Statistics Analyses Systems (SAS) software was used for the analysis of variance.

Results and discussion

The results of the influence of the type of wall material on the survival of *Beijerinckia sp.* during encapsulation and analysis of the powders are shown in table 1.

Table 1. Analysis of the powders and survival of *Beijerinckia sp* during spray drying with different wall materials.

Wall material	Moisture content (%)	Water activity (Aw)	Nº of viable cells (CFU/g solids)		Log reduction of viable cells [log (No/N)]
			Before drying	After drying	
10 DE Malt dextrin	3.19	0.253	1.25×10^9	8.4×10^8	0.17
	3.20	0.246	1.40×10^9	8.2×10^8	0.23
	3.17	0.259	1.28×10^9	8.8×10^8	0.16
Capsul	3.35	0.428	1.19×10^9	2.55×10^7	1.67
	3.37	0.431	1.45×10^9	4.0×10^7	1.56
	3.38	0.434	1.26×10^9	3.3×10^7	1.58
Glucose	2.66	0.320	1.83×10^9	4.0×10^8	0.66
	2.62	0.323	1.75×10^9	4.3×10^8	0.61
	2.60	0.322	2.12×10^9	5.0×10^8	0.63
20 DE Malt dextrin	3.25	0.349	1.58×10^9	1.96×10^8	0.91
	3.23	0.350	1.48×10^9	1.60×10^8	0.97
	3.26	0.347	1.26×10^9	1.31×10^8	0.98
Blend	3.41	0.229	2.7×10^9	8.8×10^7	1.47
(gum + 20 DE	3.46	0.232	1.3×10^9	9.5×10^7	1.14
Malt dextrin)	3.45	0.233	2.3×10^9	9.0×10^7	1.41

The viable cell count of the powders is a good index to evaluate the degree of heat damage exerted during spray drying. The traditional plate count method and serial dilutions in sterile NaCl solutions (0.85% w/v) were used to determine the bacterial counts, since quality control methods to determine the number of bacteria within a starter culture have not yet been standardized. In Brazil, starter culture quality is not regulated, nor are the existing regulations well enforced.

During spray drying, the maximum survival was obtained for bacteria encapsulated with 10 DE malt dextrin, followed, in decreasing order, by glucose, 20 DE malt dextrin, the blend and capsul. The moisture content in the fresh powders varied from 2.60 to 3.46% (Table 1). According to Prajapati et al. (1987) maximum bacterial survival

occurs in powders with moisture contents below 3.5%. The mean water activity (Aw) of the spray-dried products, in the same order as above, was 0.252 , 0.321, 0.349 0.231 and 0.431 respectively. The "available" water for microbial growth differs considerably depending on the solute. When glucose was used as the wall material, an Aw of 0.321 was obtained with only 2.6% water content, whereas with capsul, an Aw of 0.431 was reached with 3.3% water content. According to Hahn-Hägerdal (1986) different wall materials probably influence the biological reaction in different ways resulting in distinct survival rates.

The values for moisture content were lower with the lower DE (dextrose equivalent) malt dextrin as compared to the higher DE malt dextrin, and the cell viability higher. Products with higher DE malt dextrin tend to stick to the walls of the drying chamber more than those with low DE (Man et al. 1999). Wall deposition in the spray dryer influences the viability rate, because it increases the residence time and thus the thermal inactivation of the cells (Solano et al. 2000).

Powders obtained with blends of gum acacia with malt dextrin had a higher moisture content after spray drying. According to Buffo and Reineccius (2000) the highly branched structure of the gum is capable of retaining more water molecules via hydrogen bonds established with its numerous free negative groups (COO^-).

Viscosity

All feed suspensions obtained from malt dextrin and from glucose showed lower values for viscosity and higher values for cell viability (table 2). Suspensions with 10 DE malt dextrin had a slightly higher viscosity than those with 20 DE malt dextrin. According to Wang and Wang (2000) the differences in structure of the malt dextrins determine their viscosity, higher molecular weight (MW) malt dextrins showing a higher

viscosity. Modified starch in the cell suspension resulted in less viscous suspensions than those with the blend (gum acacia), in agreement with Reineccius (1991).

Table 2. Viscosity of spray dryer feed suspensions.

Wall Material	Viscosity * CP (25°C)
10 DE malt dextrin	4.8
capsul	13.6
glucose	2.5
20 DE malt dextrin	4.0
blend (gum + 20 DE MD)	14.7

*Mean of triplicates.

Study of the morphology

The wall compositions affected the structure of the spray-dried microcapsules. As seen in Figure 1, in general there were only small differences in particle structure between the products using the different malt dextrins and glucose.

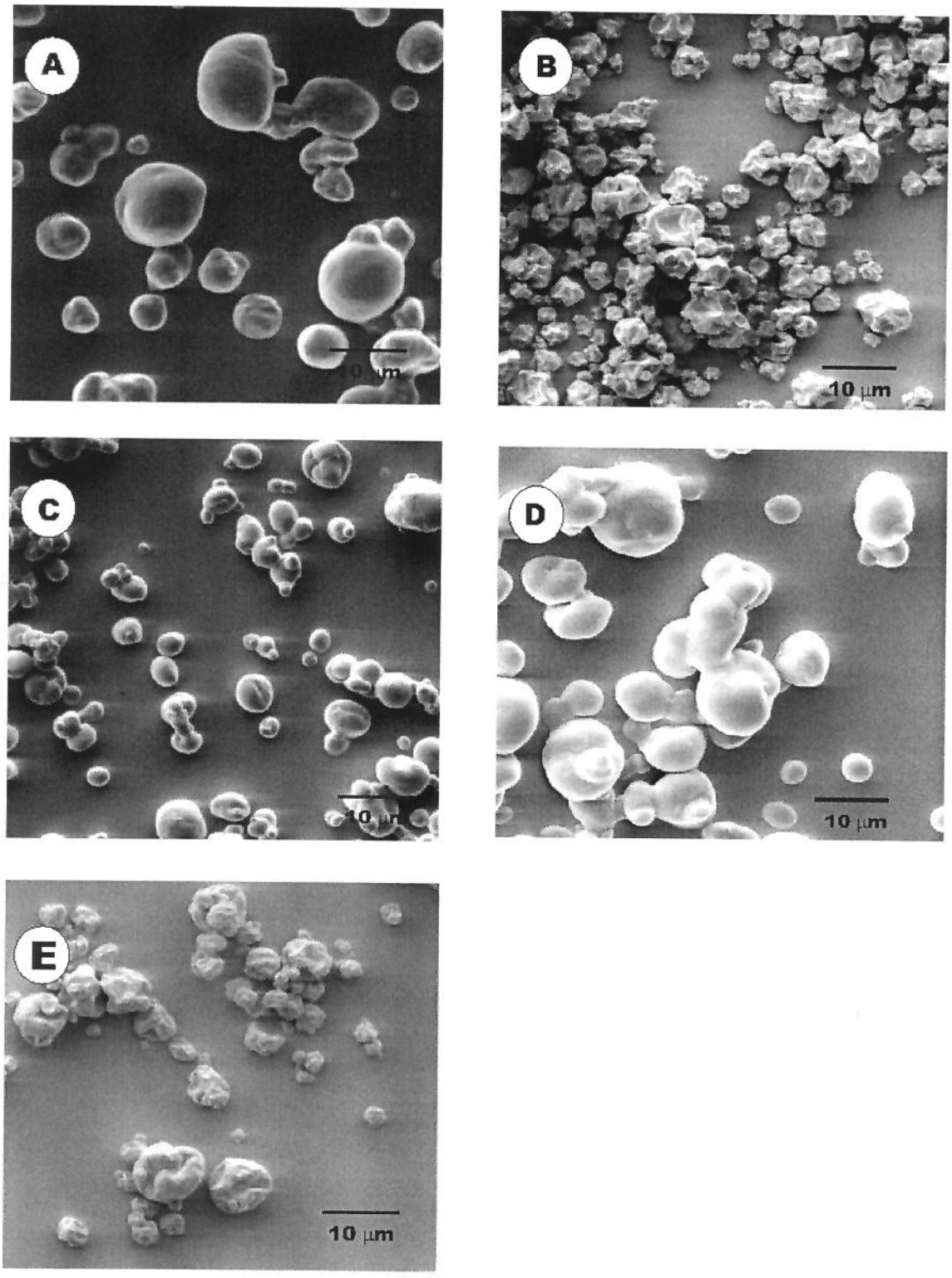

Figure1: Scanning electron photomicrographs of spray dried *Beijerinckia sp* microencapsulated in different carbohydrates: A) 10 DE malt dextrin, B) capsul, C) glucose, D) 20 DE malt dextrin and E) the blend.

The structure was more spherical with malt dextrin and with glucose, while the products obtained with capsul and with the blend (gum acacia and 20 DE maltodextrin) were more irregular. Amiet-Charpentier et al. (1998) obtained microparticles of modified starch N-LOK® containing the bacterial genus *Pseudomonas* with similar structures showed a flat "ball" effect, as if they were dehydrated.

An analysis of the surface areas of the encapsulated bacteria showed differences between products dried with different wall compositions. The powder surface area (m^2g^{-1}) obtained for glucose, 10 DE malt dextrin, 20 DE malt dextrin, the blend (gum + 20 DE MD) and capsul were 0.6218, 0.8214, 1.0104, 4.3479 and 8.0396 respectively.

Survival of *Beijerinckia sp.* during storage.

The viable cell count decreased gradually during the storage period with all the different wall compositions (table 3).

Table 3. Effect of wall material on the survival of *Beijerinckia sp.* during the storage period.

Wall Material	Moisture content (%) Storage period (days)		Log reduction of viable cells during storage [Log (No/N)] Storage period (days)			
	0	60	0	14	30	60
10 DE malt dextrin	3.19	3.47	0.17	1.00	1.89	2.95
	3.20	3.49	0.23	1.08	1.93	2.84
	3.17	3.47	0.16	1.08	2.12	2.77
Capsul	3.35	3.75	1.67	2.18	2.41	5.40
	3.37	3.76	1.56	2.17	2.44	5.57
	3.38	3.79	1.58	2.07	2.45	5.70
Glucose	2.66	2.86	0.66	0.95	1.07	1.07
	2.62	2.81	0.61	0.95	0.99	1.13
	2.60	2.79	0.63	1.06	1.06	1.12
20 DE malt dextrin	3.25	3.64	0.91	1.21	1.37	1.62
	3.20	3.60	0.97	1.07	1.31	1.75
	3.21	3.55	0.98	1.02	1.25	1.52
Blend (gum+20 DE Malt dextrin)	3.41	3.73	1.47	1.85	2.17	2.26
	3.46	3.81	1.14	1.73	1.90	1.88
	3.45	3.82	1.41	1.73	2.13	2.14

A decrease in the count was observed for the different types of powder. Capsul showed the lowest count at the end of two months and glucose the highest, followed, in decreasing order by 20 DE malt dextrin, 10 DE malt dextrin, the blend (gum + 20 DE MD) and capsul. An increase in moisture content for all powders during storage, since there was no control of the relative humidity during storage. The maximum percent increase in moisture content was for 20 DE malt dextrin followed, in decreasing order, by capsul, the blend (gum + 20 DE MD), 10 DE malt dextrin and glucose. However, the relationship between the degree of increase in moisture content and the corresponding

decrease in viable cell count of *Beijerinckia sp* for the different powders, varied with the type of wall composition.

The viability of *Beijerinckia sp* during storage was higher for the products with a more spherical particle structure and smaller surface area. According to Nei (1973) during drying three fractions of cellular water (free water, intermediate water and structural water) are eliminated with the consequent exposure of hydrophilic sites on the protein under oxidizing conditions, causing damage to the cell components. According to Waldez et al. (1985) the free water is eliminated during the first stage of drying since the hydrogen bonds are relatively weak, whilst the bound water may be partially eliminated during the next stage of drying, although the majority remains bound to the dehydrated product by electrostatic forces. The glucose wall material showed the greatest viable cell count during the spray drying process and during the storage period. According to Hahn-Nägerdal, (1986) the effect of sugars on the stabilization of enzymes against heat denaturation is proportional to the number of equatorial hydroxyl groups in the sugar molecule.

The analyses of variance of log (No/N) for each wall material (WM) and period (days) are presented in table 4. The group analysis of variance is presented in table 6.

Table 4. The analysis of variance for the polynomial regression of the data for log (No/N).

Cause of Variation	GL	QM				
		WM1***	WM2	WM3	WM4	WM5
Treatment	3	3.984922**	4.909433**	0.131822**	0.318144**	0.368431**
RL	1	11.514667**	13.92584**	0.264532**	0.899079**	0.821816**
RQ	1	0.427396**	0.636546**	0.105266**	0.00359n.s.	0.238449**
RC	1	0.012705n.s.	0.165890**	0.025667*	0.051769*	0.000025n.s.
Residue	6	0.008663	0.010100	0.002014	0.004986	0.005122

* significant difference between treatments (95% level of confidence)
** significant difference between treatments (99% level of confidence)
*** wall material: WM1 (10 DE MD), WM2 (capsul), WM3 (glucose), WM4 (20 DE MD) and WM5 (blend).
n.s. no significant difference between treatments

The F test for the treatments was significant at the 1% level of probability for WM1, WM2, WM3, WM4 and WM5. The null hypothesis (Ho) was therefore rejected and it was verified that the storage period influenced the dependent variable log (No/N) for all the wall materials used. The percent variation was clearly explained as a function of the WM used. From the coefficient of determination (R^2), it was shown that the contribution of the linear model in the explanation of the phenomenon for WM 1,2,3,4 and 5 corresponds to 96.32%, 94.55%, 67.0%, 94.20% and 74.35%, respectively. The linear regressions based on the data for log reductions of cell viability obtained with the different wall materials during storage are shown in table 5.

Table 5. Linear regressions showing the relationship between log reduction of cell viability of *Beijerinckia sp*. During storage and the type of wall material.

Wall material	Linear regressions of log reductions
WM1	0.377048 + 0.04389 (T)*
WM2	1.4199 + 0.048273 (T)
WM3	0.767015 + 0.006653 (T)
WM4	0.892758 + 0.012266 (T)
WM5	0.151126 + 0.011727 (T)

* Storage time.

Table 6. Group analysis of variance Log (No/N).

Cause of variation	GL	QM
Wall material (WM)	4	5.372167**
Period (P)	3	6.041246**
Interaction (WM x P)	12	0.917877**
Block (Wall material)	10	0.017868*
Mean residue	30	0.0061

* significant difference between treatments (95% level of confidence)
** significant difference between treatments (99% level of confidence)
n.s. no significant difference between treatments

The interaction (WM x P) was significant at the 1% level of probability, indicating that the periods have a different influence on the variable log (No/N) for each WM. One solution is to consider the individual analyses, that is, for each wall material.

Dehydrated glucose syrup was shown to be the best wall material for *Beijerinckia sp*. In the spray dryer, maintaining a greater number of viable cells during the storage period, as compared to the other wall materials tested, since greater retention of viability during storage was one of the main objectives of encapsulating the bacteria.

A review of the literature showed no report of any identical study in order to compare their results with the present data. In conclusion it appears to be essential to make an appropriate selection of the wall material for the microcapsules of *Beijerinckia sp* since

this may affect the survival of the bacteria during the spray drying process as well as their viability during the storage period.

References

Amanullah, A., Serrano, L.C., Galindo, E. and Nienow, A.W., 1996. Reproducibility of pilot scale xanthan fermentations. Biotechnology Progress, **12**, 466-473.

Amiet-Charpintier, C., Gadille, P., Digats, B. and Benoit, J.P., 1998. Microencapsulation of *rhizobacteria* by spray-drying: formulation and survival studies. Journal of Microencapsulation, **15**, 639-659.

Buffo, R. and Reineccius, G., 2000. Optimization of gum acacia/modified starch/maltodextrin blends for the spray drying of flavours. Perfumer & Flavorist, **25**, 37-51.

Hahn-Hägerdal, B., 1986. Water activity: a possible external regulator in biotechnical process. Enzyme Microbial Technology, ***8***, 322-327.

Kidby,D.; Sandford,P.;Herman,A. and Cadmus, H., 1977. Maintenance procedures for the curtailment of genetic instability: *Xanthomonas campestris* NRRL B-1459. Applied and Environmental Microbiology, **33**,840-845.

Kim, E., Zylstra, G.T., 1995. Molecular analysis of m-xilene degradation by *Beijerinckia sp.* – biphenil nyl degradation gene expression in *Pseudomonas putida* (conference abstract). Industrial Waste Disposal, **15**, 101.

Maldonade, I.R., 1996. Contribuição ao estudo dos parâmetros de fermentação por *Beijerinckia sp.* Master Thesis, Faculdade de Engenharia de Alimentos, Universidade Estadual de Campinas , Campinas, Brasil.

Man, Y.C., Irwandi, J. and Abdullah, W.J.W., 1999. Effect of different types of maltodextrin and drying methods on physico-chemical and sensory properties of encapsulated durian flavour. Journal of the Science of Food and Agriculture, **79**,1075-1080.

Mary, P., Moschetto, N. and R. Tailliez, 1993. Production and survival during storage of spray-dried *Bradyrhizobium japonicum* cell concentrates. Journal of Applied Bacteriology, ***74***, 340-344.

Muro, M.A. and Luchi, M.R., 1989. Preservação de Microrganismos. Editora da Fundação Tropical de Pesquisas e Tecnologia "André Tosello". Campinas, SP, Brasil. 70p.

Nei, T., 1973. Some aspects of freezing and drying of microorganisms on the basis of cellular water. Cryobiology, **10**, 403-408.

Prajapati, J.B., Shah, R.K. and Dave, J.M., 1987. Survival of *Lactobacillus acidophilus* in blended-spray dried acidophilus preparations. Australian Journal of Dairy Technology, 17-21.

Reineccius, G.A., 1991. Carbohydrates for flavor encapsulation. Food Technology, 144-149.

SAS INSTITUTE. Statistics Analyses Systems (SAS): versão 6-09.4.ed.Cary.NC1989. 2v. (Compact Disc).

Shaihidi, F. and Han, X., 1993. Encapsulation of food ingredients. Critical Reviews in Food Science and Nutrition, **33**, 501-547.

Solano, G.L., Cervantes, M.A.S., Alvarado, M.A.G. and Jimenes, G.R., 2000. Improved viability of spray dried brewer's yeast by using starch (grits) and maltodextrin as processing aids. Journal of Food Process Engineering, **23**, 453-462.

Teixeira, P., Castro, H. and Kirby, R., 1995. Spray drying as a method for preparing concentrated cultures of *Lactobacillus bulgaricus*. Journal of Applied Bacteriology, **78**, 456-462.

Valdez, G.F., Gior, G.S., Holgado, A.P.R. and Oliver, G., 1985 Effect of drying medium on residual moisture content and viability of freeze-dried lactic acid bacteria. Applied and Environmental Microbiology, **49**, 413-415.

Wang, Y.J. and Wang, F., 2000. Structures and properties of commercial maltodextrins from corn, potato and rice starches. Starch/Stärke, **52**, 296-304.

Webb, P.A. and Orr, C., 1997. Analytical Methods in Fine Particle Technology, Micrometrics Instrument Corporation, Norcross, GA, USA. 301p.

4. CHAPTER III - ACTIVITY AND SURVIVAL OF SPRAY DRIED *BEIJERINCKIA SP.* MICROENCAPSULATED IN DIFFERENT CARBOHYDRATES

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ACTIVITY AND SURVIVAL OF SPRAY DRIED *BEIJERINCKIA SP* MICROENCAPSULATED IN DIFFERENT CARBOHYDRATES

ABSTRACT: This study examines the possibility of preserving *Beijerinckia* cultures by encapsulation using a spray drier, for use in biotechnological processes in the production of biopolymers. An adequate choice of the wall (coating) material is one of the factors, which will determine the degree of cell survival and the maintenance of fermentative activity in the encapsulated inoculum. Malt dextrin, dehydrated glucose syrups, modified starch and acacia (gum Arabic) were used as wall materials. The results showed that spray dried *Beijerinckia* encapsulated in malt dextrin, stored for two months and inoculated into sterile must after re-hydration, presented the greatest stability with respect to fermentative activity, although the glucose encapsulated cells showed the greatest percent viability during spray drying and during the storage period.

Index Entries : fermentation;spray drying;*Beijerinckia;*encapsulation;biopolymer.

INTRODUCTION

Microbial polysaccharides have been compared with traditional plant polysaccharides. The advantages of microbial polysaccharides are new, constant functionality, reproducible chemical and physical properties and a stable cost and supply. They offer a potentially new source of functional biopolymers for food, industrial and medical applications. Bacterial polysaccharides are incorporated into food as thickeners, suspending or gelling agents, in order to improve food quality and texture. They are also used in metal recovery, water clarification and oil well drilling (1).

Though exo-polysaccharides are produced by a large number of microorganisms, reports on the *Beijerinckia* species are very scarce. Pure culture inoculation of broth is not always a convenient means of inducing polysaccharide fermentation on a commercial scale because it is difficult to prepare a consistent inoculum with high fermenting activity. A new approach to this problem is the use of dried bacteria as a starter. The development of dried concentrated cultures for inducing gum fermentations could eliminate many of the problems customarily involved in the preparation and maintenance of starter cultures in the industrial plant. According to Teixeira et al. (2) culture concentrates can be evaluated and standardized for activity before shipment to the processor, making it possible to produce consistently high-quality products. Dried preparations have the advantages of long term preservation and convenience in handling, storage, marketing, and consumption.

Bacterial cells are likely to lose their viability and activity (gum producing capacity) during drying and the subsequent storage period. The choice of an appropriate suspending medium may be the most important factor in increasing the survival rate and activity of the microorganisms during drying and subsequent storage .The use of spray dried cultures may be promising, having similar advantages to freeze dried cultures, but much less costly to produce. However, special attention must be given to the operation of the spray dryer in order to provide viable cultures with rapid gum production on re-hydration. This requires the use of mechanisms to minimize cellular injury during spray drying. Reports found in the literature show that the loss of cell viability during spray drying may be in the order of 3 logarithmic cycles (3).

The amount of water remaining after drying affects not only the viability of the bacteria, as determined immediately after the process, but also the rate of loss of viability during subsequent storage (4). The physiological reactions of organisms depend on the

mobility of the bound water, not of the free water. The optimum residual moisture content varies with the composition of the fluid in which the organisms are dried, with the storage atmosphere, with the species and physiological state of the organisms.

Spray drying can be used as an encapsulation process when it entraps "active" material within a protective matrix or carrier (wall material) formed from a polymer (5). The encapsulation of viable bacterial cells has several advantages over encapsulation of isolated enzymes. The stability of enzymes in intact cells is greater than in extracts and the production achieved by cells is easily manipulated (6).

Successful microencapsulating is the result of a judicious choice of wall material composition for a determined core material, and the result of a well-managed process design. The carbohydrates, due to their diversity, low cost and widespread use in foods, have become the preferred choice for encapsulation. Sugars, malt dextrin, modified starches and gums, are all used as wall materials (7).

Our objectives were to determine the effect of different carbohydrates on cell survival and gum production during spray drying and the subsequent storage period.

MATERIALS AND METHODS

Materials

Malt dextrin and dehydrated glucose syrups (Corn Products International, Mogi Guaçu, Brazil), modified starch (Capsul®, National Starch, São Paulo, Brazil) and acacia (Spray Gum®, Colloids Naturels Brasil Commercial Ltda., São Paulo, Brazil). The materials used were all of commercial food grade.

Organism

Beijerinckia sp. isolated from sugar cane roots, maintained at 4°C on YM agar slants and transferred every 30 days according to Muro and Luchi (8).

Pre-culture and production of cell concentrates.

Beijerinckia sp was grown in shaker flasks containing YM medium. The medium was adjusted to pH 6.5, and sterilized at 121°C for 15 min. The pre-culture was inoculated with two loops of culture from a slant and incubated at 25°C and 200 rpm for 24 hours in a New Brunswick model G25 shaker. This pre-culture was then used to inoculate a second broth (5% w/v). The second broth contained sugar cane molasses (1.5% w/v) and brewers yeast autolyzate (2.0% w/v) (9). All fermentation broths were made using the same lot numbers of components. The cultures were incubated at 25°C and 200 rpm for 18 h. Stationary phase cells were harvested by centrifugation (22.300xg for 15 min. at 5°C) and washed twice with sterile distilled water.

Spray drying experiments.

A cell pellet from 4L of culture was re-suspended in 390 mL of a sterile solution of 10% (w/v) skim milk in accordance with Mary et al. (10). This medium was aseptically supplemented with 15% (w/v) non-sterilized wall material. Bacterial suspensions (450 mL) were incubated at 25°C and 100 rpm for 30 min to allow for cell adaptation and

then sprayed. In preliminary experiments, this ratio resulted in a suspension with a total viable count of $\geq 10^9$ cells g^{-1}. The total solids content was 25% (w/v) based on the dry weight of the bacterial cells. The proportion of the different ingredients in the formulation of the spray-dried cultures was decided on from the results of the preliminary trials. Five different wall materials were employed: dehydrated glucose syrups, 10 and 20 dextrose equivalent (DE) malt dextrin, Capsul® and a blend (20 DE malt dextrin and acacia in the ratio 3:2) according to Reineccius (11). A magnetic stirrer was used for all the bacterial suspensions to maintain a homogeneous feed during drying. The viscosity of the bacterial suspensions was determined with all the wall materials.

A LabPlant model SD-04 (Leeds, UK) spray dryer was used. The entrance and exit temperatures, liquid flow rate, air pressure and nozzle diameter were respectively: 135°C, 75°C, 10 mL/min, 5 kgf/cm^2 and 1 mm. The processing conditions were standardized from the results of the preliminary trials. Microencapsulating of the bacteria in five different wall materials was performed in triplicate, maintaining the same drying conditions. The resulting spray dried bacteria were homogenized and stored separately in 2g quantities in sealed sterile glass bottles at 4°C. Viability and activity assays were performed on two bottles of each different powder.

Viability assays were carried out on the powders dried under the different test conditions just before drying on the feed solution, after drying and during storage at 4°C (after 14, 30 and 60 days). Each sample of spray-dried bacteria was tested after re-hydration with respect to viability and biological activity (in terms of fermentative activity). As the re-hydrating conditions were based on the results of the preliminary trials, these gave re-hydrated products with a total viable count between 10^7 and 10^8 cells per mL, a level of inoculum comparable to that obtained with the traditional loop

transfer in the pre-culture method, according to Kidby et al. (12). For all encapsulated materials, 1g of spray dried bacteria was re-hydrated at 25°C and 100 rpm for 24h in a shaker in 10 mL of a sterile solution of skim milk (10% w/v). One milliliter of the cell suspension was then subjected to serial dilutions with a sterile solution of NaCl (0.85% w/v) before transferring to YM agar plates, and incubating at 25°C for 36h. The total viable cell number was expressed as colony forming units per gram (FCU/g) of dry powder. Survival was defined as the ratio of viable cells before and after drying at each storage time, converted to the base 10 logarithm (log No/N). Physico-chemical analyses of the encapsulated samples included sugar and moisture contents and water activity.

Assay for fermentative activity

A one gram sample of powder was re-hydrated as described above and inoculated into sterile gum production media in a ratio of 5% (v/v). The broth containing sucrose (1% w/v) and brewers yeast autolyzate (1% w/v), pH previously adjusted to 6.5 prior to sterilization (121° C, 15 min) according to Maldonade (9) was inoculated and incubated in a New Brunswick model G25 shaker at 25°C and 200 rpm for 24h. The culture broth was centrifuged at 22.300Xg at 5°C for 30 min to remove the cells. The supernatant was kept for substrate (residual sugars) and product (polysaccharide) analysis, as described below.

Analytical Procedures

Determination of dry cell weights

Microbial dry weights were monitored gravimetrically. The organisms collected by centrifugation were re-suspended in distilled water to remove undesired soluble material and re-centrifuged. The final pellet was suspended in water again and transferred to a pre-weighed Pyrex dish that was dried to constant weight at 105°C. The results were expressed in biomass of concentration (g dry cell mass per liter of substrate).

The dry weight of spray dried *Beijerinchia sp.*, microencapsulated in different carbohydrates, was determined in the following manner. The pre-weighed microencapsulated cells were washed with distilled water (in a ratio of 1 g of powder per 200 mL of water) to remove undesirable soluble material, and centrifuged at 22,300 X g at 5°C for 30 min. The resulting pellet was dried to constant weight at 105°C. All powders were analyzed twice. The supernatant was kept for sugar analyses as described below.

Polysaccharide concentration

The estimation of polysaccharide concentration was carried out by gravimetric analysis. The supernatant remaining after centrifuging the bacterial cells was precipitated with 4 volumes of ethanol. The precipitates were washed twice with ethanol. The precipitated gum was dried at 45°C to constant weight and the results

expressed as g gum per liter. The yield of gum was reported as g gum per g sugar consumed.

Sugar concentration

The sugar concentration in the cell free broth and substrate was measured using the dinitrosalicylic acid method (13) modified according to Giordano (14) and expressed as gram sugar consumed (initial sugar – residual sugar) per liter.

Moisture content and Water activity

The moisture content was determined by drying in a vacuum oven at 100°C and 0.6 atm, to constant weight. The water activity was determined in an Aqualab Mod CX2 (Decagon, Pullman, Washington) at 25°C.

Statistical analysis

The analysis of variance by orthogonal polynomial regression was applied to the data for each variable, in order to verify if the bacteria encapsulated in different wall materials (WM) behaved distinctly with respect to the following variables: viability (log No/N) and fermentative activity (concentration of cell and polysaccharide mass, biopolymer yield and sugar consumption) during storage at the different time intervals (periods). Subsequently a group analysis of variance was carried out, combining the 5 individual experiments in a single analysis. The Statistics analyses systems (15)

software was used for the analyses of variance. All the experiments were performed in triplicate.

RESULTS AND DISCUSSION

Viability

The experimental results for the survival of spray dried *Beijerinckia sp.* during encapsulation in different wall materials and during storage for various times (0, 14, 30 and 60 days) at room temperature and a_w are shown in Tables 1 and 2 respectively.

Table 1

Powder analyses and survival of *Beijerinckia sp* during spray drying in different wall materials.

Wall Material (WM)	Moisture content (%)	Water activity (aw)	No of viable cells (CFU/g solids)		Log reduction of viable cells [log (No/N)]
			Before drying	After drying	
10 DE Malt dextrin (WM1)	3.19	0.253	1.25×10^9	8.4×10^8	0.17
	3.20	0.246	1.40×10^9	8.2×10^8	0.23
	3.17	0.259	1.28×10^9	8.8×10^8	0.16
Capsul (WM2)	3.35	0.428	1.19×10^9	2.55×10^7	1.67
	3.37	0.431	1.45×10^9	4.0×10^7	1.56
	3.38	0.434	1.26×10^9	3.3×10^7	1.58
Glucose (WM3)	2.66	0.320	1.83×10^9	4.0×10^8	0.66
	2.62	0.323	1.75×10^9	4.3×10^8	0.61
	2.60	0.322	1.12×10^9	5.0×10^8	0.63
20 DE Malt dextrin (WM4)	3.25	0.349	1.58×10^9	1.96×10^8	0.91
	3.23	0.350	1.48×10^9	1.60×10^8	0.97
Blend (gum + 20 DE MD) (WM5)	3.26	0.347	1.26×10^9	1.31×10^8	0.98
	3.41	0.229	2.7×10^9	8.8×10^7	1.47
	3.46	0.232	1.3×10^9	9.5×10^7	1.14
	3.45	0.233	12.3×10^9	9.0×10^7	1.41

The total number of viable bacteria decreased during spray drying and storage in the different wall materials. From the results (Table 2) it can be seen that the wall material DE 20 malt dextrin, resulted in a greater retention of viability of the bacteria during storage. The decrease in viable cell counts of *Beijerinckia sp* in the different powders varied according to the wall material. Such a varied behavior of the different powders could be attributed to the effect of the original chemical composition and/or their interaction products.

Table 2

Effect of wall material on the survival of *Beijerinckia sp*. during storage.

WM	Moisture content (%) Storage period (days)		Log reduction of viable cells after storage [log (No/N)] Storage periods (days)			
	0	60	0	14	30	60
10 DE Malt dextrin	3.19	3.47	0.17	1.00	1.89	2.95
	3.20	3.49	0.23	1.08	1.93	2.84
	3.17	3.37	0.16	1.08	2.12	2.77
Capsul	3.35	3.75	1.67	2.18	2.41	5.40
	3.37	3.76	1.56	2.17	2.44	5.57
	3.38	3.79	1.58	2.07	2.45	5.70
Glucose	2.65	2.86	0.66	0.95	1.07	1.07
	2.62	2.81	0.61	0.95	0.99	1.13
	2.60	2.79	0.63	1.06	1.06	1.12
20 DE Malt dextrin	3.25	3.64	0.91	1.21	1.37	1.62
	3.20	3.60	0.97	1.07	1.31	1.75
	3.21	3.55	0.98	1.02	1.25	1.52
Blend (gum+ 20 DE MD)	3.41	3.73	1.47	1.85	2.17	2.26
	3.46	3.81	1.14	1.73	1.90	1.88
	3.45	3.89	1.41	1.73	2.13	2.14

The analyses of variance of the log reduction in viability (No/N) of the bacteria encapsulated in each wall material (WM) for the different storage periods (days), are presented in Table 3. The group analysis of variance is presented in Table 4.

Table 3

Analysis of variance for the polynomial regression study of the data for log(No/N).

Cause of Variation	GL	QM***				
		WM1	WM2	WM3	WM4	WM5
Treatment	3	3.984922**	4.909433**	0.131822**	0.318144**	0.368431**
RL	1	11.514667**	13.92584**	0.264532**	0.899079**	0.821816**
RQ	1	0.427396**	0.636546**	0.105266**	0.00359n.s.	0.238449**
RC	1	0.012705n.s.	0.165890**	0.025667*	0.051769*	0.000025n.s.
Residue	6	0.008663	0.010100	0.002014	0.004986	0.005122

* significant difference between treatments (95% of confidence).
** significant difference between treatments (99% of confidence).
*** wall material: WM1 (10 DE MD), WM2 (capsul), WM3 (glucose), WM4 (20 DE MD) and WM5 (blend).
n.s. non-significant difference between treatments.

For the treatments, the F test was significant at the 1% level of probability for WM1, WM2, WM3, WM4 and WM5. Thus we rejected the null hypothesis (Ho) and showed that the storage period influenced the dependant variable log (No/N) for all the wall materials used, the percent variation being distinctly explained as a function of the wall material used. From the coefficients of determination (R^2), it was shown that the contribution of the linear model to the explanation of the phenomenon for WM 1, 2, 3, 4 and 5 was 96.32%, 94.55%, 67.0%, 94.20% and 74.35% respectively. The regression equations, where Yn was log (No/N) of the WM n and x the storage time, were as follows:

$Y1 = 0.377048 + 0.04389\ x$

$Y2 = 1.4199 + 0.048273\ x$

$Y3 = 0.767015 + 0.006653\ x$

$Y4 = 0.892758 + 0.012266\ x$

$Y5 = 1.5126 + 0.011727x.$

Table 4

Group analysis of variance. Log (No/N).

Cause of variation	GL	QM
Wall material (WM)	4	5.372167**
Period (P)	3	6.041246**
Interaction (WM x P)	12	0.917877**
Block (Wall material)	10	0.017868*
Average residue	30	0.0061

* significant difference between treatments (95% of confidence).
** significant difference between treatments (99% of confidence).
n.s. non-significant difference between treatments.

The interaction (WM x P) was significant at the 1% level of probability, indicating that the storage period had an influence on the viability of the encapsulated bacteria as a function of the wall material used. The solution found was to consider the analyses individually, that is, for each wall material.

Substantial differences in survival during spray drying and the shelf life were observed for the different carbohydrates. During spray drying the maximum survival was obtained for bacteria encapsulated with 10 DE malt dextrin, followed, in decreasing order, by glucose, 20 DE malt dextrin, the blend and capsul. The average water activity (aw) of the spray dried products, in the same order as above, was 0.252; 0.321; 0.349; 0.231 and 0.431, respectively. However, at the end of two months, the bacteria encapsulated in glucose showed a greater level of survival, which was followed, in

decreasing order, by 20 DE malt dextrin, the blend, 10 DE malt dextrin and capsul. The experiments showed that the survival rates were not linearly related to aw.

It is interesting to find ways to store microbial cultures with a high retention of viability for use as starter cultures. The "available" water for the growth of microorganisms differs considerably depending on the solute. When glucose was used as the wall material, an aw reading of 0.321 was obtained with only 2.6% moisture content whereas with capsul, an aw of 0.431 was obtained with 3.3% moisture content. However, 20 DE malt dextrin and the blend produced powders with similar aw values but different degrees of survival, in agreement with Hahn-Hägerdal (16) who suggested they probably influenced the biological reactions in different ways, resulting in distinct levels of survival.

Amiet-Charpentier et al (17) obtained similar results microencapsulating *rhizobacteria* in modified starch, but these did not survive more then a week. The maximum bacterial survival at the end of two months occurred in powders with moisture contents below 3.5%, in agreement with Prajapati et al. (18).

The moisture content is an important parameter in the stability of dried cultures. Microbial biomass contains 70-90% of water. Biopolymers and membranes are dispersed in a water medium, and life takes place only in the presence of water. Water is a structural component of both biopolymers and bio-membranes. In addition, water as a substance is directly involved in a number of biochemical reactions. The term bound water is understood as that part of the intracellular water, which combines directly with proteins, nucleic acids, membranes or other substances and which is responsible for maintaining their structural organization (19).

The studies reported here indicated that the loss of cellular viability appeared to be related to damage to the following cell components: cell membrane, cell wall and DNA.

In the cell membrane it is supposed that the loss of water hydrated layers causes disorientation of the phospholipid molecules, at least in some membrane parts. Under these conditions, membrane lipids may change their configuration from lamellar to hexagonal. Such disorientation of the phospholipids in the membranes of dried organisms increases the danger of irreversible damage under these conditions. This may be due to oxygen leakage and the presence of oxidizing reactions, with peroxidation of the lipids and with the presence of phospholipases and enzymatic degradation of the main bio-membrane components. A consequence of membrane phospholipid disorientation is the destruction of the bio-membrane barrier function, increasing permeability (19).

A comparison between glucose and malt dextrin, showed that the low DE malt dextrin and glucose showed a greater protective effect of the cells against damage during spray drying, although greater protection (survival) was observed in the higher DE malt dextrin during storage. Studies with encapsulated orange peel and trans-β-carotene in malt dextrin, showed that increasing the DE extended the shelf-life of the encapsulated compound, probably because a more dense, oxygen impermeable matrix, was formed (20). The presence of glucose in the encapsulation system had a considerable effect due to its antioxidant properties (6). However, products encapsulated in the higher DE malt dextrin could affect the survival of cells during spray drying because of their higher hygroscopicity (21). The greater the hygroscopicity of the powder, the longer it tends to stay in the drying chamber, so the drying process is relatively slower, increasing the thermal inactivation of the cells, according to Solano et al. (3).

Fermentative activity

The effects of the different types of wall material (WM) on the fermentative activity of *Beijerinckia sp* at the different storage times are shown in Table 5.

Table 5

Fermentative activity of *Beijerinckia sp* encapsulated in different carbohydrates, during storage.

Period	Wall material	Block	Dry mass g/L	Yield	Sugar consumed g/L	Conc. of Polysacc. g/L
0	1	1	2.02	0.42	11.25	4.70
0	1	2	2.07	0.46	11.75	5.41
0	1	3	2.14	0.50	13.70	6.85
0	2	1	1.37	0.40	7.85	3.14
0	2	2	1,57	0.45	8.25	3.71
0	2	3	1.55	0.47	8.15	3.83
0	3	1	1.95	0.17	6.15	1.04
0	3	2	1.55	0.22	7.95	1.75
0	3	3	2.34	0.26	9.30	2.42
0	4	1	3.50	0.42	12.10	5.05
0	4	2	3.86	0.45	10.80	4.86
0	4	3	3.78	0.37	11.15	4.23
0	5	1	2.63	0.37	9.10	3.37
0	5	2	2.57	0.41	8.45	3.46
0	5	3	2.60	0.44	7.65	3.37
14	1	1	1.07	0.43	7.65	3.29
14	1	2	1.15	0.45	7.95	3.58
14	1	3	1.23	0.47	8.40	3.95
14	2	1	1.30	0.26	7.40	1.92
14	2	2	1.33	0.35	8.00	2.80
14	2	3	1.42	0.41	7.85	3.22
14	3	1	0.95	0.22	9.30	2.05
14	3	2	0.85	0.29	12.25	3.55
14	3	3	0.89	0.31	13.15	4.08
14	4	1	4.46	0.44	9.25	5.07
14	4	2	3.00	0.47	10.60	4.98
14	4	3	3.80	0.47	9.75	4.61
14	5	1	2.56	0.33	9.25	3.05
14	5	2	2.54	0.34	8.60	2.92
14	5	3	2.56	0.32	8.25	2.64
30	1	1	0.95	0.40	9.60	3.84
30	1	2	1.05	0.38	9.95	3.78
30	1	3	0.85	0.46	8.45	3.89
30	2	1	1.18	0.26	7.20	1.87
30	2	2	1.08	0.31	7.45	2.31

30	2	3	1.17	0.29	6.95	2.01
30	3	1	0.79	0.39	13.65	5.32
30	3	2	0.71	0.29	12.90	3.74
30	3	3	0.84	0.34	12.20	4.15
30	4	1	2.25	0.50	12.70	6.35
30	4	2	1.95	0.39	11.60	4.52
30	4	3	1.30	0.36	11.00	3.96
30	5	1	2.47	0.33	10.77	3.55
30	5	2	2.50	0.29	11.60	3.36
30	5	3	2.53	0.35	11.00	3.85
60	1	1	0.45	0.40	9.80	3.92
60	1	2	0.50	0.37	9.45	3.50
60	1	3	0.70	0.41	8.90	3.65
60	2	1	0.59	0.26	7.35	1.91
60	2	2	0.55	0.30	7.20	2.16
60	2	3	0.50	0.29	6.75	1.95
60	3	1	0.76	0.43	8.80	4.35
60	3	2	0.67	0.62	11.50	7.13
60	3	3	0.81	0.58	10.35	6.00
60	4	1	1.66	0.55	12.70	6.98
60	4	2	1.45	0.52	11.60	6.03
60	4	3	1.18	0.46	11.00	5.06
60	5	1	2.50	0.30	12.50	3.75
60	5	2	2.46	0.38	11.60	4.41
60	5	3	2.52	0.31	11.30	3.50

Although the average dry cell weights of the spray dried *Beijerinckia sp.*, microencapsulated in the different carbohydrates was 0.0508 per gram of powder and the standard deviation was 0.007, after re-hydration the initial number of viable cells in the inoculum differed according to the type of carbohydrate and storage days. The whole study was conducted after the reactivation period.

The results of the fermentations (Table 5) on a laboratory scale, under conditions similar, using inoculae of *Beijerinckia sp* encapsulated in the different wall materials, showed that the type of wall material directly influenced the bacterial fermentative activity, stored for different periods of time, with respect to sugar consumption, cell growth and gum production.

According to Becking (22) the main sources of carbon for the cellular growth of *Beijerinckia sp.* are glucose, sucrose and fructose. Maldonade (9) observed that the amount of cell mass produce by *Beijerinckia sp* during fermentation, increased from 1.75 gL^{-1} to 4.8 gL^{-1}, when the glucose concentration was increased from0.5% to 10.0%, with a fixed nitrogen concentration. They also showed that sucrose was a better carbon source than glucose, when used at the same concentration.

In fermentations using *Beijerinckia sp* encapsulated in malt dextrin, there is a great variety of sugars, since according to Dokic et al. (23) industrially produced malt dextrin normally consists of a broad spectrum of saccharides. Malt dextrin can be produced by acid, acid/enzyme or double enzyme hydrolysis and the composition of the malt dextrin is influenced by the type of hydrolysis and conditions used .All fermentation broths were made using the same lot numbers for the components.

After drying, the fermentations carried out with bacteria encapsulated in 20 DE malt dextrin showed relatively greater cell growth, followed, in decreasing order, by the bacteria encapsulated in the blend (gum + 20 DE WM), 10 DE malt dextrin, glucose and capsul. According to the results in Table 5, even in the cells which survived 60 days of storage, a gradual decrease in the production of cell mass during fermentation occurred, using bacteria encapsulated in the different wall materials. On the other hand, there was a distinct behavior of the encapsulated bacteria during the same period, with respect to polysaccharide yield.

The analyses of variance for dry mass produced by the bacteria encapsulated in each WM after the different storage periods, are presented in Table 6. The group analysis of variance is presented in Table 7.

Table 6

Analysis of variance of the polynomial regression study of the dry weight data.

Cause of variation	GL	QM				
		WM1	WM2	WM3	WM4	WM5
Treatments	3	1.254700**	0.533231**	0.985208**	4.499142**	0.007511**
RL	1	3.089570**	1.537676**	1.626817**	11.043783*	0.018099**
RQ	1	0.503902**	0.031758n.s	1.093588**	0.307416ns	0.004196*
RC	1	0.170619**	0.000257n.s	0.235213*	2.146282*	0.000239n.s.
Residue	6	0.008542	0.006197	0.030358	0.205942	0.000536

* significant difference between treatments (95% of confidence)
** significant difference between treatments (99% of confidence)
n.s. non-significant difference between treatments

For the treatments, the F test was significant at the 1% level of probability for WM1, WM2, WM3, WM4 and WM5. Thus we rejected the null hypothesis (Ho) and showed that the storage period distinctly influenced the dependent variable dry weight for all the wall materials used. From the coefficients of determination (R^2) it was verified that the contribution of the linear model in the explanation of the phenomenon for WM 1, 2, 3, 4 and 5 corresponded to 82.10%, 96.12%, 55.0%, 82.0% and 80.32%, respectively. The regression equations where Yn is the weight of dry mass produced by the bacteria encapsulated in WMn and x the storage time, were the following:

$Y1 = 1.772845-0.02274\ x$

$Y2 = 1.551228- 0.0160\ x$

$Y3 = 1.521483 -0.0165\ x$

$Y4 = 3.80024- 0.04299\ x$

$Y5 = 2.581914-0.00174\ x$

Table 7

Group analysis of variance. Dry weight.

Cause of variation	GL	QM
Wall material (WM)	4	7.859878**
Period (P)	3	4.327584**
Interaction (WM x P)	12	0.735519**
Block (Wall material)	10	0.067735n.s.
Mean residue	30	0.050315

* significant difference between treatments (95% of confidence).
** significant difference between treatments (99% of confidence).
n.s. non-significant difference between treatments.

From the group analysis of variance, it was verified that the interaction (WM x P) showed a significant effect on the variable dry mass at the 1% level of probability, indicating that the storage period has a different effect on the dry mass produced by bacteria encapsulated in each WM. The wall material DE 20 malt dextrin conferred relatively greater retention of cell viability and stability with respect to cell activity (production of polysaccharide) during the storage period. The cell growth in the bio-polymer producing medium, showed that the encapsulated bacterial cells were capable of regenerating. With respect to the numbers of viable cells, the differences observed between the re-hydrated inocula encapsulated in the different wall materials after 24 hours of incubation remained, due to the kinetics of distinct growth patterns.

The carbon/nitrogen ratio (C/N ratio) in the growth medium plays an important role in polysaccharide production. The concentrations of total sugars in the dry weight of spray dried *Beijerinckia sp* microencapsulated in glucose, 10 DE malt dextrin, 20 DE malt dextrin, the blend (gum + 20 DE MD) and capsul were (% w/w): 88.0; 94.0; 94.0; 70.0 and 94.0 respectively (standard deviations of 0.042; 0.021; 0.015; 0.015 and 0.02). The initial concentration of total sugars in the medium for the production of bio-polymers by fermentation with spray dried *Beijerinckia sp* microencapsulated in the

different wall materials varied from 15.4 to 16.7 gL^{-1} (values lower than 0.2% w/v) and a fixed nitrogen concentration.

The analyses of variance for the amount of sugar consumed by the bacteria encapsulated in each wall material (WM) at the different storage times (days), are presented in Table 8. The group analysis of variance is presented in Table 9.

Table 8.

Analysis of variance with a polynomial regression study of the data for sugar consumption.

Cause of variation	GL	QM				
		WM1	WM2	WM3	WM4	WM5
Treatments	3	9.536875**	0.648333**	14.285833*	2.441875*	8.751742**
RL	1	5.013866n.s.	1.636743**	4.967599ns	1.70364n.s.	22.74513**
RQ	1	12.418432**	0.25319n.s.	37.59787**	0.32615n.s.	0.85114n.s.
RC	1	11.178272*	0.05506n.s.	0.292008ns	5.295873*	2.659041*
Residue	6	0.851042	0.063333	1.645833	0.491458	0.205992

* significant difference between treatments (95% of confidence).
** significant difference between treatments (99% of confidence).
n.s. non-significant difference between treatments.

For the treatments, the F test was significant at the 1% level of probability for WM1, WM2 and WM5, and for WM3 and WM4 at the 5% level of probability. From the coefficients of determination (R^2) it was verified that the contribution of the linear model in the explanation of the phenomenon for WM 2 and 5 corresponded to 84.15% and 86.63%, respectively, whilst the quadratic model contributed 87.73% to the explanation of the phenomenon in the case of WM 3. The regression equations, where Yn is the amount of sugar consumed by the bacteria encapsulated by WM n and x the storage time, were the following:

$Y2 = 7.9636-0.001655\ x$

$Y3 = 7.897994+0.307863\ x - 0.004495\ x^2$

Y5 = 8.401804+0.061693 x.

For the bacteria encapsulated in WM 1 and WM 4, a contribution by the cubic model in the explanation of the sugar consumption during fermentation was verified, although this has no practical justification.

Table 9

Group analysis of variance. Sugar consumption.

Cause of variation	GL	QM
Wall material (WM)	4	23.364727**
Period (P)	3	4.745259**
Interaction (WM x P)	12	7.729849**
Block (Wall material)	10	1.169298n.s.
Average residue	30	19.545950

* significant difference between treatments (95% of confidence).
** significant difference between treatments (99% of confidence).
n.s. non-significant difference between treatments.

In the group analysis of variance (Table 9), it was shown that both the type of wall material (WM) and the interaction (WM x Period) influenced the concentration of sugar consumed by the encapsulated bacteria, at a 1% level of probability.

Vendruscolo (24) showed that the type of sugar influenced the yield and quality of bio-polymer produced by *Beijerinckia sp* , the bio-polymer yield using sucrose in the fermentation being 2% greater than that obtained with glucose. The viscosity of the 6% aqueous solution of the biopolymer, measured at 25°C, was greater when sucrose was used in the fermentation.

The cell free fermented must (Figure 1) was viscous and cloudy for the 10 DE and 20 DE malt dextrin wall materials and for glucose, although for capsul and the blend (20 DE MD + gum Arabic), it was clear and transparent.

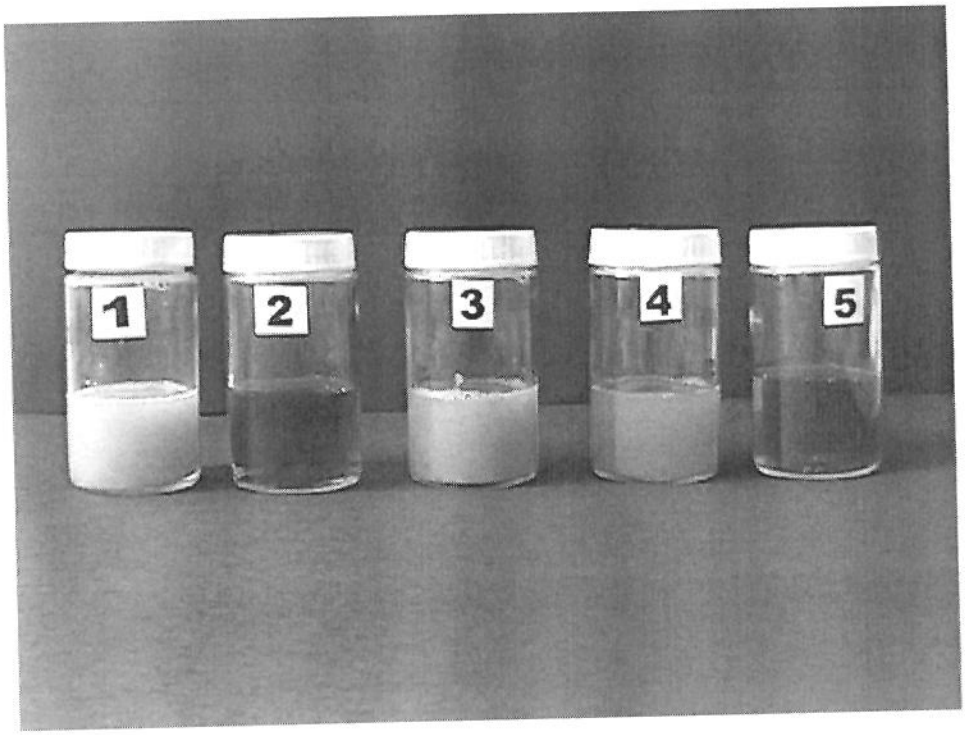

Fig. 1. Cell free must fermented by cells encapsulated in the different wall materials: 1) 10 DE malt dextrin 2) capsul, 3) glucose, 4) 20 DE malt dextrin and 5) the blend (gum Arabic + 20 DE malt dextrin).

The experiments carried out using the blend (20 DE malt dextrin + gum Arabic) resulted in inoculum with reasonable stability with respect to cell viability and fermentative activity, although after 24 hours of incubation, the characteristics of the polymers produced were distinct, a fact also observed with the wall material capsul. In the fermentation with *Beijerinckia sp* encapsulated in malt dextrin, there was a great diversity of sugars, and thus the pattern of polysaccharide production can vary, since

in general, the production of biopolymers depends on the type of microorganism and the composition of the medium.

Different growth phases and alterations of the growth medium, for example by using different substrates and limiting nutrients, do not influence the primary structure, but do affect the molecular mass and the yield of gum. Therefore the biopolymer product recovered from a culture represents a mixture of the gums produced in the different growth phases (25). According to Busta (26) the duration of the lag phase prior to the logarithmic growth phase of the culture, varies as a function of the degree of repair required, due to reversible cellular injuries suffered by the action of the spray-drier and subsequent storage. Starter cultures of bacteria prepared by spray drying show a longer lag phase before the onset of growth. Injured cells have extended lag periods before they start to grow, which means that injured cells will take longer to start their desirable activities in fermentations.

According to Casas et al. (27) both xanthan gum concentrations and the average molecular weight of xanthan increase during fermentation, under any conditions, and the molecular weight of a polymer has a direct influence on its rheological behavior, with larger molecules having higher viscosity. The biopolymer was recuperated from the fermentation liquid after 24 hours of incubation by precipitation with ethanol (Figure 2). From the characteristics of the ethanol precipitates, it was shown that the wall materials directly influenced the solubility/precipitation of the biopolymers formed after 24 hours of incubation. All the fermentation liquids were precipitated using the same lot numbers of solvent and the same conditions.

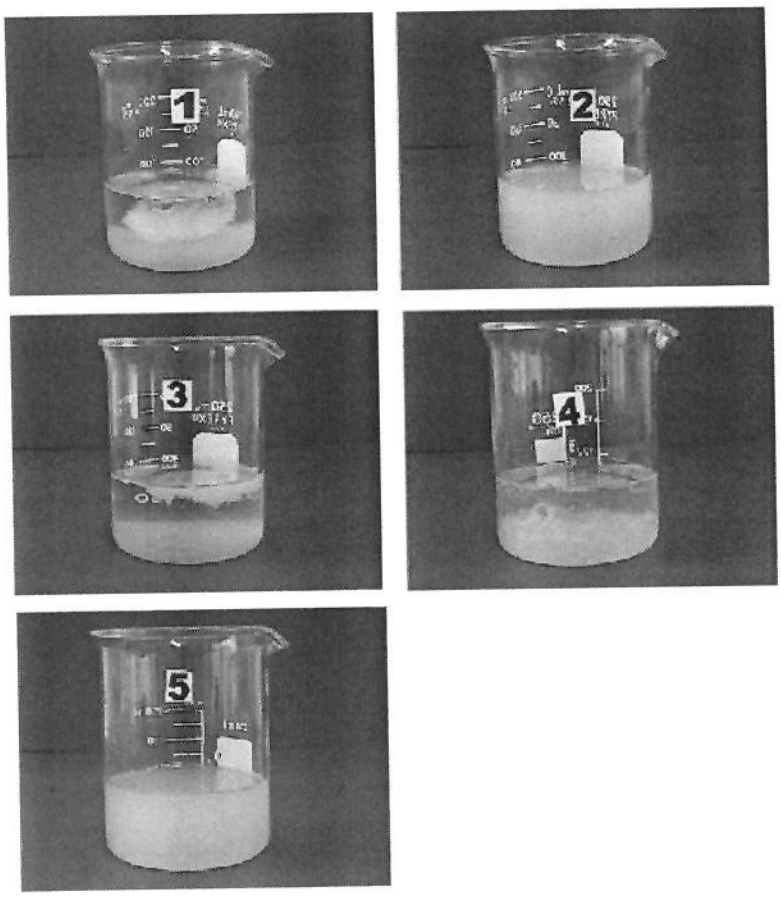

Fig. 2. Recuperation of biopolymer from the fermentation by bacteria encapsulated in the different wall materials: 1) 10 DE malt dextrin 10; 2) capsul; 3) glucose; 4) 20 DE malt dextrin; 5) the blend (gum Arabic + 20 DE malt dextrin).

The growing cultures of *Beijerinckia* entered the stationary phase after about 16 – 20 h. According to FU and Etzel (28) after the lag phase the production of metabolites by spray dried inoculum, obtained under different conditions of spray drying, are equivalent. The bacterial cells encapsulated in capsul and the blend (gum + malt dextrin) produced polysaccharides with distinct characteristics, probably due to the relatively longer lag phase. The polysaccharides obtained from the fermentation with bacteria encapsulated in capsul and in the blend (gum Arabic + 20 DE malt dextrin)

were recuperated using the following procedure: the polysaccharide was precipitated with ethanol after removing the biomass by centrifugation, dialyzed against water, re-precipitated and freeze-dried.

The analyses of variance for the amount of polysaccharide produced by the bacteria encapsulated in each wall material (WM) at the different storage periods (days), are shown in Table 10. The group analysis of variance is shown in Table 11.

Table 10

Analysis of variance with a polynomial regression study, for the polysaccharide data.

Cause of variation	GL	QM				
		WM1	WM2	WM3	WM4	WM5
Treatments	3	2.856288*	1.560031**	9.057433*	1.06517n.s.	0.547453*
RL	1	3.684124*	3.446763**	26.17998**	2.83408n.s.	0.842598*
RQ	1	3.145989*	1.231072**	0.97771n.s.	0.30918n.s.	0.15608n.s.
RC	1	1.738728n.s.	0.00225n.s.	0.01460n.s.	0.05223n.s.	0.643693*
Residue	6	0.296164	0.085847	1.029042	0.256292	0.096519

* significant difference between treatments (95% of confidence).
** significant difference between treatments (99% of confidence).
n.s. non-significant difference between treatments.

For the treatments, the F test was significant at the 1% level of probability for WM2 and WM3, and at the 5% level of probability for WM1 and WM5. It was not significant for WM4. Thus the storage period influenced the dependent variable production of biopolymer for the bacteria encapsulated in WM1, 2, 3 and 5. From the coefficients of determination (R^2), it was shown that the contribution of the linear model in the explanation of the phenomenon for WM 2, 3 and 5 corresponded to 73.65%, 96.35% and 51.30%, respectively, whilst the quadratic model contributed to the explanation of the phenomenon in the case of WM1. The regression equations, where Yn is the production of biopolymer by the bacteria encapsulated in WM n and x is the storage time, were the following:

$Y1 = 5.414213 - 0.10554\,x + 0.0013\,x^2$

$Y2 = 3.193584 - 0.02402\,x.$

$Y3 = 2.077443 - 0.066188\,x.$

$Y5 = 3.127105 - 0.011874\,x.$

Table 11

Group analysis of variance. Polysaccharides.

Cause of variation	GL	QM
Wall material (WM)	4	10.802913**
Period (P)	3	1.796393**
Interaction (WM x P)	12	3.322495**
Block (Wall material)	10	0.795788*
Average residue	30	0.352773

* significant difference between treatments (95% of confidence).
** significant difference between treatments (99% of confidence).
n.s. non-significant difference between treatments.

In the group analysis of variance it was shown that both the wall material (WM) and the interaction (WM x P) exerted an influence on the polysaccharide content at a level of 1% probability. The analyses of variance for yield with each WM at the different storage periods are shown in Table 12. The group analysis of variance is presented in Table 13.

Table 12

Analysis of variance with a polynomial regression study for the data of polymer yield.

Cause of variation	GL	QM				
		WM1	WM2	WM3	WM4	WM5
Treatments	3	0.002919*	0.160305**	0.060955**	0.00615n.s.	0.00470n.s.
RL	1	0.008199**	0.033837**	0.179588**	0.01131n.s.	0.00614n.s.
RQ	1	0.000155n.s	0.014086**	0.00313n.s.	0.00155n.s.	0.00670n.s.
RC	1	0.000403n.s.	0.00017n.s.	0.00014n.s.	0.00561n.s.	0.00125n.s.
Residue	6	0.000436	0.000831	0.003831	0.001797	0.001164

* significant difference between treatments (95% of confidence).
** significant difference between treatments (99% of confidence).
n.s. non-significant difference between treatments.

The F test was significant at the 1% level of probability for WM1, WM2 and WM3. The null hypothesis (Ho) was rejected and it was verified that the period influenced the variable dependent, yield of biopolymer, for these three wall materials. For WM4 and WM5, there was no significant for storage period of the variable under study. From the coefficients of determination (R^2), it was shown that the contribution of the linear model in the explication of the phenomenon for WM 1, 2 and 3, corresponded to 93.63%, 80.10% and 98.20%, respectively. The regression equations were as follows:

$Y1 = 0.459622 - 0.00117\ x$

$Y2 = 0.399367 - 0.00238\ x.$

$Y3 = 0.200803 - 0.005482\ x\ .$

Table 13

Group analysis of variance. Biopolymer yield.

Cause of variation	GL	QM
Wall material (WM)	4	0.034539**
Period (P)	3	0.008659**
Interaction (WM x P)	12	0.020524**
Block (wall material)	10	0.003698*
Average residue	30	0.001612

* significant difference between treatments (95% of confidence).
** significant difference between treatments (99% of confidence).
n.s. non-significant difference between treatments.

With respect to the yield of biopolymer, it was shown that both the wall material and the interaction (Wm x Storage period) were significant at the 1% level of probability, although the storage period had a distinct effect for each wall material.

An alternative method for the maintenance of *Beijerinckia sp* inoculum is the encapsulation with carbohydrates such as malt dextrin or glucose in a spray drier, which presented relatively greater viability and fermentative activity. Encapsulation technology offers new market opportunities for inoculum use in the fermentation biopolymer industry, but the total costs of the spray dried encapsulated powder will vary depending on the wall material chosen. Thus this factor must be one of the determining factors in the choice.

REFERENCES

1. Gandhi, H.P., Ray, R.M. and Patel, R.M.(1997), Carbohdyrate Polymers, **34**, 328-327.
2 . Teixeira, P.C., Castro, M.H. and Kirby, R.M.(1995), Journal of Food Protection , **57**, 934-936.
3 . Solano, G.P., Cervantes, M.A.S., Alvarado, M.A.G. and Jimenes, G.R. (2000), Journal of Food Process Engineering, **23**, 453-462.
4 . Valdez, G.F., Giori, G.S., Holgado, A.P.R. and Oliver, G.(1985), Applied and Environmental Microbiology, **49**, 413-415.
5. Diziezak, J.D.(1988), Food Technology, 136-141.

6 . Shaihidi, F. and Han, X.(1993), Critical Reviews in Food Science and Nutrition, **33**, 501-547.
7 . Zeller, B.L., Saleeb, F.Z. and Ludescher, R.D.(1999), Trends in Food Science & Technology, **9**, 389-394.
8 . Muro, M.A. and Luchi, M.R.(1989), Preservação de Microrganismos. Editora da Fundação Tropical de Pesquisas e Tecnologia "André Tosello". Campinas, SP, Brasil. 70p.
9 . Maldonade, I.R.(1996), Dissertation, Faculty of Food Engineering, Unicamp, Campinas, Brazil.
10 . Mary, P., Ochin, D. and Tailliez, R.(1985), Applied and Environmental Microbiology, **50**, 207-211.
11 . Reineccius, G.A.(1991), Food Technology, 144-149.
12 . Kidby, D., Sandford, P., Hernan, A. and Cadmus, M. (1977), Applied and Environmental Microbiology, **33**, 840-845.
13 . Miller, G.L.(1959), Analytical Chemistry, **31**, 426-428.
14 . Giordano, R.L.(1992), PhD thesis, Escola Politécnica da USP, Universidade de São Paulo, São Paulo, Brazil.
15 . SAS INSTITUTE Statistics Analyses Systems (SAS) (1989) : version 6-09.4.ed.Cary.NC. 2v. (Compact Disc).
16 . Hahn-Hägerdal, B.(1986), Enzyme Microbiology and Technology, **8**, 322-326.
17. Amiet-Charpentier, C., Gadille, P., Digats, B. and Benoit, J.P.(1998) Journal of Microencapsulation, **15**, 639-659.
18 . Prajapati, J.B., Shah, R.K. and Dave, J.M.(1987), Australian Journal of Dairy Technology, 17-21.
19 . Lievense, L.C., Riet K.(1994), Advances in Biochemical Bioengineering/Biotechnology, **51**, 71-89.
20. Desobry, S.A., Netto, F.M. and Labuza, T.P.(1999), Journal of Food Processing Preservation, ***23***, 39-55.
21 . Man, Y.C., Irwandi, J. and Abdullah, W.J.W.(1999), Journal of the Science of Food and Agriculture , 79,1075-1080.
22 . Becking, J.H.(1974) Soil Science, ***118***, 196-212.
23 . Dokic, P., Jakovljevic, J. and Dokic-Baucal, L.(1998) Colloids and surfaces A: Physicochemical and Engineering Aspects, **141**, 435-440.
24 . Vendruscolo, C.T.(1995) ,PhD thesis , Faculty of Engineering, Unicamp, Campinas, Brazil.
25 . Becker A.,Katzen F.,Puhler,A. and Ielpi L. (1998), Applied Microbiology and Biotechnology **50**,145-152.
26. Busta, F.F.(1976) Journal of Milk an Food Technology, **39**, 138-145.
27. Casas, J.A., Santos, V.E. and Garcia-Ochoa, F.(2000), Enzyme and Microbial Technology, **26**, 282-291.
28 . Fu, W., Etzel, M.R.(1995) Journal of Food Science, **60**, 195-200.

5. CHAPTER IV - EFFECT OF SPRAY-DRYING ON THE QUALITY OF ENCAPSULATED CELLS OF *BEIJERINCKIA SP.*

PROCESS BIOCHEMISTRY-Encaminhado para publicação

Effect of spray-drying on the quality of encapsulated cells of *Beijerinckia sp*

ABSTRACT

The survival and fermentative activity of *Beijerinckia sp* cultures encapsulated in malt dextrin by spray drying were determined under various conditions of spray drying and during the subsequent storage period of the products. The survival of *Beijerinckia sp* was greater with lower outlet air temperatures and with lower solids contents in the feed suspension during spray drying. Increasing either the spray dryer outlet air temperature or the feed solids content resulted in a decrease in the moisture content of the spray dried powders. A gradual decrease in viability was observed during storage for all treatments, although there was a relatively greater retention of fermenting activity in the inoculae obtained with lower outlet air temperatures and lower solids contents in the feed suspension.

Key words: Fermentation; Spray drying; *Beijerinckia*; Encapsulation.

1. Introduction

Heat treatments and drying processes are widely used in food technology in order to destroy and inhibit microorganisms and their spores. Drying is also used to maintain the viability of microorganisms before their use in fermentations or other biotechnologies [1].

Microbial polysaccharides of economic interest are usually produced at the industrial level by batch fermentations, and variations in the properties are frequently observed due to difficulties in preparing a consistent inoculum with high fermentative activity [2]. A new approach to this problem is to use dried bacteria as starters. These offer several advantages: they are easy to use when dealing with large quantities and they store well for long periods.

Dehydration decreases water availability inside the encapsulated cells obtained by spray drying such that the cells reach a dormant state during which the metabolism is slowed down and even stopped completely [3]. The spray drying of microorganisms dates back to 1914 to the work of Rogers [4] who dried milk cultures of lactic acid bacteria. Since then much research has been performed on the use of spray drying of bacteria in order to overcome the work involved in maintaining liquid stock cultures without loss in activity. Although this process proved promising, it has small been developed commercially. Reasons for this are mainly low survival rates during drying of the cultures, low stability under storage and the difficulty in re-hydrating the product. The encapsulation of microorganisms in carbohydrates can limit heat transfer when inoculae are dried by spray drying, minimizing the deleterious effects [5].

Microencapsulation by spray drying is widely used in the food industry due to its low cost. Spray drying is useful for the coating of heat sensitive food ingredients, since drying is very rapid and the core is heated to temperatures much lower than 100°C. The advantages of spray drying include the diversity and availability of the machinery and the variety of particle sizes, and one of its disadvantages is the oxidation of constituent compounds.

The encapsulation of selected bacteria has been investigated for use in soil environments in agriculture and bioremediation. However, elevated temperatures can be stressful to the microbial cell during encapsulation. Microbial cell survival during the spray drying procedure is dependent on many factors, including the strain of organism, the age of the culture, growth conditions and the spray drying conditions [6]. According to Johnson and Etzel [7] the spray drying parameters affecting cell survival include the air inlet and outlet temperatures and the type of atomization.

The overall objectives of this study were: (1) to evaluate the effects of the spray drying conditions on the survival and activity of *Beijerinckia*, and (2) to establish processing conditions to produce a spray dried inoculum of *Beijerinckia* with maximum fermenting activity.

2. Materials and methods

2.1. Materials

Malt dextrin (Corn Products International, Mogi Guaçu, Brazil). The malt dextrin used was of a commercial food grade.

2.2. Organism

Beijerinckia sp. isolated from sugar cane roots was maintained at 4°C on YM agar slants and transferred every 30 days according to Muro and Luchi [8].

2.3. Pre-culture and Production of Cell Concentrates.

Beijerinckia sp was grown in shaker flasks containing YM medium. The medium was adjusted to pH 6.5 and sterilized at 121°C for 15 min. The pre-culture was inoculated with two loops of culture from a slant and incubated at 25°C and 200 rpm for 24 hours in a New Brunswick model G25 shaker. This pre-culture was then used to inoculate a second broth (5% v/v). The second broth contained sugar cane molasses (1.5% w/v) and brewers yeast autolyzate (2.0% w/v) [9]. All fermentation broths were made using the same lot numbers of components. The cultures were incubated at 25°C and 200 rpm for 18 h. Stationary phase cells were harvested by centrifugation (22.300xg for 15 min. at 5°C), washed twice with sterile distilled water and re-centrifuged.

2.4. Spray Drying Experiments.

A cell pellet from 4L culture was re-suspended in 390 mL of a sterile solution of 10% (w/v) skim milk in accordance with Mary et al. [10]. This basal medium was aseptically supplemented with 15% (w/v) non-sterilized 20 dextrose equivalent (DE) malt dextrin. The bacterial suspension (450 mL) was incubated at 25°C and 100 rpm for 30 min to allow for cell adaptation and then sprayed. In preliminary experiments, this ratio resulted in a suspension with a total viability of $\geq 10^9$ cells/mL. The total solids content was 25% (w/v) on a dry weight basis of the bacterial cells. The proportion of the different ingredients in the formulation of the spray-dried cultures was determined from the results of the preliminary trials. A magnetic stirrer was applied to the bacterial suspensions to maintain a homogeneous feed during drying. Similar procedures were used to prepare bacterial suspensions with total solids contents of 33% (w/v) in which the basal medium was supplemented with 23% (w/v) of malt dextrin. The viscosity of the bacterial suspensions was determined.

A LabPlant model SD-04 (Leeds, UK) spray dryer was used. In studying the effect of various temperatures, all the conditions were fixed (atomizing air pressure 5 Kgf/cm^2, flow rate of the peristaltic pump 10 mL/min, nozzle diameter 1 mm). The inlet and outlet air temperatures were 135, 155, 175°C and 80, 90, 100°C respectively. The powder was collected in a single-cyclone separator. Similar procedures were used to obtain bacterial suspensions with total solids contents of 33% w/v.

The microencapsulating of bacteria under different conditions of encapsulation was performed in triplicate. The resultant spray-dried bacteria were homogenized and stored separately in 2g quantities in sealed sterile glass bottles at 4°C. Viability assays were performed on two bottles of each different powder.

2.5. Viability assays

Viability assays were carried out on the feed solutions just before drying, and on the various powders dried under different conditions immediately after drying, and during storage (after 21 and 60 days). Each sample of spray-dried bacteria was tested for its viability and fermentation activity after re-hydrating the powder. As the re-hydration conditions were determined from the results of the preliminary trials, these gave re-hydrated products with total viable counts between 10^7 and 10^8 cells per mL, a level of inoculum comparable to that obtained with the traditional loop transfer in the pre-culture method according to Kidby *et al.* [2]. For all treatments, 1g of spray-dried bacteria were re-hydrated in 10mL of a sterile solution of skim milk (10% w/v) at 25°C and 100 rpm in a shaker for 24h. One milliliter of the cell suspension was then subjected to serial dilutions in a sterile NaCl (0.85% w/v) solution, before transferring onto YM agar plates, and incubating at 25°C for 36h. The total viable cell number was expressed as colony forming units per gram (CFU/g) of dry powder. Survival was defined as the ratio of viable cells before and after drying at each storage time, converted to the base 10 logarithm (log No/N). The analyses carried out on the encapsulated samples included moisture, water activity and morphology.

2.6. Fermenting activity assay

A one-gram sample of powder was re-hydrated as described above and inoculated into sterile medium for gum production, at a rate of 5% (v/v). The broth, containing sucrose (1% w/v) and brewers yeast autolyzate (1% w/v), and pH previously adjusted to 6.5 prior to sterilization (121° C, 15 min) in accordance with Maldonade [9] was inoculated and incubated in a New Brunswick model G25 shaker, at 25°C and 200 rpm for 24h.

The culture broth was centrifuged at 22.300 x g at 5°C for 30 min to remove the cells. The supernatant was kept for substrate (residual sugars) and product (polysaccharide) analyses as described below.

2.7. Determination of cell dry weights

Microbial dry weights were monitored gravimetrically. The organisms harvested by centrifugation were re-suspended in distilled water to remove undesirable soluble material and re-centrifuged. The final pellet was again suspended in water, transferred to a pre-weighed Pyrex dish and dried to constant weight at 105°C. The results were expressed in concentration of biomass (g dry cell mass per liter of substrate).

The dry weight of spray dried *Beijerinchia* sp. microencapsulated in malt dextrin was determined in the following manner: The pre-weighed microencapsulated cells were washed with distilled water (at a ratio of 1 g of powder per 200 mL of water) to remove undesirable soluble material, and centrifuged at 22,300 x g at 5°C for 30 min. The resulting pellet was dried to constant weight at 105°C. All powders were analyzed twice. The supernatant was kept for sugar analyses as described below.

2.8. Polysaccharide concentration

The estimate of polysaccharide content was carried out by gravimetric analysis. The supernatant remaining after centrifuging the bacterial cells was precipitated with 4 volumes of ethanol. The precipitates were washed twice with ethanol. The precipitated gum was dried at 45°C to constant weight and the results expressed as g gum per liter of substrate. The yield of gum was given as g gum per g sugar consumed.

2.9. Sugar concentration

The sugar concentration in the cell free broth and substrate was measured using the dinitrosalicylic acid method [11] modified according to Giordano [12] and expressed as gram sugar consumed (initial sugar – residual sugar) per liter.

2.10. Moisture Content and Water Activity

The moisture content was determined in a vacuum oven at 100°C and 0.6atm, until constant weight was reached. The water activity was determined using an Aqualab Mod CX2 (Decagon, Pullman, Washington) at 25°C.

2.11. Scanning Electron Microscopy (SEM)

The powder samples were analyzed in a SEM in order to examine the external appearance of the particles. The encapsulated samples were fixed in stubs on an adhesive carbon paper and gold plated (metallizer MED 020 BALZERS, Lichtenstein, Austria). Images of the specimen were obtained at an accelerating voltage of 20 Kv (Scanning Electron Microscope DSM 900 Zeiss), and taken at 2,000 magnifications.

2.12. Analysis of Particle Size and Distribution

Samples were evaluated using the Anton Paar Lumosed PhotoSedimentometer (model A-8054, Retsch, Haan, Germany) with a sedimentation medium of isopropanol p.a..

2.13. Viscosity

The viscosity of the bacterial suspensions obtained using the different test formulations, was determined as described by Man et al. [13] using the Haake Rotovisco CV20 coaxial cylinder viscometer at 25°C.

2.14. Statistical Analysis

To analyze the bacteria encapsulated by spray drying in malt dextrin, obtained under the different processing conditions, with respect to the reduction in cell viability (log No/N) and fermentation activity during drying and storage, an analysis of variance was applied to the data obtained for each variable, using a polynomial orthogonal regression. A compound analysis of variance was then carried out, uniting

the individual experiments in a single analysis. The Statistics Analyses Systems (SAS) [14] software was used for the variance analyses.

3.Results and discussion

The data in table 1 show the effect of three different drying temperatures and two different total solids contents on the survival of *Beijerinckia* sp.

Table 1. Survival of *Beijerinckia sp* after spray drying at three different outlet-drying temperatures and two different total solids contents (levels of 20 DE malt dextrin).

Treat-ment	Total Solids content(%)	Drying temperatures Air in	content (%)	Moisture content (%)	Water activit (aw)	N° of viable cells Before drying (CFU/g solids)	After drying (CFU/g solids)	Log reduction of viable cells [log (N°/N)]
1	25	135	80	3.74	0.358	$1.40x10^9$	$1.28x10^8$	1.04
1				3.60	0.353	$1.58x10^9$	$1.46x10^8$	1.03
1				3.80	0.360	$1.75x10^9$	$2.49x10^8$	0.85
2	25	155	90	3.13	0.230	$1.49x10^9$	$2.78x10^7$	1.73
2				3.28	0.225	$1.30x10^9$	$1.80x10^7$	1.86
2				3.30	0.240	$1.81x10^9$	$2.50x10^7$	1.50
3	25	175	100	2.99	0.229	$1.80x10^9$	$1.69x10^7$	2.03
3				3.04	0.235	$2.15x10^9$	$1.98x10^7$	2.04
3				2.85	0.230	$1.62x10^9$	$1.45x10^7$	2.05
4	33	135	80	3.69	0.254	$1.50x10^9$	$6.90x10^6$	2.33
4				3.80	0.250	$1.90x10^9$	$9.80x10^6$	2.29
4				3.70	0.257	$1.42x10^9$	$5.80x10^6$	2.39
5	33	155	90	3.06	0.218	$1.58x10^9$	$3.50x10^6$	2.65
5				3.23	0.210	$2.10x10^9$	$5.90x10^6$	2.55
5				2.89	0.217	$1.70x10^9$	$4.20x10^6$	2.61
6	33	175	100	2.76	0.176	$1.94x10^9$	$8.30x10^7$	1.37
6				2.16	0.178	$1.35x10^9$	$5.40x10^7$	1.40
6				2.45	0.182	$1.70x10^9$	$7.60x10^6$	1.35

In terms of the log reductions of viable cells (log No/N), it can be seen from the data (table 1) that the reductions were lower at 25% solids than at 33% solids for outlet temperatures of for 80°C and 90°C. With an inlet air temperature of 175°C, a

greater decrease in the number of survivors at 25% solids content was observed even in those assays with relatively higher initial cell counts. It has been reported that higher cell concentrations in the feed suspension generally exhibit higher survival rates, a possible explanation for this effect being the release of intracellular components from damaged cells that could protect other cells and dead bacteria reducing the interfacial area between viable cells and the external medium, thus exerting a "shielding effect" against severe conditions of the external medium [6]. An attempt was made to standardize the initial cell count by producing cell concentrates using the same lot numbers of the medium components and similar procedures to those used in the fermentations. However, the differences in the initial viable cell counts in the suspensions used in the different treatments, do not explain the differences in survival of *Beijerinckia* obtained between the different treatments.

From Table 1 it can be seen that each 20°C increase in the inlet air temperature produced a 10°C increase in the outlet air temperature. At 25% solids content, 20°C increases in the inlet and 10°C increases in the outlet air temperatures, treatments 2 and 3 respectively, resulted in the populations of *Beijerinckia* suffering reductions of 1 logarithmic cycle in treatment 2 and 2 logarithmic cycles in treatment 3.

In treatment 6, which used the greatest solids content and highest temperatures (175°C/100°C inlet/outlet) the encapsulated *Beijerinckia* presented survival levels similar to those of treatments 1, 2 and 3 (25% total solids content). However, an increase of 20°C at the outlet temperature (treatment 6) presented a less deleterious effect on the survival of *Beijerinckia* than the same increase at the inlet temperature (treatment 5).

According to Elizondo and Labuza [15] during the spray drying of yeast with an outlet temperature above 80°C, a 10°C rise in temperature only caused a 30%

increase in the rate of kill, although an increase in temperature of 30°C resulted in a reduction of two log cycles.

Cell attenuation during spray drying may result from dehydration and thermal inactivation. The ratio of thermal inactivation to dehydration inactivation during spray drying decreases with decreasing outlet air temperature [7]. In a spray drying process, high inlet temperatures are necessary, precisely because the specific heat of evaporation must be supplied in a very short time. As a result of this, significant thermal inactivation of the cells can occur, despite the short residence time. Due to the evaporative cooling in the first part of the drying process, the survival of bacterial cells during spray drying is strongly correlated to the outlet temperature and not directly to the inlet temperature of the dryer. The highest survival rate is found at the lowest outlet temperatures .In the first stage of the drying process the particle surface remains wet (constant drying rate) and due to the evaporating water, the temperature will not exceed the 'wet-bulb' temperature. The thermal inactivation will be limited at this stage, because the high evaporation rate and the resulting wet-bulb temperature protect the cells from the higher air temperatures in the dryer. At the next stage of the drying process, the particle surface becomes dry and the temperature increases maximally to the inlet temperature of the dryer. In this stage the evaporative cooling is no longer available and the thermal inactivation will increase, but due to the lower water concentrations, the cells will show higher heat resistance [16].

In this experiment, the residual cell viability decreased with increasing solids content of the spray-drying medium. These results are qualitatively similar to those obtained by Johnson and Etzel [17] in their study on the effects of spray drying on two bacteria: *Serratia marcescens* and *Staphylococcus*, in which the authors showed that decreasing the outlet air temperature increased the percent cell viability, whereas

altering the inlet air temperature had little effect. Increasing the total solids content of the feed solution decreased the percentage of surviving bacteria. This last finding was unexpected because batch-kinetics studies demonstrated that heat resistance increased with increasing total solids content. They attributed the decrease in cell viability to a decrease in the drying rate with feed solutions of higher solids content, resulting in a higher moisture content at the center of the dried particle, since higher moisture contents decrease heat resistance.

Samples dried at lower temperatures had greater moisture contents and aw than those dried at higher temperatures. According to To and Etzel [18] increasing the temperature increases heat inactivation. However, increasing the solids content decreases the water activity, and thus the water may not be sufficiently available for the inactivation reactions. Thus dehydration may protect the cells from thermal inactivation through such a mechanism.

Figure 1 shows the scanning electron photomicrographs of the microstructure of the spray dried bacterial powders. In general, the structure was more or less spherical, with small differences in particle structure between the products obtained using different conditions of spray drying.

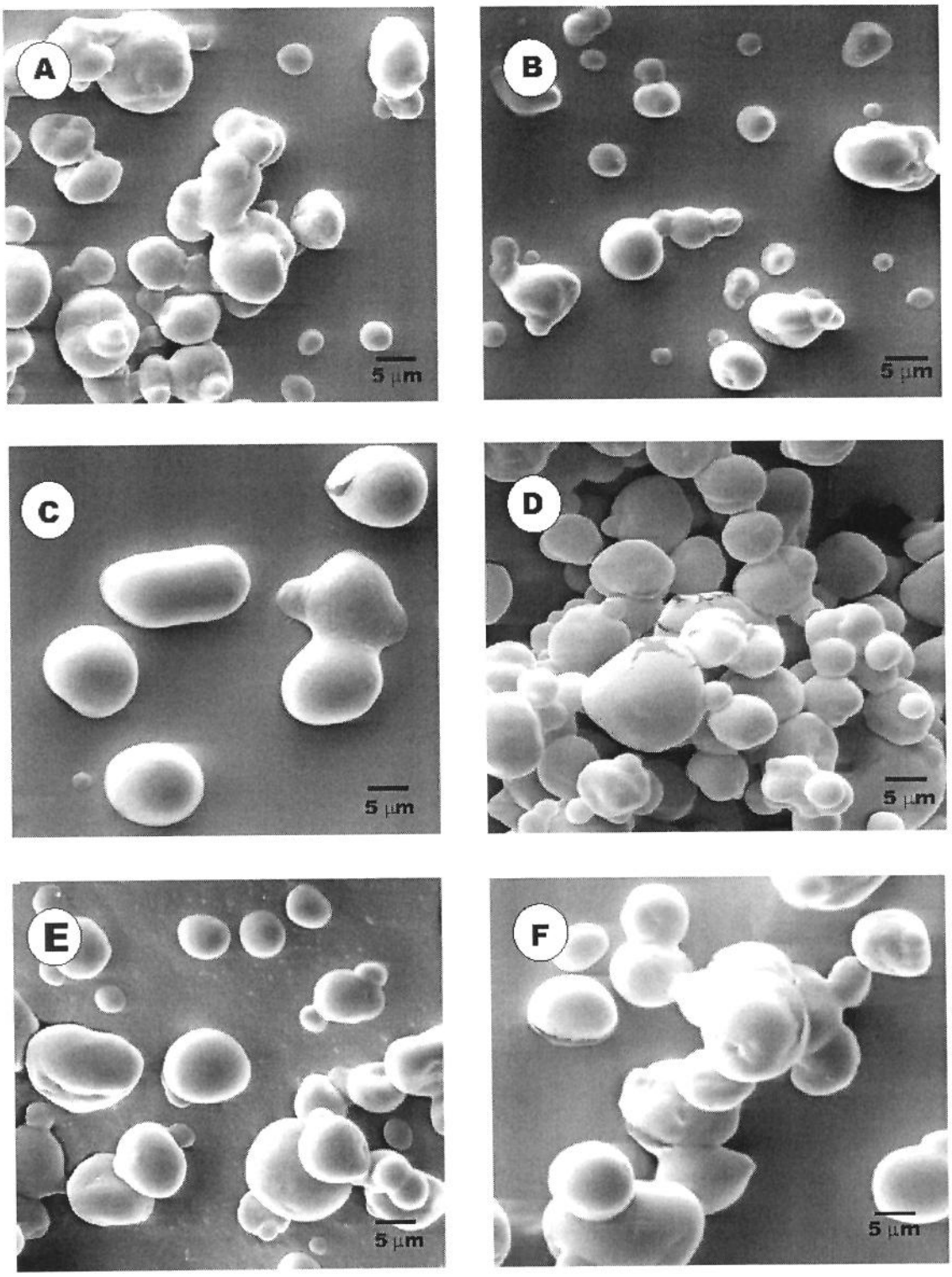

Fig.1. Scanning electron photomicrographs of encapsulated spray-dried *Beijerinckia* powder obtained under different conditions. Treatments: at 25% solids (a) 135°C; (b) 155°C and (c) 175°C, and at 33% solids (d) 135°C; (e) 155°C and (f) 175°C.

During microencapsulation, the capsule size distribution is a function of the process parameters and the characteristics of the cell suspension. The viscosity is the characteristic of the suspension considerably altered by changing the solids concentration that an increase in solids concentration in the suspension results in larger particles [16]. Table 2 shows the final characteristics of the bacterial feed suspensions and spray dried microencapsulated cells.

Table 2. Process parameters, characteristics of bacterial feed suspensions and spray dried microencapsulated cells.

Temperat. air inlet	% solids suspension	Viscosity of suspension 20°C (cp)	Mean Size (μm)	Particles of size range (capsules)	Log reduction of viable cells
135	25	3,8	20.60	5 – 50	0.97
155	25	4,2	21.45	5 – 50	1.70
175	25	3,7	20.90	2.5 – 45	2.04
135	33	6,1	19.50	10 – 50	2.34
155	33	5,5	22.40	5 – 50	2.60
175	33	6,6	17.00	2.5 – 45	1.37

Capsules from all powder samples showed the same general size distribution. The average size of the particles was 20 μm with variations from 5 to 50 μm. The mean particle sizes at 25% solids were 20.6; 21.45 and 20.9 μm for inlet air temperatures of 135, 155 and 175°C respectively. The mean particle sizes at 33% solids were 19.5; 22.4 and 17.0 μm for inlet air temperatures of 135, 155 and 175°C respectively.

Studies carried out by Daemen and Stege [19] on the effects of particle size on the destruction of bacteria and enzymes in spray dried skim milk, showed that the drying time approximately increases with the square of the particle size, allowing more

time for inactivation. They expected the destruction of bacteria and enzymes to be greater in larger particles. Instead, larger particles had greater vacuole volumes which protected the bacteria and enzymes against high temperatures during drying. Thus inactivation during spray drying results from a complicated balance between the vacuole volume and the size of the particle.

The results presented in table 2 show that the average particle sizes with 25% and 33% solids, increased with increase in inlet air temperature from 135 to 155°C, together with a reduction in viable cells after drying. However, with an inlet air temperature of 175°C, there was a decrease in particle size.

Increases in solids content result in increases in suspension viscosity. According to Ré [20] more concentrated feed suspensions produce larger particles. These particles present relatively smaller ratios between the surface area and the volume and greater core retention, thus requiring longer drying times, which can reduce cell viability due to the longer contact time of the particles with the hot air. On the other hand, increasing the air inlet temperature increases drying speed. Thus the retention of bacterial viability during drying under different processing conditions, may be a result of the effects of increases in viscosity and in drying speed.

Effects of storage time on bacterial survival

Storage tests were carried out over a two-month period on powders stored at 4°C. Significant differences in the viable cell counts were observed, as shown in Table 3.

Table 3. Changes in cell survival in the spray dried powders during storage at 4°C.

	log (No/N) Period (days)		
Treatment	1 (0)	2 (21)	3 (60)
1	1.04	1.59	2.08
	1.03	1.48	1.92
	0.85	1.22	1.80
2	1.73	2.12	2.40
	1.86	2.36	2.75
	1.50	1.93	2.01
3	2.03	2.50	3.00
	2.04	2.49	2.93
	2.05	2.56	3.07
4	2.33	3.50	3.63
	2.29	3.45	3.54
	2.39	3.53	3.69
5	2.65	3.93	4.21
	2.55	3.65	3.92
	2.61	3.95	4.13
6	1.37	2.43	2.60
	1.40	2.59	2.87
	1.35	2.48	2.69

All samples, irrespective of treatment, showed a gradual decrease in cell survival during storage. Generally, as the inlet air temperature increased, there was a more rapid decrease in relative survival during storage. The greatest decrease in relative survival occurred with the cells spray-dried at the highest inlet air temperature (175°C).

According to Fu and Etzel [21] two factors may have combined to cause these cells to have the greatest decrease in relative survival during storage: having the greatest extent of un-repairable injuries with the greatest percent of death after drying, and also having the lowest moisture contents. According to Champagne et al. [22] the

accentuated removal of water may expose cracks and void spaces in the dried particles to air, causing oxidative degeneration of the proteins and cellular compounds. In treatment 6 there was greater bacterial survival during drying and a lower aw, thus these factors could have determined the relatively greater retention of cell viability.

Fermenting Activity

The sugar consumption and gum production by the encapsulated inoculae obtained from the different treatments and stored for different intervals were determined, the retention of fermenting activity during storage being compared with the reference value of the activity after drying (zero time). All experiments were conducted after a period of reactivation, since the lag period of the cells increases as a result of the drying process. According to Teixeira et al.[23] starter cultures of lactic acid bacteria prepared by spray drying cannot be used for direct inoculation in dairy fermentations. This is largely due, not to a decrease in viability, but to an increase in the lag phase before the onset of growth. Injured cells have extended lag periods before they start to grow, which means that injured cells will take longer to start their desirable activities in fermentations. The results obtained for the fermentation activity of *Beijerinckia* encapsulated in malt dextrin under different conditions of spray drying, during storage, are summarized in table 4.

Table 4. Fermenting activity of *Beijerinckia*, encapsulated by different treatments, during storage.

Treatment	Period (days)								
	1 (0)			2 (21)			3 (60)		
	Cell mass g L^{-1}	Sugar g L^{-1}	Yield gum	Cell mass g L^{-1}	Sugar g L^{-1}	Yield gum	Cell mass g L^{-1}	Sugar g L^{-1}	Yield gum
1	3.71	11.50	0.46	1.70	9.11	0.50	1.49	10.00	0.53
	3.77	12.33	0.42	1.88	9.73	0.46	1.67	8.87	0.49
	4.25	12.86	0.40	2.27	11.05	0.42	1.91	10.50	0.47
2	3.07	8.52	0.32	3.13	6.85	0.18	3.62	7.00	n.d.
	3.11	8.63	0.34	3.16	7.00	0.23	3.72	7.20	n.d.
	3.27	8.85	0.28	3.55	8.05	0.16	3.74	8.00	n.d.
3	3.37	11.15	0.14	3.65	8.61	0.16	3.82	7.86	n.d.
	3.35	10.95	0.15	3.53	6.85	0.15	3.85	7.07	n.d.
	3.60	12.10	0.18	3.83	9.05	0.17	3.98	8.27	n.d.
4	3.75	9.81	0.44	2.31	8.85	n.d.	2.27	8.79	n.d.
	3.67	9.15	0.39	2.28	8.50	n.d.	2.27	8.47	n.d.
	3.79	10.20	0.45	2.35	8.98	n.d.	2.32	8.85	n.d.
5	2.65	9.80	0.60	2.15	8.15	n.d.	1.83	7.23	n.d.
	2.40	8.91	0.48	1.89	7.20	n.d.	1.63	6.50	n.d.
	2.58	9.13	0.53	2.05	7.66	n.d.	1.69	6.90	n.d.
6	3.30	3.98	n.d.	2.98	3.70	n.d.	2.98	3.42	n.d.
	5.10	4.73	n.d.	4.60	3.99	n.d.	3.54	3.80	n.d.
	4.50	4.15	n.d.	4.33	4.10	n.d.	3.21	3.54	n.d.

The results for the fermentative activity of the inoculae encapsulated under different conditions of spray drying and then stored, show that they presented different patterns of cell growth and polysaccharide production during the storage period.

The *Beijerinckia* encapsulated at 25% solids content in the feed suspension and using the lowest drying temperature, presented the greatest retention of fermentative activity during storage, although there was a decrease in cellular growth. In treatments

2 and 3 (25% solids content) the encapsulated bacteria showed greater stability with respect to cell growth during fermentation, but there was a reduction in the production of biopolymers and consequent decrease in sugar consumption.

According to Gassem et al. [24] the association of cell mass with polysaccharide production can be explained as follows: when the cells grow slowly, the synthesis of polymers in the cell wall is lower, increasing the availability of the isoprene phosphate available for exopolymer synthesis.

The inoculae obtained in treatments 4 and 5 presented a more accentuated reduction in polysaccharide and biomass production as compared to the reduction in sugar consumption. Although in experiment 6, the bacteria encapsulated with 33% solids content and a higher drying temperature, presented relatively greater stabity in the production of biomass during fermentation, the same was observed with respect to the levels of sugar consumption, the production of biopolymer was not detected under the conditions used for fermentation and polymer recuperation.

The variance analyses for the viability and fermentative activity of the bacteria encapsulated under different conditions after the different storage periods (days), are shown in tables 5 and 6 respectively.

Table 5. Summary of the individual variance analyses after the different storage periods for the variable log (No/N).

		QM		
Cause of variation	GL	Period 1	Period 2	Period 3
Blocks	2	0.0093	0.3350	0.0159
Temperature	2	0.4424*	0.2459 $^{n.s.}$	0.3604 $^{n.s.}$
% Solids	1	1.2853*	5.9168*	4.8257*
% Solids x Temperature	2	1.7012*	1.6908*	1.9471*
Residues	10	0.0083	0.0599	0.0385

* significant difference between treatments (99% confidence level)
$^{n.s.}$ non-significant difference between treatments

From the results of the variance analyses, a significant effect can be observed at the 1% level of probability for the solids level and interaction (% solids x temperature) with respect to cell viability after the three storage periods. The factor air-drying temperature was shown to have a significant effect on cell viability only during encapsulation.

Table 6. Summary of the individual variance analyses after the different storage periods (P_n), for fermentative activity of the encapsulated bacteria with respect to: a) cell mass content; b) sugar consumption; c) biopolymer concentration and d) biopolymer yield.

(a)

Cause of variation	GL	QM		
		P_1	P_2	P_3
Blocks	2	0.2036	0.2542	0.0009
Temperature	2	2.0033**	4.4815**	0.10005**
% Solids	1	$0.0032^{n.s.}$	$0.1721^{n.s.}$	0.0394**
% Solids x Temperature	2	0.8517*	1.2523**	0.0825**
Residues	10	0.1549	0.1360	0.0009

(b)

Cause of variation	GL	.....QM		
		P_1	P_2	P_3
Blocks	2	0.3642	1.3525	0.7231
Temperature	2	15.0909**	16.6348**	19.4947**
% Solids	1	40.5900**	12.7849**	16.5696**
% Solids x Temperature	2	22..6618**	8.2495*	5.7024**
Residues	10	0.2325	0,3884	0.1679

(c)

Cause of variation	GL	QM		
		P_1	P_2	P_3
Blocks	2	0.1952	0.0066	0.0387
Temperature	2	21.7910**	5.0575**	11.7451**
% Solids	1	1.7174*	26.0642**	11.7451**
% Solids x Temperature	2	3.1255**	5.0575**	11.7451**
Residues	10	0.1243	0.0220	0.0387

(d)

Cause of variation	GL	QM		
		P_1	P_2	P_3
Blocks	2	0.0014	0.0004	0.0002
Temperature	2	0.2415**	0.0409**	0.1233**
% Solids	1	$0.0022^{n.s.}$	0.3280**	0.1233**
% Solids x Temperature	2	0.0547**	0.0409**	0.1233**
Residues	10	0.0011	0.0005	0.0002

* significant difference between treatments (95% confidence level)
** significant difference between treatments (99% confidence level)
$^{n.s.}$ non-significant difference between treatments

From the results of the variance analyses presented in table 6, a significant effect can be seen for the drying temperature and the interaction (% solids x temperature) with respect to the fermentative activity (cell and polymer concentrations, sugar consumption and biopolymer yield) during both encapsulation and storage. The solids content had a significant effect on the variables sugar consumption and biopolymer concentration during encapsulation and storage, but with respect to the variable cell concentration, the effect of the solids content was only significant during storage and for the variable biopolymer yield, the effect of the solids content was only significant during encapsulation.

Group analysis of the 4 variables after the 3 periods.

From the individual variance analyses of fermentative activity after the three storage periods (Table 6), it can be seen that the cell concentration did not show homogenous variance, so the data were transformed to the logarithmic scale for the variance to become more stable. Subsequently group variance analyses were carried out for the variables log No/N, sugar consumption and biopolymer concentration and yield, after the three storage periods, as shown in table 7.

Table 7. Summary of the group variance analyses for the variables cell viability (log No/N) and fermentative activity (biopolymer conc., sugar consumption and yield) of the encapsulated bacteria after the 3 storage periods.

Cause of variation	GL	QM			
		log No/N	Sugar consumption	Biopolymer Concentrat.	Biopolymer yield
Period	2	5.8991**	19.2144**	22.8737**	0.2549**
Blocks	6	0.0196 n.s.	0.8133 n.s.	0.0802 n.s.	0.0007 n.s.
Temperature	2	1.0347**	51.0189**	32.1092**	0.2788**
% Solids	1	11.0704**	65.4941**	32.2944**	0.2563**
% Solids x	2	5.2333**	33.0884**	13.4864**	0.1260**
Temperature	4	0.079 n.s.	0.1008 n.s.	3.2422**	0.0635**
Period x Temperature	2	0.4787**	2.2252*	3.6162**	0.0987**
Period x %Solids	4	0.0529 n.s.	1.7626*	3.2209**	0.0465**
Per. x % Sol. x Temp.	30	0.0356	0.22495	0.0617	0.0006
Residue					

* significant difference between treatments (95% confidence level).
** significant difference between treatments (99% confidence level).
n.s. non-significant difference between treatments.

From the results shown in table 7, it can be seen that the storage time exerted a significant effect at the 1% level of probability, on the viability (log No/N) and the fermentative activity (sugar consumption, biopolymer concentration and yield) of the *Beijerinckia* encapsulated in malt dextrin in the different treatments. The air drying temperature, percent solids in the cell suspension and the interaction (storage time x percent solids) showed significant effects at a level of probability equal or lower than 5%, on the fermentative activity and viability of the inoculae encapsulated in malt dextrin.

Tukey's test was applied to the factors shown to be significant in the group variance analyses, to check at which levels these factors showed significant differences, as can be seen in table 8. Tukey's test was not applied to the solids content, since only 2 levels of concentration were studied and these presented

significantly different effects on the viability and fermentative activity of the encapsulated bacteria.

Table 8. Summary of Tukey's test for the different levels of the variables.

		Variables			
Variation	Levels	log No/N	Sugar consumption	Biopolymer concentrat.	Biopolymer yield
Temperature	135	B	A	A	A
	155	A	B	B	B
	175	B	C	C	C
Solids	30	A	A	A	A
	40	B	B	B	B
Period	0	C	A	A	A
	21	B	B	B	B
	60	A	B	C	C

It was shown that the three storage periods differed from each other for the variables log No/NF, and biopolymer concentration and yield. The level of sugar consumption for the encapsulated cells obtained from the different treatments, only differed after the first storage period. The air drying temperatures of 135°C and 175°C did not differ with respect to the cell viability of the encapsulated cells obtained and stored, whilst the fermentative activity of the encapsulated inoculae at the three air drying temperatures, did vary between them.

The lowest air-drying temperature and lowest solids content during the encapsulation of *Beijerinckia* in 20 DE malt dextrin conferred relatively greater retention of viability and fermentative activity on the cultures. According to Paul et al. [3] water loss changes the overall quality of the cells by physical and structural modifications as well as by degradation of molecules via enzymatic and chemical reactions, since all the reaction rates are greatly influenced by water activity (aw).

However, the different experimental conditions did not result in such great differences in aw, sufficient to explain the differences in viability and fermentative activity determined.

According to the same authors, based on studies of the characteristics of *Azospirillum lipoferum*, it was shown that cell survival during storage was not affected by aw values in the range 0 – 0.55. Thus more studies are required to identify heat damage to the different inoculae, resulting from the different treatments, which determined the different physiological reactions observed with respect to the fermentative activity and cell viability.

4.Conclusion

The bacterial cells are likely to lose their viability and activity (biopolymer production capacity) during spray drying and the subsequent storage period. The choice of appropriate encapsulation conditions for the spray drying of the cell cultures is one of the most important factors determining the degree of survival and activity of the microorganisms during and after spray-drying of *Beijerinckia*. However, according to Reineccius [25] the choice of the spray drying encapsulation conditions depends on the microorganism to be dehydrated and the encapsulation material.

REFERENCES

[1] MARECHAL, P.A., MARNAÑON, M.I., POIRIER, I., GERVAIS, P. The importance of the kinetics of application of physical stresses on the viability of microorganisms: significance for minimal food processing. Trends in Food Science & Technology, 1999; 10:15-20.

[2] KIDBY, D., SANDFORD, P., HERMAN, A. and CADMUS, H.Maintenance procedures for the curtailment of genetic instability: *Xanthomonas campestris* NRRL B-1459. Applied and Environmental Microbiology, 1977; 33: 840-845.

[3] PAUL, E.; FAGES, J.; BLANC, P.; GOMA, G. and PAREILLEUX, A. Survival of alginate-entrapped cells of *Azospirillum lipoferum* during dehydration and storage in relations to water properties. Applied Microbiology and Biotechnology., 1993; 40:34-39.

[4] ROGERS, L.A. The preparation of dried cultures. Journal of Infectious Diseases, 1914; 14:100-123.

[5] TEIXEIRA, P.; CASTRO, H. and KIRBY, R. Spray drying as a method for preparing concentrated cultures of *Lactobacillus bulgaricus*. Journal of Applied Bacteriology, 1995;78: 456-462.

[6] LIEVENSE, L.C. and RIET, K. van't Convective Drying of Bacteria II. Advances in Biohemical Enginerring/Biotechnology, 1994; 51:72-89.

[7] JOHNSON, J.A.C. and ETZEL, M.R. Inactivation of lactic acid bacteria during spray drying. Aiche Symposium, 1993; 89: 89-107.

[8] MURO, M.A. and LUCHI, M.R.. Preservação de Microrganismos. Editora da Fundação Tropical de Pesquisas e Tecnologia "André Tosello". Campinas, SP, Brasil , 1989, 70p.

[9] Maldonade, I.R., Contribuição ao estudo dos parâmetros de fermentação por *Beijerinckia* sp. Dissertation , Faculdade de Engenharia de Alimentos, Unicamp, Campinas, Brasil,1996.

[10] Mary, P., Moschetto, N. and Tailliez, R., Production and survival during storage of spray-dried *Bradyrhizobium japonicum* cell concentrates. Journal of Applied Bacteriology, 1993, 74: 340-344.

[11] Miller, G.L., Use of dinitrosalicylic acid reagent for determination of reducing sugar. Analytical Chemistry, 1959, 31: 426-428.

[12] Giordano, R.L., Estudo da coimobilização de glicoamilase e levedura para a fermentação alcoólica contínua de matéria-prima amilácea. PhD Thesis, Escola Politécnica, Universidade de São Paulo, São Paulo, Brazil, 1992.

[13] Man, Y.C., Irwandi, J. and Abdullah, W.J.W., Effect of different types of maltodextrin and drying methods on physico-chemical and sensory properties of encapsulated durian flavour. Journal of the Science of Food and Agriculture, 1999, 79:1075-1080.

[14] SAS INSTITUTE. Statistics Analyses Systems (SAS): version 6-09,4ed. , 2v. (Compact Disc) ,Cary,NC, 1989.

[15] ELIZONDO, H. and LABUZA, T.P.V., Death kinetics of yeast in spray drying. Biotechnology and Bioengineering, 1974, 16 :1245-1259.

[16] LIEVENSE, L.C. and RIET, K. van't , Convective Drying of Bacteria I. Advances in Biochemical Enginerring/Biotechnology, 1993, 50: 46-63.

[17] JOHNSON, J.A.C. and ETZEL, M.R., Properties of *Lactobacillus helveticus* CNRZ-32 attenuated by spray-drying, freeze-drying, or freezing. Journal of Dairy Science, 1995, 78:761-768.

[18] TO, B.C.S. and ETZEL, M.R., Survival of *Brevibacterium linens* (ATCC 9174) after, spray drying, freeze drying, or freezing. Journal of Food Science, 1997, 62 :167-170, 189.

[19] DAEMEN, A.L.H. and STEGE, H.J. van der, The destruction of enzymes and bacteria during the spray-drying of milk and whey. Netherland Milk Dairy Journal, 1982, 36:211-229.

[20] RÉ, M.I., Microencapsulation by spray drying. Drying Technology, 1998, 16, 413-425.

[21] FU, W.-Y. and ETZEL, M.R., 1995. Spray of *Lactococcus lactis spp.* C_2 and cellular injury. Journal of Food Science, 60, 195-200.

[22] CHAMPAGNE, C.P.; GARDNER, N.; BROCHU, E.; BEAULIEU, Y., 1991. The freeze-drying of lactic acid bacteria. Canadian Institute of Food Science and Technology Journal, 24,118-128.

[23] TEIXEIRA, P.; CASTRO, H.; MOHÁCSI-FARKAS, C. and KIRBY, R., 1997. Identification of sites of injury in *Lactobacillus bulgaricus* during heat stress. Journal of Applied Microbiology, 83, 219-226.

[24] GASSEM, M.A.; SCHIMIDT, K.A. and FRANK, J.F., 1997. Exopolysaccharide production from whey lactose by fermentation with *Lactobacillus delbrueckii spp. bulgaricus*. Journal of Food Science, 62, 171-173, 200.

[25]REINECCIUS, G.A., 1989. Flavor Encapsulated on Food. Reviews International, 5, 147-176.

6. CHAPTER V - EXOPOLYSACCHARIDE PRODUCTION BY ENCAPSULATED *BEIJERINCKIA* CULTURES.

Process Biochemistry - Encaminhado para publicação

EXOPOLYSACCHARIDE PRODUCTION BY ENCAPSULATED *BEIJERINCKIA* CULTURES.

ABSTRACT

It is desirable that spray-dried microorganisms maintain their viability and activity during encapsulation, a possibility that depends on the strain and the conditions of encapsulation. A wide variety of modifications may occur in cells after encapsulation The intention of this work was to examine the effects of encapsulation in malt dextrin on the production of biopolymer, comparing encapsulated and non-encapsulated *Beijerinckia* cells. From the characterization of the biopolymers and study of the fermentation kinetics, it was shown that the encapsulation of *Beijerinckia* mainly influenced cell growth and biopolymer production.

Keywords: Fermentation, Spray drying, *Beijerinckia*, Encapsulation.

1. INTRODUCTION

Microbial polysaccharides of prominent economic interest are usually produced at the industrial level by batch fermentation, which is difficult to control. Variations in the biopolymer properties, e.g., rheological parameters, are frequently observed. The lack of reproducibility in gum fermentations was associated with inconsistencies in inoculum development and strain instability, factors which increase with the number of culture propagation steps required for larger scales of operation. Problems associated with fermentation reproducibility at the industrial scale may result in inconsistent productivity, yield and quality, all of which can

translate into financial losses. However reproducible results can be obtained by optimizing the inoculation methodology, the medium composition and the operational conditions. Several investigators have considered the possibility of drying large quantities of bacterial cultures, with the idea of using the dried product to replace the usual liquid bulk starter in the production of gum by fermentation. For this purpose spray drying would be preferable to freeze drying because of the lower cost [1]. Spray drying is a technology widely used in the food industry, and it is the most common micro-encapsulation technique in use. Many biochemical processes use encapsulated cells. A typical process would be that of a concentrated starter culture in the presence of a protective additive, being suspended in a medium composed of the encapsulating matrix and solvent. This suspension is pumped into a chamber where it is atomized into a very fine spray of droplets. At the same time a flow of very hot dry air is injected together with the droplets and dries them almost instantaneously. The dried particles are collected and cooled. In most spray driers, the total contact time of the particles with the hot air is less than 20-30 sec, thus giving excellent retention of the components [2].

Previous studies have demonstrated changes in microbial growth and substrate utilization activity. It is desirable that spray dried microorganisms maintain their viability and activity during encapsulation, a possibility that depends on the strain and the conditions of encapsulation. A wide variety of modifications may occur in cells after encapsulation, for example, the conditions for optimal growth are often different from those of non-encapsulated cells, while product yields and lag time may be affected [3].

The aim of this study was to examine the effects of encapsulation in malt dextrin on the production of biopolymer by *Beijerinckia*. The study focused on a comparison of growth, substrate utilization and polysaccharide production between encapsulated and non-encapsulated *Beijerinckia*.

2. Material and methods

2.1. *Materials*

The malt dextrin (Corn Products International, Mogi Guaçu, Brazil) used was of commercial food grade.

2.2. *Organism*

Beijerinckia sp isolated from sugar cane roots by Vendruscolo [4], was maintained at 4°C on YM agar slants and transferred every 30 days according to Muro and Luchi[5].

2.3. *Pre-culture and Cell Production*

Beijerinckia was grown in shaker flasks containing YM medium. The medium was adjusted to pH 6.5 and sterilized at 121°C for 15 min. The pre-culture was inoculated with two loops of culture from a slant and incubated at 25°C and 200 rpm for 24 hours in a New Brunswick model G 25 shaker. This pre-culture was then used to inoculate a second broth in the proportion of 5% v/v. The second broth contained sugar cane molasses (1.5% w/v) and brewers yeast autolyzate

(2.0% w/v) [6]. All fermentation broths were made using the same lot numbers of components. The cultures were incubated at 25°C and 200 rpm for 18 h. Stationary phase cells were concentrated by centrifugation (22.300xg for 15 min. at 5°C) and washed twice with sterile distilled water.

The production of *Beijerinckia* cells for the fermentation assays with non-encapsulated cells was accomplished using the same conditions used for the production of encapsulated cells, as described above. After 18 hours of incubation, aliquots were removed for the viability assay and then used for inoculation into the biopolymer-producing medium in the fermenting activity assay.

2.4. *Spray Drying Experiments*

A cell pellet from 4L of culture was re-suspended in 390 mL of a 10% (w/v) sterile aqueous solution of skim milk according to Mary et al. [7]. This basal medium was aseptically supplemented with 15% (w/v) non-sterilized 20 dextrose equivalent (DE) malt dextrin. The bacterial suspension (450 mL) was incubated at 25°C and 100 rpm for 30 min to allow for cell adaptation and then sprayed. In preliminary experiments this ratio resulted in a suspension with a total viable count of $\geq 10^9$ cells/mL and total solids content of 25% (w/v). The proportions of the different ingredients in the formulation of the spray-dried cultures were decided from the results of the preliminary trials. A magnetic stirrer was applied to the bacterial suspensions to maintain a homogeneous feed during drying.

A Lab Plant model SD-04 (Leeds, UK) spray dryer was used. The entrance and exit temperatures, liquid flow rate, air pressure and nozzle diameter were

respectively: 135°C, 80°C, 10 mL/min, 5 kgf/cm^2 and 1 mm. The processing conditions were standardized from the results of the preliminary trials. The resultant spray-dried bacteria were homogenized and stored in sterile sealed glass bottles at 4°C. The experiment was carried out in duplicate. Viability assays were performed on the re-hydrated inoculum before inoculating into the biopolymer-producing medium.

Spray dried bacteria were re-hydrated in the following way: 20g of spray-dried bacteria were re-hydrated at 25°C and 100 rpm for 24h in a shaker in 200 mL of a sterile solution of skim milk (10% w/v) in a 1L conical flask. After re-hydration, aliquots of the suspension were removed for the viability assay and the suspension then used to inoculate the biopolymer-producing medium. For the viability assay, one milliliter of the cell suspension was subjected to serial dilutions with a sterile solution of NaCl (0.85% w/v), before transferring onto YM agar plates and incubating at 25°C for 36 h. The total viable cell number was expressed as colony forming units per milliliter of suspension. The viability assay for non-encapsulated *Beijerinckia* was carried out under the same conditions.

2.5. Fermenting activity assay

Two types of experiment were carried out: one with encapsulated cells and one with non-encapsulated cells. An attempt was made to follow the same procedures during these experiments so that a valid comparison could be made between the kinetics of the encapsulated cells and the non-encapsulated cells. The broth containing sucrose (1% w/v) and brewers yeast autolyzate (1% w/v), the pH previously adjusted to 6.5 prior to sterilization (121°C, 15 min) according to

Maldonade [6], was inoculated with 90 mL of inoculum at a rate of 5% (v/v), and incubated in a New Brunswick model G 25 shaker at 25°C and 200 rpm for 25 h. Similarly 20 mL samples were withdrawn after different lengths of time and analyzed for pH, polysaccharide content, sugar content and dry weight. The polysaccharides produced after 24 hours of incubation, by encapsulated and non-encapsulated cells of *Beijerinckia,* were compared with respect to their structural characteristics and physical and chemical properties. All kinetic experiments were carried out in duplicate. The fermentative assays with non-encapsulated *Beijerinckia* were also carried out in gum producing medium containing 1.5% w/v sucrose, maintaining the remaining conditions and procedures the same as those used in the assays with the encapsulated inoculum.

Samples withdrawn from the culture broths were centrifuged at 22,300 X g at 5°C for 30 min to remove the cells. The supernatants were analyzed with respect to their sugar and biopolymer concentrations as described below. The cell precipitates were analyzed immediately for their cell mass (dry weight) as described below.

2.6. pH

The pH values of the samples of fermentation media were determined using standard pH electrodes and a Digimed mod. DMPH-2 pH-meter.

2.7. Determination of the dry cell weights

Microbial dry weights were monitored gravimetrically. The organisms collected by centrifugation were re-suspended in distilled water to remove

undesired soluble material and re-centrifuged. The final pellet was again suspended in water and then transferred to a pre-weighed Pyrex dish, which was dried to constant weight at 105°C. The results were expressed in g dry cell mass per liter of substrate.

The dry weight of microencapsulated spray dried *Beijerinckia* sp. was determined in the following manner. The pre-weighed microencapsulated cells were washed with distilled water (in a ratio of 1 g of powder per 200 mL of water) to remove undesired soluble material and centrifuged at 22,300 X g at 5°C for 30 min. The resulting pellet was dried to constant weight at 105°C. The powder was analyzed twice. The supernatant was kept for sugar analyses as described below.

2.8. Polysaccharide concentration

The estimation of polysaccharide content was carried out by a gravimetric analysis. The supernatant remaining after centrifuging the bacterial cells was precipitated using 4 volumes of ethanol. The precipitates were washed twice with ethanol and the precipitated gum dried to constant weight at 45°C, the results being expressed as g gum per liter of substrate and the yield in gum as g gum per g sugar consumed.

2.9. Sugar concentration

Sugar concentration in the cell free broth and substrate was measured using the dinitrosalicylic acid method [8], modified according to Giordano [9],and expressed as gram sugar consumed (initial sugar – residual sugar) per liter of substrate.

2.10. Structural characteristics and physicochemical properties of the polysaccharide

A comparative study of the polysaccharides obtained in batch fermentations with the different inoculum was made by way of analyses of their structures (composition and molecular weight distribution) and their rheological behavior. The polysaccharides were isolated according to the experimental procedure of Galindo and Albiter [10] as described above and purified from the protein by precipitation with trichloroacetic acid (TCA) according to Druzian [11] and Clarke [12].

Composition of the polysaccharides

Monomers of the polysaccharides were detected by hydrolyzing samples (0.01g) of the purified polymers with 2M trifluoracetic acid (TFA) at 100°C for 16 h, and evaporating to dryness with nitrogen. The hydrolyzed samples were dissolved in 1 mL water, filtered through a 0.45□m membrane filter and the sugars determined by HPLC on a polystyrene lead divinilbenzene-sulfonate column (Polyspher®CH Pb-Merck, Darmstad, Germany) (0.79 x 30cm) at 80°C eluting with de-ionized water with a flow rate of 0.4 mL/minute and a refractive index detector. The sugars were identified from the retention times of corresponding standards [13].

Measurement of the molecular weight distribution

Polysaccharide samples were prepared by dispersion in water plus 0.01% w/v of a 0.02% solution of sodium azide, stirring constantly at room temperature for 20h.

The molecular weights of the polysaccharides were determined by size-exclusion gel permeation chromatography (GPC) using columns connected in series, eluting with de-ionized water containing 0.02% sodium (NaN_3) with a flow rate of 1.0 mL/min at 55°C and identification using the refractive index detector according to Pereira [14].

Rheological properties

The polysaccharide samples (300 mg) were prepared by dispersion under stirring at 30°C in 10 mL of de-ionized water. The measurements of viscosity and the dynamic rheological tests were carried using a Haake rheometer model CV 20, Q45 sensor system, and shear rate of 10 s^{-1} at 25°C under the following conditions: parallel plates with 45 mm in diameter, 0.5 mm gaps between the plates to obtain shear rates of 300 s^{-1} .

2.11. Statistical analysis

Firstly linear regressions were made between the fermentation time and the following variables: pH, cell mass and biopolymer concentrations, biopolymer yield and sugar consumption. Secondly Pearson's correlation was applied between the residues for each variable. The Statistics Analyses Systems (SAS) software [15] was used for the analyses.

3. Results and discussion

Results from the encapsulated and suspended cell fermentations are plotted in Figure 1, 2 and 3. Figure 1 shows the changes in pH during the fermentations and Figures 2 A and 2 B show the production of cell mass and of biopolymer

during the batch fermentation with encapsulated and non-encapsulated cells respectively. Figure 3 the data for sugar consumption in an experiment with non-encapsulated and encapsulated cells.

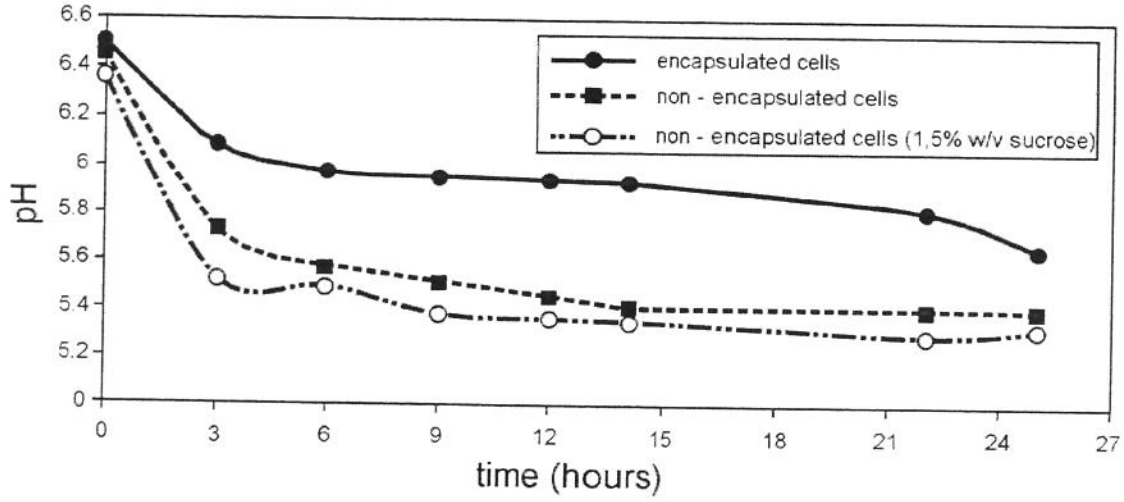

Fig. 1. Kinetics of polysaccharide production – changes in pH.

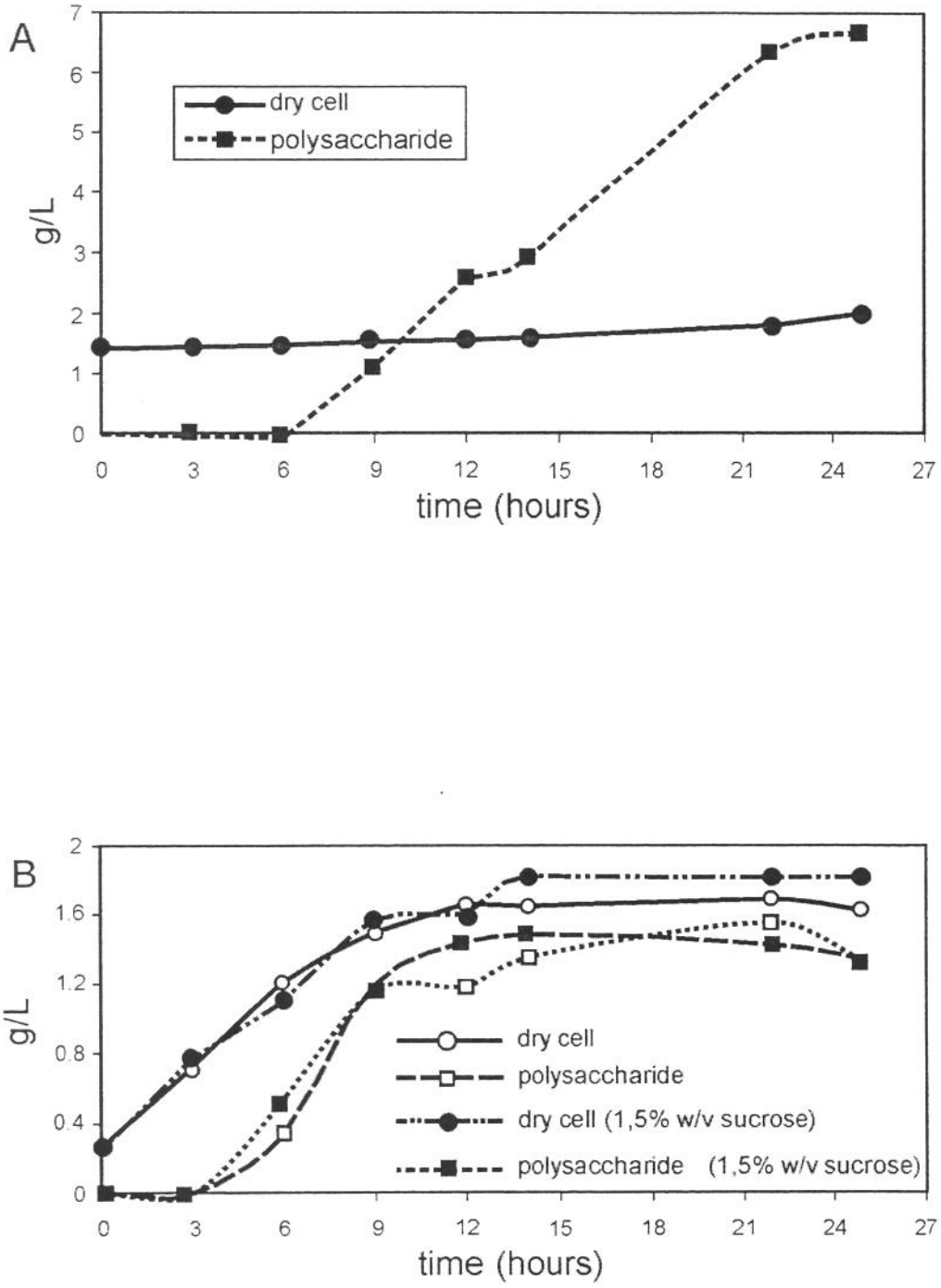

Fig. 2. Polysaccharide production – Dynamic changes of cell and polysaccharide concentrations. Encapsulated *Beijerinckia* (A) and non-encapsulated cells (B).

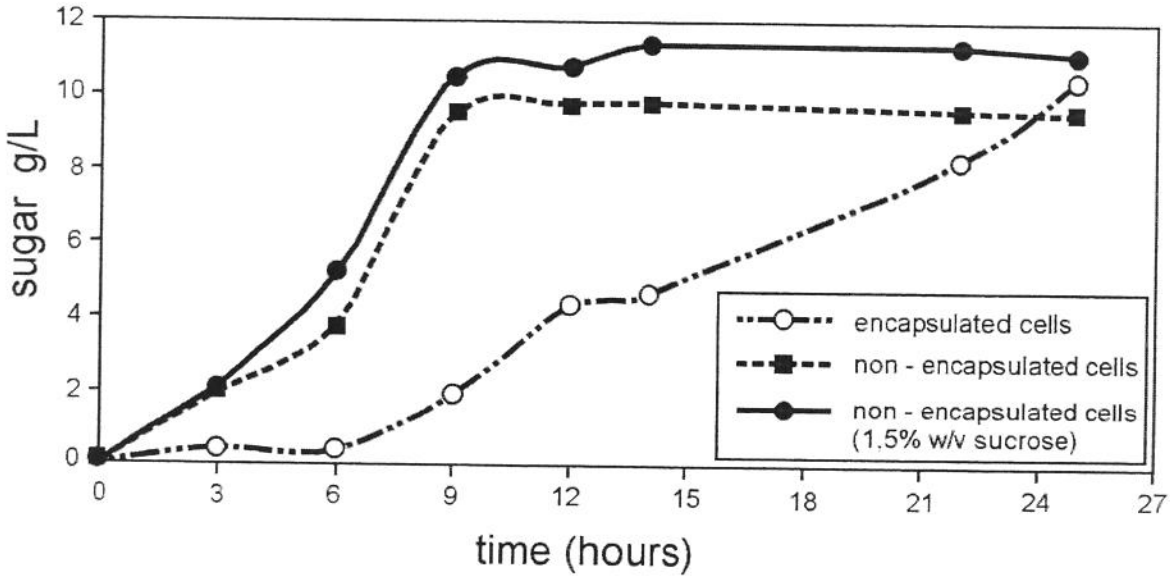

Fig. 3. Polysaccharide production – Dynamic changes of concentrations sugar.

In a comparison of the fermentations (Figures 1, 2A and 2B) by the two types of inoculum (encapsulated and non-encapsulated cells) appreciable differences were noted with respect to the productions of cell mass and polysaccharide and the extent of the lag phase. The encapsulated cultures of *Beijeirinckia* showed slight growth after a lag phase of about 12h, growth continuing for up to 25 hours of incubation, whilst with non-encapsulated *Beijerinckia*, there was no lag phase, exponential growth occurring immediately and continuing for approximately 14 hours of incubation. Exponential growth then ceased, the population having reached the stationary phase, a growth profile similar to that obtained by Maldonade [6].

According to Madigan et al. [16] when a microbial population is inoculated into a fresh medium, growth does not usually begin immediately but only after a

period of time called the lag phase, the extension of which depends on the history of the culture and growth conditions. A lag also ensues when the inoculum consists of cells that have been damaged by heat or radiation, because of the time required for the cell to repair the damage, before the cells can start to grow, meaning that injured cells will take longer to start their desirable activities in biopolymer fermentations.

Although the initial number of viable cells was similar in both types of experiment, the initial dry masses (zero time) differed. After 25h of incubation at 25°C and 200 rpm, the encapsulated cells exhibited a lower cellular yield than the non-encapsulated cells. The cell yield (Yx/s) % (g/g) was calculated by using Yx/s (% g/g) = [final cell dry weight (g/L) – initial cell dry weight (g/L)] / sugar consumption (g/L)] x 100, giving values of 5.6% and 14.0% for the encapsulated cells and non-encapsulated cell fermentations respectively. According to Bratbak and Dundas [17] the dry-matter content of the cells of a given strain may vary with the growth conditions. According to Sikyta [18] the bacterial composition varies according to the stage in batch cultures, due to changes in the physiological state of the cells.

According to Madigan [16] in a batch culture the pH can change during growth as a result of metabolic reactions that consume or produce acidic substances. According to Barbosa and Garcia-Canedo [19] in fermentations with *Beijerinckia* the pH of the medium decreases due to the production of acetic acid. After 25h at 25°C, the pH of a sample fermented by encapsulated and non-encapsulated *Beijerinckia* decreased from 6.50 and 6.36 to 5.66 and 5.36 respectively (Figure1).

Figures 2A and 2B show that the production of biopolymer by non-encapsulated cells occurred during the growth phase of the fermentation, and that

with the decrease in cell growth, a decrease in polysaccharide production was also observed, suggesting that the production of polysaccharide is growth related, a fact not observed for the encapsulated cells. The maximum biopolymer concentration achieved at the end of the incubation period in non- encapsulated fermentations was much less that the maximum concentration achieved in encapsulated-cell fermentations 1.35 g/L^{-1} versus 6.71 g/L^{-1}.

The mean polysaccharide yield obtained was 12% and 63% in the conversion of sugar to polymer, for the non-encapsulated cell and encapsulated cell fermentations respectively, when incubated under similar conditions. The encapsulated inoculum, after reactivation, may contain dead cells showing injuries or non-reversible inactivation, damaged cells and normal cells [20]. In the experiments, the initial viable bacterial populations were similar, being about 10^8 cells/mL, although, according to Klibanov [21] from a biotechnological standpoint a dead cell can be considered to be a bag filled with enzymes, which can merely maintain the required enzymatic activity, although being incapable of reproduction, whilst the non-encapsulated cells can utilize part of the available sugar as a source of energy (for microbial growth and maintenance) and other metabolic products, which can decrease the formation of the desired economic product.

Pearson's correlation coefficients between the time with the variables of fermentations (pH, dry mass, sugar consumption, concentration and production of biopolymer) by encapsulated and non-encapsulated cells presented in Table 1 .

Table 1. Pearson's correlation coefficients between the residues of the linear regressions of time with the following variables: pH, dry mass, sugar consumption, concentration and production of biopolymer.

Variable 1	Variable 2	Correlation coefficient (Prob* > R)		
		A	B	C
Dry mass	pH	-0.2489 (0.3524 ^{ns})	0.85352 (<0.0001)	0.6840
Dry mass	Sugar consum.	-0.33109 (0.2103 ^{ns})	0.95571 (<0.0001)	(0.3862 (<0.0001)
Dry mass	Conc. biopolymer	-0.28778 (0.2798 ^{ns})	0.86758 (<0.0001)	0.85670 (<0.0001)
Dry mass	Yield Biopolymer	-0.51546 (0.0410)	0.90482 (<0.0001)	0.66178 (0.0052)
Conc. Biopolymer.	pH	-0.40299 (0.1217 ^{ns})	0.53911 (^{ns})	0.9951 (0.0433)
Conc. Biopolymer	Sugar consum.	0.80452 (0.0002)	0.76060 (^{ns})	0.77646 (0.0004)
Conc. Biopolymer	Yield Biopolymer	0.87353 (<0.0001)	0.93069 (<0.001)	0.86687 (<0.001)
Yield Biopolymer	pH	-0.09148 (0.7362 ^{ns})	0.62342 (0.0099)	0.51062 (0.0433)
Yield Biopolymer	Sugar consum.	0.77189 (0.0005)	0.77951 (0.0004)	0.68181 (0.0036)
Sugar consum.	pH	0.06977 (0.7974 ^{ns})	0.92459 (<0.0001)	0.90483 (<0.0001)

* Values lower than 0.05 indicate significant correlation.

A,B correspond to the coefficients obtained in the fermentations in gum producing medium containing 1% w/v sucrose, by encapsulated and non-encapsulated cells respectively; C are the coefficients obtained in gum producing medium containing 1.5% w/v sucrose, by non-encapsulated cells.

During incubation, the non-encapsulated cells presented a high correlation between the cell concentrations (dry mass) and the biopolymer concentration, at a 0.05 level of significance. For the encapsulated cells there was no such correlation between these two variables. The non-encapsulated cells presented growth associated polysaccharide production, whereas the encapsulated cells did not.

For the non-encapsulated cells, the fermentation pH showed higher correlations with the variables dry mass and sugar consumption and relatively lower correlations with the variables biopolymer concentration and yield. For the encapsulated cells there was no significant correlation with the variables analyzed. For the variable sugar consumption during fermentation, the non-encapsulated cells showed high correlations with the variables dry mass and biopolymer concentration and yield. During fermentation, the encapsulated inoculum presented high correlations with sugar consumption and the variables biopolymer concentration and yield.

According to Becking [22] *for Beijerinckia* sp. the main carbon sources for cellular growth are glucose, sucrose and fructose. Maldonade [6] observed that the amount of cell mass produced by *Beijerinckia* strain 7070 during fermentation increased from 1.75 gL^{-1} to 4.80 gL^{-1} with increased glucose concentration from 0.5% to 10% and fixed nitrogen concentration. He also observed that sucrose was a better source for cellular growth than glucose, when using the same concentrations and conditions.

It was shown that the non-encapsulated cells showed similar fermentative activities when the carbon source increased from 1.0% to 1.5% w/v sucrose with a fixed nitrogen concentration, presenting no substantial effect on cellular growth or polysaccharide production. Studies on the composition of the medium for xanthan gum production found that a low carbon/nitrogen ratio improved bacterial growth and that a high ratio improved xanthan production, and they suggested working in two steps. Most of these studies only analyzed the results in the stationary phase of microbial growth [23].

Gandhi et al. [24] reported that the type of carbon source had an important function in both growth and polysaccharide production, a carbon source giving excellent results for growth not necessarily giving good polysaccharide yields. The fermentations with *Beijekinckia* encapsulated in malt dextrin, inoculating into gum producing medium containing 1% w/v sucrose, presented better cellular growth and biopolymer yield than the non-encapsulated inoculum under similar incubation conditions. At the end of the incubation period, the encapsulated cells showed an average polysaccharide yield of 63% with an average sugar consumption of 10.51 $g\ L^{-1}$. From these results, it was presumed there was a contribution in sugar to the

substrate composition from the re-hydrated inoculum encapsulated in malt dextrin. The composition of malt dextrin is influenced by the type of hydrolysis and conditions used, so industrially produced malt dextrin normally consists of a wide range of saccharides [25].

Vendrusculo [4] showed that the type of sugar influenced the yield and quality of biopolymer produced by *Beijerinckia*, the yield being 2% greater with sucrose than with glucose. Also at 25°C, the viscosities of 6% aqueous solutions of the biopolymers produced from sucrose fermentation were greater than those of biopolymers produced from glucose fermentation.

Becker et al. [26] reported that in the production of xanthan gum, different growth phases of the microorganisms and alterations of the growth medium, for example by using different substrates and limiting nutrients, did not influence the primary structure, but did affect the molecular mass and the yield of gum.

According to Casas et al. [27] the average gum molecular weight increases during the fermentation time under any conditions and the molecular weight of a polymer has a direct influence on its rheological behavior, with larger molecules showing higher viscosities. In this experiment it was shown that with non-encapsulated inoculum, gum production was associated with cell growth, and low efficiency was obtained for the conversion of sugar to polymer or biomass. However the polysaccharide yields may have been underestimated in these experiments, since, according to Galindo and Albiter [10] polysaccharide yields measured by precipitation may be lower than the true values due to low-molecular weight material failing to precipitate, since the yield in polysaccharide depends to a certain extent on the degree of polymerization.

The macromolecular characterization of the excreted polysaccharides by structural analysis and rheological studies revealed that the encapsulation of *Beijerinckia* did not influence the basic sugar components in the polysaccharides obtained. The monomeric composition of the polysaccharides obtained from both encapsulated and non-encapsulated inoculum was galactose, fucose and glucose as shown in Figure 4, similar results being obtained by Scamparini et al. [13].

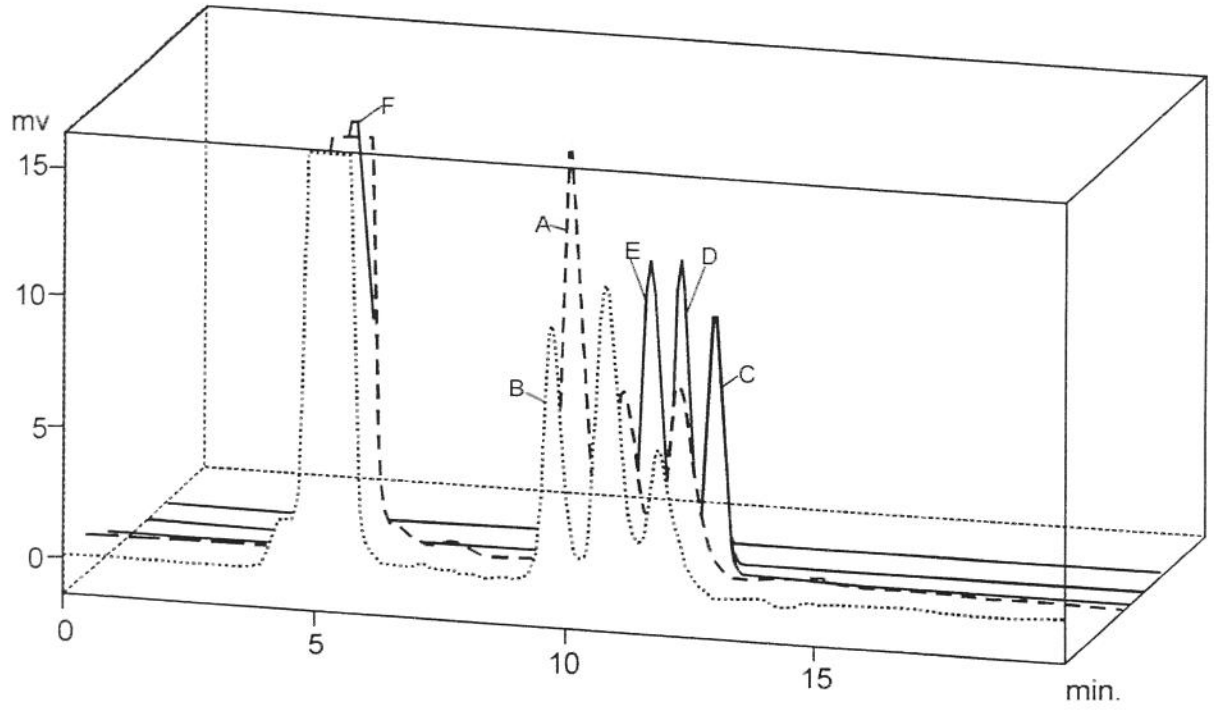

Fig. 4. Composition of the polysaccharide produced by *Beijerinckia* analyzed by HPLC using a refractive index detector. (A) encapsulated, (B) non- encapsulated, (C) fucose (Rt = 11.772), (D) galactose (Rt = 10.668), (E) glucose (Rt = 9.662), (F) TFA

Chromatographic profiles (Figure 5) of the molecular size distribution of the biopolymers produced by encapsulated and non-encapsulated *Beijerinckia* showed that the biopolymers presented differences in the distribution of eluted species, this

difference in elution profiles reflecting differences in the structure and size of the polysaccharides, which could have implications in the rheological properties of the solutions of these polysaccharides.

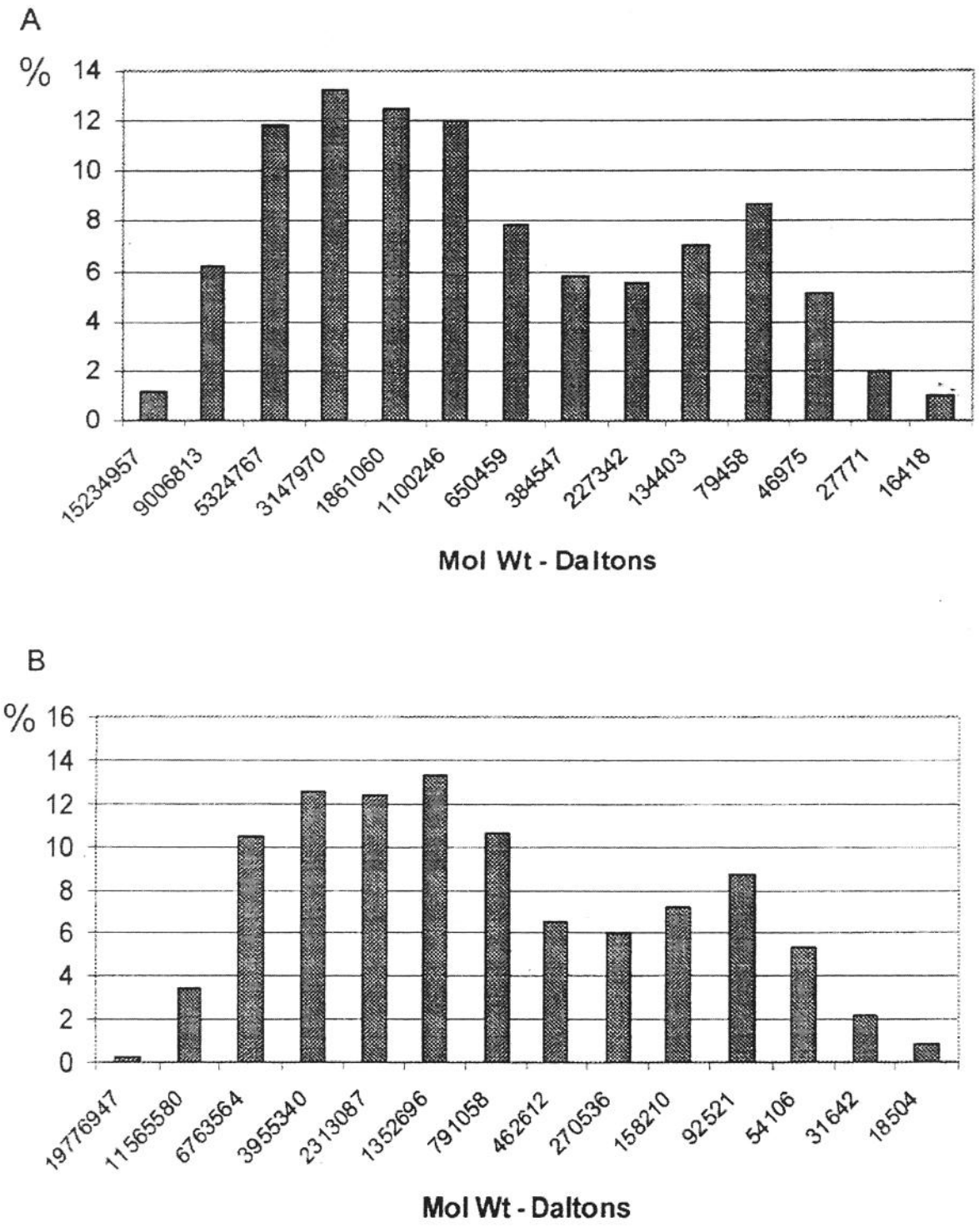

Fig. 5 – Chromatographic profile (GPC) of the molecular weight distribution of the biopolymers produced by *Beijerinckia* (A) encapsulated cells and (B) non-encapsulated cells.

RHEOLOGICAL BEHAVIOUR

Measurements were performed on a controlled strain Haake rheometer model CV20, sensor system Q45, shear rate of 10 s^{-1}, at 25°C. The rheograms (Figure 6) demonstrated that the biopolymers has pseudoplastic behaviour, described by the model of Ostwald, the results showed that biopolymer obtained encapsulated cells of *Beijerinckia* have a smaller apparent viscosity than the non-encapsulated cells.

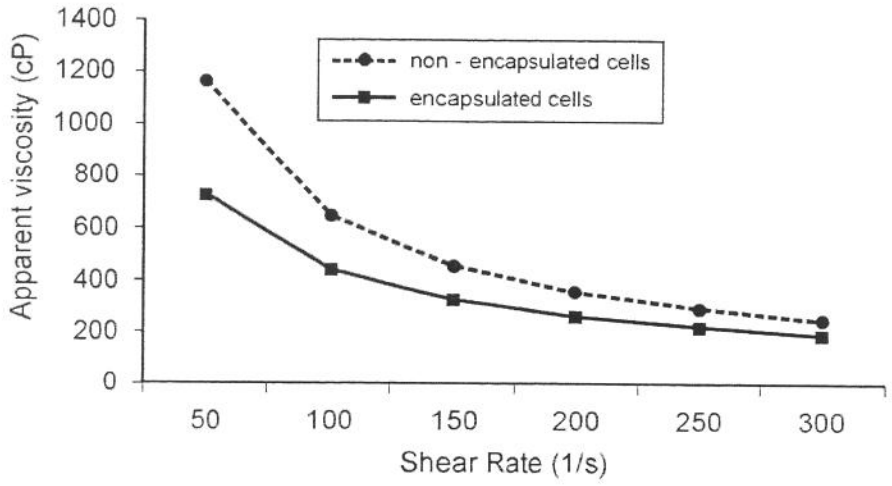

Figure 6 - Apparent viscosity of aqueous solutions with 3% of the biopolymers of *Beijerinckia* obtained by non- encapsulated and encapsulated cells. The measurements were performed in a Haake rheometer CV20, sensor system Q45, at 25°C.

OSCILLATORY MEASUREMENTS

Viscoelastic behaviour of the samples confirmed by the oscillatory test, gel-like properties are shown in the Figure 7A and 7B.

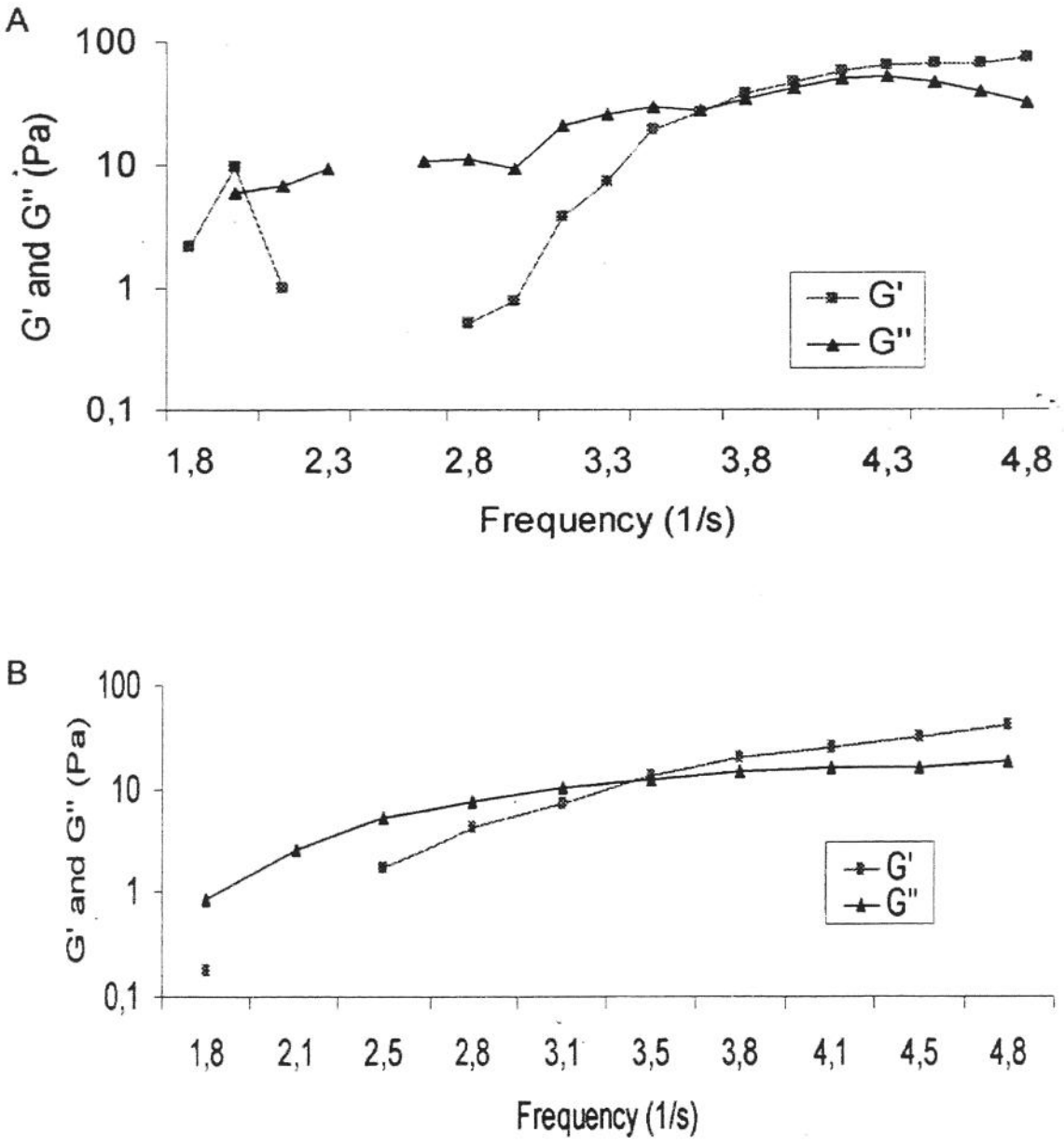

Fig.7. Mechanical spectra of aqueous solutions with 3% of the biopolymer of the *Beijerinckia sp* obtained by non- encapsulated (A) and encapsulated cells (B). The measurements were performed in a Haake rheometer CV20, sensor system Q45, at 25°C.

The storage modulus (G') was superior to the loss modulus (G"), when they were applied superior frequency to 3,5 (1/s). Starting from this point the elastic response became superior the viscous and independent of the oscillatory frequency.

Comparing the Figures 7 A and 7 B, was observed that the transition area among a structure predominantly viscous and the elastic structure of the aqueous solutions of biopolymer obtained by non- encapsulated cells ,it showed the smallest conformation by deformation .Fact this, verified qualitatively by the profile of the curve and for the resistance of the handling of the sample.

4. Conclusion

From the characterization of the biopolymers by way of a structural analysis and rheological study, it was shown that the process of encapsulating *Beijerinckia* mainly influenced cell growth and biopolymer production, due to *physiological* alterations resulting from the encapsulation process using a spray dryer. According to Marechal et al. [28] the temperature and aw have a considerable influence on the viability of the microorganisms, although cell damage is not simply an on/off phenomenon, depending on the intensity of hydric or thermic stresses. Such stresses would induce the cells to react passively or actively to avoid loss of cellular integrity or biological activity, the active responses normally being metabolic modifications involving the synthesis of intracellular molecules, whilst the passive responses consist of physical changes such as membrane permeability. Based on qualitative and quantitative aspects of the production of polysaccharides

by *Beijerinckia* encapsulated in malt dextrin, it was concluded that this was a viable technique to obtain inoculum of *Beijerinckia* for use in fermentations.

REFERENCES

[1] TEIXEIRA, P.; CASTRO, H. and KIRBY, R., 1995. Spray drying as a method for preparing concentrated cultures of *Lactobacillus bulgaricus*. Journal of Applied Bacteriology, 78: 456-462.

[2] ELIZONDO, H. and LABUZA, T.P.V., 1974. Death kinetics of yeast in spray drying. Biotechnology and Bioengineering, 16: 1245-1259.

[3] DORAN,P.M. and BAILEY,J.E. 1986.Effects of immobilization on growth ,fermentation proprieties and macromolecular composition of *Saccharomyces cerevisiae* attached to gelatin .Biotechnology and Bioengineering,28,73 –87.

[4] VENDRUSCOLO, C.T., 1995. Produção e caracterização do biopolímero produzido por *Beijerinckia sp.* isolada do solo cultivado com cana-de-açúcar da região de Ribeirão Preto – São Paulo – Brasil. Master Thesis, Faculdade de Engenharia de Alimentos, Unicamp, Campinas, Brazil.

[5] MURO, M.A. and LUCHI, M.R., 1989. Preservação de Microrganismos. Editora da Fundação Tropical de Pesquisas e Tecnologia "André Tosello". Campinas, SP, Brasil. 70p.

[6] MALDONADE, I.R., 1996. Contribuição ao estudo dos parâmetros de fermentação por *Beijerinckia sp.* Master Thesis, Faculdade de Engenharia de Alimentos, Unicamp, Campinas, Brasil.

[7] MARY, P., MOSCHETTO, N. and R. TAILLIEZ, 1993. Production and survival during storage of spray-dried *Bradyrhizobium japonicum* cell concentrates. Journal of Applied Bacteriology, 74, 340-344.

[8] MILLER, G.L., 1959. Use of dinitrosalicylic acid reagent for determination of reducing sugar. Analytical Chemistry, 31, 426-428.

[9] GIORDANO, R.L., 1992. Estudo da coimobilização de glicoamilase e levedura para a fermentação alcoólica contínua de matéria-prima amilácea. Master Thesis, Escola Politécnica da USP, Universidade de São Paulo, São Paulo, Brazil.

[10] GALINDO, E. and ALBITER, V., 1996. High-yield recovery of xanthan by precipitation with isopropyl alcohol in a stirred tank. Biotechnology Progress, 12, 540-547.

[11] DRUZIAN, J.I., 2000. Estudo da estrutura de polissacarídeos produzidos por duas espécies de *Rhizobium* e uma de *Bradyrhizobium* isolados de solo de cultivar de feijão de corda (*Vigna unguiculata*). Master Thesis, Faculdade de Engenharia de Alimentos, Unicamp, Campinas, Brazil.

[12] CLARKE, M.A., 1995. Sugars and sugars products, sugar processing Research Institute Inc., A.O.A.C., Official Methods of Analysis, cap. 44, 2-3, vol. 2, part II, 16th Edition.

[13] SCAMPARINI, A., VENDRUSCOLO, C., MALDONADE, J., DRUZIAN, J., MARIUZZO, D., 2000. New biopolymers produced by nitrogen fixing microorganisms for use in foods. In: Hydrocolloids. Part 1. Physical Chemistry Industrial Application on Gels Polysaccharides. Edited by Nishinari, K. Osaka University, Osaka, Japan, 169-178.

[14] PEREIRA, A.M., 1996. Estudo da influência da glicose na síntese "in vitro" de dextrana. Master Thesis, Faculdade de Engenharia de Alimentos, Unicamp, Campinas, Brazil

[15] SAS INSTITUTE. Statistics Analyses Systems (SAS) : version 6-09.4.ed.Cary.NC1989. 2v. (Compact Disc).

[16]MADIGAN, M.T.;MARTINKO, J.M. and Parker,J. Brock biology of microorganisms.9 th ed. New Jersey, Prentice Hall Inc., 2000. chapter 5.

[17] BRATBAK, G. and DUNDAS, T., 1984. Bacterial dry matter content and biomass estimations. Applied and Environmental Microbiology, 48, 755-757.

[18] SIKYTA, B., 1995. Kinetics of microbial growth and product formation. In: Techniques in Applied Microbiology. Elsevier Science Ed., Amsterdan, Netherlands, 33-45.

[19] BARBOSA, H.R. and GARCIA-CANEDO, A.M.D., 1989. A concentração de fosfato no crescimento de *Beijerinckia* derxii cultivada com N_2 ou NH^+_4 como fonte de nitrogênio. Revista Brasileira de Ciência do Solo, 13, 399-402.

[20] TEIXEIRA, P.; CASTRO, H.,MOAHÄCSI-FARKAS,C.and KIRBY, R., 1997.Identification of sites of injury in *Lactobacillus bulgaricus* during heat stress. Journal of Applied Microbiology,83, 219-226.

[21] KLIBANOV, A.M., 1983. Immobilized enzymes and cells as practical catalysts. Science, 219, 722-727.

[22] BECKING, J.H., 1974. Nitrogen-fixing bacteria of the genus *Beijerinckia*. Soil Science, 118, 196-212.

[23] GARCÍA-OCHOA, F., SANTOS, V.E. and FRITSCH, A.P., 1992. Nutritional study of *Xanthomonas campestris* in xanthan gum production by factorial design of experiments. Enzyme Microbiology and Technology, 14, 991-996.

[24] GHANDI, H.P., RAY, R.M. and PATEL, R.M., 1997. Exopolymer production by *Bacillus species*. Carbohydrate Polymers, 34, 328-327.

[25] DORIK, P., JAKOVLJEVIC, J. and DOKIC-BAUCAL, L., 1998. Molecular characteristics of maltodextrins and rheological behaviour of diluted and concentrated solutions. Colloids and surface A: Physicochemical and Engineering Aspects, 141, 435-440.

[26] BECKER, A., KATZEN, F., PÜHLER, A. and IELPI, L., 1998. Xanthan gum biosynthesis and application: a biochemical/genetic perspective. Applied Microbiology and Biotechnology, 50, 145-152.

[27] CASAS, J.A., SANTOS, V.E. and GARCIA-OCHOA, F., 2000. Xanthan gum production under several operational conditions: molecular structure and rheological properties. Enzyme and Microbial Technology, 26, 282-291.

[28] MARECHAL, P.A.; MARNAÑON, M.I.; POIRIER, I.; GERVAIS, P., 1999. The importance of the kinetics of application of physical stresses on the viability of microorganisms: significance for minimal food processing. Trends in Food Science & Technology, 10: 15-20.

7. CONCLUSÕES GERAIS

- O material de parede é um dos fatores determinantes do grau de sobrevivência das células de *Beijerinckia* encapsuladas, durante a secagem em spray –drier e posterior período de estocagem.
- A retenção da atividade fermentativa das células de *Beijerinckia* encapsuladas, utilizando spray-drier ,durante a estocagem depende da composição do material de parede.
- A sobrevivência e a atividade fermentativa das células de *Beijerinckia*, encapsuladas em maltodextrina por spray-drier, durante a secagem e posterior período de estocagem, depende das condições operacionais do spray-drier (temperatura de secagem) e do teor de sólidos da suspensão de alimentação.
- Menores temperatura de saída no spray –drier e teor de sólidos na suspensão de alimentação resultam em maior retenção da viabilidade das células de *Beijerinckia* encapsuladas em maltodextrina.
- O processo de encapsulaçao de *Beijerinckia* em maltodextrina por spray-drier influe no crescimento celular e na produção de biopolímero.