Universidade Federal do Rio Grande do Sul

Centro de Biotecnologia

Programa de Pós-graduação em Biologia Celular e Molecular

PRODUÇÃO DE PEPTÍDEOS ANTIMICROBIANOS POR *Bacillus* sp. P11

TESE DE DOUTORADO

Fernanda Leal Leães

Orientador: Prof. Dr. Adriano Brandelli

Porto Alegre, abril de 2012.

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Tese submetida ao Programa de Pós-graduação em Biologia Celular e Molecular da UFRGS como requisito parcial para obtenção do Grau de **Doutor** em Ciências.

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"... quaisquer que sejam os favores que da vida recebam nossos esforços, é preciso, ao aproximarmo-nos do grande termo, ficarmos com o direito de dizer: 'Fiz o que pude'".

Louis Pasteur

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LISTA DE ABREVIATURAS

µL: Microlitro
ANOVA: *Analysis of Variance*
ATCC: *Americam Type Culture Collection*
BHI: *Brain Heart Infusion*
BLS: *Bacteriocin-like Substance*
CFU: *Colony Forming Unit*
EUA: Estados Unidos da América
FDA: *Food and Drugs Administration*
GRAS: *Generally Regarded as Safe*
kDa: kiloDalton
LAB: *Lactic Acid Bacteria*
mg: Miligrama
min: Minuto
mL: Mililitro
mM: Milimolar
MSR: Metodologia de Superfície de Reposta
ng: Nanograma
NRPS: Peptídeo Sintetase Não-ribossomal
PKS: policetídeo sintetase
qRT-PCR: Reação em Cadeia de Polimerase em Tempo Real
RNA: Ácido Ribonucleico
rpm: Rotações por Minuto
s: Segundo
UA: Unidades Arbitrárias

RESUMO

A produção de atividade antimicrobiana por *Bacillus* sp. P11 foi testada em diferentes subprodutos da indústria de alimentos. Farelo de soja foi o meio escolhido para determinar o efeito de três variáveis (temperatura, pH inicial e concentração do substrato) sobre a atividade antimicrobiana pela metodologia de superfície de resposta, utilizando um experimento fatorial 2^3. Na faixa estudada, temperatura e pH inicial do meio apresentam efeito significativo sobre a produção de atividade antimicrobiana, já a concentração do substrato não teve nenhum efeito significativo. Os dados mostraram produção máxima em pH inicial entre 7,0 e 8,5 e temperatura entre 39 e 42ºC. Nessas condições a produção de atividade antimicrobiana por *Bacillus* sp. P11 em farelo de soja foi comparada com a produção em meio de cultivo comercial (caldo BHI). Os resultados obtidos para os dois meios de cultura foram semelhantes, indicando que o farelo de soja pode ser um substrato de baixo custo para produção de antimicrobianos por *Bacillus* sp. P11. Para demostrar o efeito da temperatura e do pH na produção de peptídeos antimicrobianos, verificou-se a expressão de *sboA* (subtilosina A) e *ituD* (iturina A) em *Bacillus* sp. P11, utilizando a metodologia de qRT-PCR. A produção de subtilosina A e iturina A foi confirmada por espectrometria de massas. A expressão de *sboA* e *ituD* foi fortemente afetada pelo pH e pela temperatura e qRT-PCR provou ser uma poderosa ferramenta para investigar o potencial da cepa em produzir subtilosina A e iturina A. Além disso, a produção de peptídeos antimicrobianos por *Bacillus* sp. P11 foi investigada na presença de células inativadas termicamente de bactérias e fungos. A cepa exibiu maior atividade antimicrobiana na presença de células inativadas de *Staphylococcus aureus* ou *Aspergillus parasiticus.* Nessas condições a expressão de genes essenciais relacionadas com a biossíntese dos peptídeos antimicrobianos surfactina, iturina A, subtilosina A e fengicina foi investigada por qRT-PCR. Os resultados sugerem que o aumento da atividade antimicrobiana foi relacionado com a produção de iturina A, um peptídeo antimicrobiano que mostra forte atividade antimicrobiana contra um amplo espectro de fungos e pode ser usado no controle biológico de doenças de plantas. Por fim, foram testadas diferentes metodologias para a purificação parcial dos peptídeos antimicrobianos produzidos por *Bacillus* sp. P11. Os melhores resultados foram obtidos por partição em sistema aquoso bifásico com o sal sulfato de zinco, porém estudos adicionais são necessários.

ABSTRACT

Production of antimicrobial activity by *Bacillus* sp. P11 was tested in different byproducts of food industry. Soybean meal was the selected medium to determine the effect of three variables (temperature, pH and substrate concentration) on the antimicrobial activity by response surface methodology, using a 2^3 factorial design. In the range studied, temperature and initial pH of the medium had a significant effect on the production of antimicrobial activity and substrate concentration had no significant effect. The data showed maximum production at initial pH between 7.0 and 8.5 and temperature between 39 and 42°C. Under these conditions the production of antimicrobial activity by *Bacillus* sp. P11 in soybean meal was compared with the commercial medium (BHI). The results for both culture media were similar, indicating that soybean meal can be a low cost substrate for the production of antimicrobial activity of *Bacillus* sp. P11. To demonstrate the effect of temperature and pH on the production of antimicrobial peptides, we studied the expression of *sboA* (subtilosin A) and *ituD* (iturin A) in *Bacillus* sp. P11, using qRT-PCR methodology. The production of subtilosin A and iturin A was confirmed by mass spectrometry. The expression of *sboA* and *ituD* was strongly affected by pH and temperature and qRT-PCR proved to be a powerful tool to investigate the potential of the strain to produce subtilosin A and iturin A. In addition, the production of antimicrobial peptides by *Bacillus* sp. P11 was investigated in the presence of thermally inactivated cells of bacteria and fungi. The strain exhibited higher antimicrobial activity in presence of inactivated cells of *Staphylococcus aureus* or *Aspergillus parasiticus*. Under these conditions the expression of essential genes related to biosynthesis of antimicrobial peptides surfactin, iturin A, subtilosin A and fengycin was investigated by qRT-PCR. The results suggest that the increase in antimicrobial activity was related to the production of iturin A, an antimicrobial peptide that shows strong antimicrobial activity against a broad spectrum of fungi and can be used in biological control of plant diseases. Finally, we tested different methods for partial purification of antimicrobial peptides produced by *Bacillus* sp. P11. The best results were obtained by partition in aqueous two-phase system with the salt zinc sulfate, but further studies are needed.

1 INTRODUÇÃO

As indústrias farmacêutica, alimentícia e agrícola têm enfrentado novos desafios nas últimas décadas no que se refere ao combate a microrganismos patogênicos e deteriorantes.

A busca por novos compostos que possam controlar estes microrganismos é de grande importância e, devido aos avanços da biotecnologia, já se utilizam bactérias produtoras de peptídeos antimicrobianos. Também chamados metabólitos secundários ou especiais, por não estarem relacionados ao crescimento do organismo produtor, esses antibióticos constituem um grupo diverso de substâncias em relação à sua forma e função. Com relação à biossíntese, podem ser produzidos via ribossomal (bacteriocinas) ou não-ribossomal.

Na indústria de alimentos, uma das principais dificuldades na manufatura de determinados produtos relaciona-se com a presença de microrganismos deteriorantes e patogênicos, trazendo consideráveis prejuízos para a saúde humana. As bacteriocinas e outros peptídeos antimicrobianos vêm recebendo muita atenção principalmente pelo seu potencial como conservante “natural” de alimentos. No entanto, a nisina e a pediocina são as únicas bacteriocinas empregadas atualmente na preservação de alimentos, sendo sintetizadas por bactérias láticas e, normalmente, empregadas na produção de alimentos fermentados. Devido às bacteriocinas terem potenciais aplicações práticas como conservantes naturais de alimentos, um número diverso de bacteriocinas tem sido recentemente identificado e caracterizado.

De uma perspectiva clínica, o desenvolvimento e a proliferação de bactérias resistentes a antibióticos, que diminuem as opções terapêuticas, tem se tornado um grave problema de saúde no mundo todo. A rápida escalada da resistência microbiana pode fazer com que algumas infecções bacterianas não possam ser tratadas com os antimicrobianos existentes. Desta forma, a descoberta de patógenos resistentes a drogas faz a identificação de novos antimicrobianos ainda mais importante.

O emprego de microrganismos para fins de controle biológico de doenças de plantas vem sendo amplamente estudado no mundo. Neste contexto, o uso de microrganismos produtores de substâncias antibacterianas e antifúngicas contra fitopatógenos tem se mostrado uma alternativa crescente no combate a doenças de plantas. Porém, os processos envolvidos na obtenção de peptídeos antimicrobianos para uso em escala industrial são bastante dispendiosos, o que leva à necessidade da busca por formas de diminuir os custos da produção. Uma forma para se obter as substâncias antimicrobianas com menor custo, seria a utilização de resíduos agroindustriais como fonte de nutrientes para os microrganismos produtores destes compostos.

Dentro deste contexto, este trabalho se justifica pela necessidade de estudar formas menos dispendiosas de produção de compostos antimicrobianos, investigar a influência de condições ambientais e de condições indutoras na produção destes compostos, bem como pela importância de buscar mais informações sobre antimicrobianos produzidos por isolados bacterianos da Região Amazônica.

Para isso, buscou-se estudar as melhores condições ambientais para a produção destes compostos, bem como selecionar um meio de cultivo alternativo mais viável para a produção destes compostos. Além disso, buscou-se verificar a influência de fatores ambientais como pH, temperatura e substrato para crescimento, bem como de fatores indutivos, como a presença de células inativadas de bactérias ou fungos, na produção de atividade antimicrobiana por *Bacillus* sp. P11, isolado do ambiente aquático da Amazônia. Também foram testadas metodologias para purificação parcial dos peptídeos antimicrobianos produzidos por *Bacillus* sp. P11.

2 REVISÃO BIBLIOGRÁFICA

2.1 Peptídeos antimicrobianos

Os peptídeos antimicrobianos são substâncias produzidas por microrganismos ou por síntese química total ou parcial que, em baixas concentrações, inibem o crescimento de outros microrganismos. Estes compostos geralmente possuem natureza anfifílica e caráter catiônico, podendo ser cíclicos ou lineares, com tamanho variando entre 30 e 100 resíduos e uma grande variedade de aminoácidos em sua composição (DE SIMONE & SOUZA, 2002).

Além disso, os peptídeos antimicrobianos são produzidos na fase estacionária ou no final da fase de crescimento e são denominados metabólitos secundários, sendo extremamente diversificados em termos funcionais. Muitos atuam como hormônios ou fatores liberadores destes, enquanto outros são neuropeptídios, neurotransmissores, toxinas, pigmentos ou antibióticos naturais (DEMAIN, 1998; KOLTER & MORENO, 1992; MOFFIT & NEILAN, 2000).

Esses metabólitos secundários são compostos por aminoácidos, no entanto, mostram pouca semelhança com os outros polipeptídeos, tanto em relação a sua estrutura, quanto à biossíntese, pois, além de conterem aminoácidos com configuração D e possuírem, em geral, outros constituintes em sua estrutura (geralmente um β-amino ou β-hidroxi-ácido graxo), também podem sofrer modificações pós-traducionais para tornarem-se biologicamente ativos (VATER, 1986; ZUBER, NAKANO & MARAHIEL, 1993).

A produção destes peptídeos antimicrobianos pode ocorrer tanto por síntese não-ribossomal como ribossomal. A síntese não-ribossomal inclui peptídeos drasticamente modificados e amplamente produzidos por bactérias, utilizando uma grande variedade de substratos, como aminoácidos não protéicos, hidroxiácidos e substâncias policetídicas, especialmente elaboradas para serem incorporadas na estrutura desses peptídeos (HANCOCK & CHAPLE, 1999). A síntese ribossomal envolve a produção de peptídeos por todas as espécies de vida, incluindo bactérias, como o componente principal do sistema de defesa do hospedeiro (KOLTER & MORENO, 1992).

Dentre estes peptídeos destacam-se as bacteriocinas, que são sintetizadas no ribossomo e liberadas no meio extracelular, apresentando ação bactericida ou bacteriostática sobre outros microrganismos. As bacteriocinas podem ser consideradas de espectro estreito, quando atuam sobre a mesma espécie, ou de espectro amplo, quando atuam sobre diferentes gêneros de microrganismos (COTTER et al., 2005; HERNÁNDEZ et al., 2005; SWE et al., 2007).

Os primeiros registros sobre bacteriocinas datam de 1925, quando André Gratia publicou um estudo referente ao antagonismo promovido por uma linhagem de *Escherichia coli* sobre outras linhagens da mesma espécie. As substâncias responsáveis por esse efeito inibitório foram denominadas de ‘colicinas’ em referência ao microrganismo produtor original. Com a descoberta de que a produção desses compostos não se limitava ao grupo dos coliformes, Jacob et al. em 1953 (apud REEVES, 1972) propuseram o termo ‘bacteriocina’ para as proteínas antimicrobianas produzidas por microrganismos Gram-negativos e Gram-positivos.

A classificação das bacteriocinas não está bem estabelecida e é ainda objeto de debate. Apesar de datar de 1993, a classificação das bacteriocinas definida por Klaenhammer (1993) ainda é a mais citada. Uma atualização foi proposta por Cotter et al. (2005) e debatida por Heng & Tagg (2006). Bacteriocinas são geralmente classificadas combinando vários critérios, sendo os principais a família bacteriana produtora, sua massa molecular e, finalmente, suas sequências de aminoácidos homologos e/ou organização do grupo de genes.

Na Figura 01 um esquema "universal" é apresentado e é construído sobre os alicerces do esquema de classificação original para bacteriocinas de bactérias ácido láticas, proposto por Klaenhammer (1993), e incorporando elementos do esquema proposto por Cotter et al. (2005).

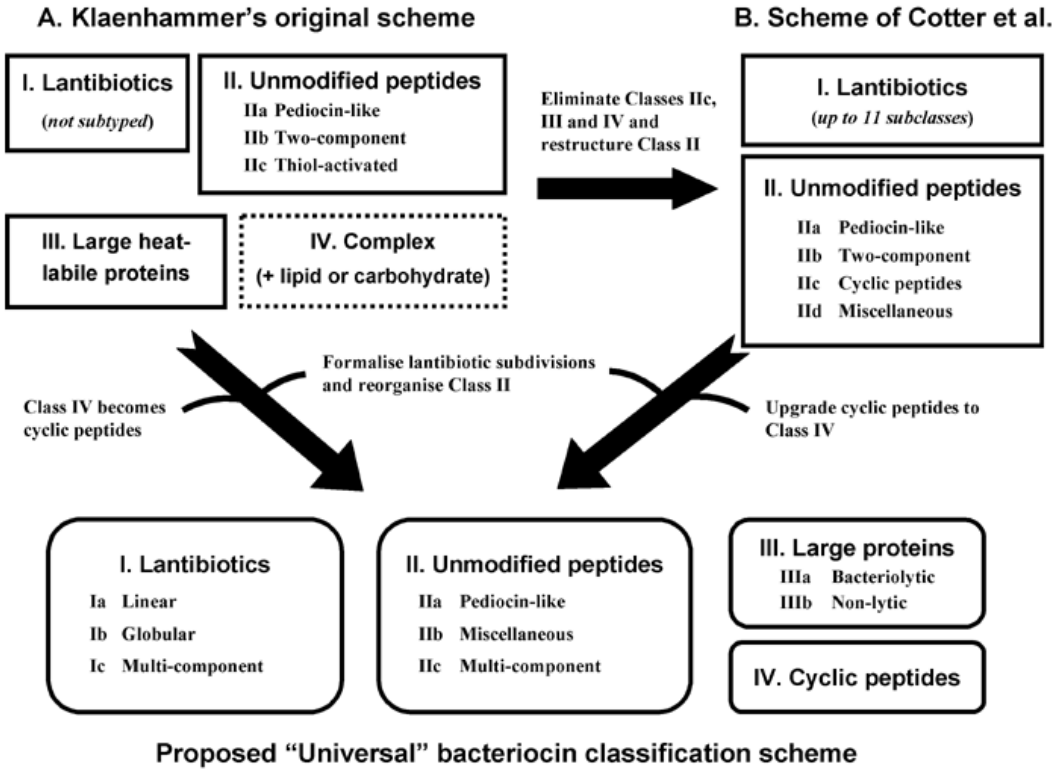

Figura 01: Esquema de classificação proposto para bacteriocinas

Fonte: Cotter et al., 2006.

Esta classificação “universal” propõe formalizar as subdivisões de lantibióticos em tipos Ia (linear), Ib (globular) e Ic (multi-componente). A primeira divisão documentada de lantibioticos em tipos A e B para moléculas lineares e globulares, respectivamente, foi proposta por Jung (1991).

2.2 Biossíntese

2.2.1 Síntese ribossomal

Os peptídeos antimicrobianos de síntese ribossomal são representados pelas bacteriocinas lantibióticas e estudos recentes mostram que a biossíntese dos lantibióticos e sua regulação ocorre de uma maneira dependente da fase de crescimento ou da densidade celular (*growth-phase-dependent*) e está regulada por um sistema duplo-componente formado por um sensor histidina quinase e um regulador de resposta. A nisina é uma das bacteriocinas mais estudados e sua biossíntese está relacionada a um sistema *quorum sensing* e inclui a própria nisina como um peptídeo feromônio (auto-indutor) e uma transdução de sinal pelo sistema regulatório duplo-componente correspondente NisRK (KLEIN et al., 1993; PARKINSON, 1993; ROSS et al., 1993; ENGELKE et al., 1994; KUIPERS et al., 1995; RA et al., 1996; MCLAUGHLIN et al., 1999; ALTENA et al., 2000; MCAULIFFE et al., 2001).

As bacteriocinas são geralmente sintetizadas como um pré-peptídeo inativo que inclui uma sequência líder N-terminal a qual mantém a bacteriocina na forma inativa dentro da célula produtora facilitando a interação com o transportador. Usualmente esta sequência é clivada durante a exportação da

bacteriocina por um sistema dedicado de transporte ou, menos frequentemente através de uma rota de secreção geral da célula (*Sec-dependente*). A exportação da bacteriocina é usualmente mediada por um transportador ABC que pode conter um domínio proteolítico N-terminal pertencente à família das cisteína proteases e que é responsável pela clivagem do peptídeo líder (VENEMA et al., 1995; DIEP & NES, 2002; COTTER et al., 2005).

Os genes para a produção da bacteriocina ativa estão geralmente organizados em operons. Estes podem estar localizados no cromossomo ou em um plasmídeo ou transposon. De uma maneira geral, os microrganismos possuem genes codificando o peptídeo estrutural, proteínas que auxiliam no processamento para a forma ativa, proteínas que auxiliam o transporte da bacteriocina através da membrana, proteínas regulatórias e proteínas que conferem imunidade ao organismo produtor (CLEVELAND et al., 2001).

Genes que codificam proteínas de imunidade geralmente estão situados dentro ou muito próximos do operon que contém os genes requeridos para a biossíntese da bacteriocina. Produtos com funções relacionadas com a imunidade têm sido identificados como peptídeos pequenos, fortemente carregados e associados à membrana plasmática. O mecanismo de imunidade das bactérias produtoras de bacteriocinas tem a capacidade de fazer a distinção entre a bacteriocina produzida pela própria cultura e aquelas sintetizadas por outras bactérias (CLEVELAND et al., 2001; HOFFMANN et al., 2004; DRIDER et al., 2006; MAQUEDA et al., 2008).

A indução da expressão do gene é ativada por um acúmulo do peptídeo feromônio quando há uma baixa produção constitutiva. Quando um limiar de concentração do feromônio é atingido os peptídeos se ligam aos seus

receptores (histidina proteína quinase), seguido por uma cascata de fosforilação que leva à fosforilação do regulador de resposta o qual se liga e ativa promotores regulados levando a expressão dos genes com consequente produção da bacteriocina (NES et al., 1999).

2.2.2 Síntese não-ribossomal

Os peptídeos antimicrobianos de síntese não ribossomal são sintetizados por um mecanismo chamado Mecanismo Multienzimático, o qual está sob o controle do operon *srf A* (MARAHIEL et al., 1993) e é composto por várias subunidades de polipeptídeos de peso molecular variando entre 100.000 e 600.000 Da (ZUBER & MARAHIEL, 1997). Esses polipeptídeos catalisam a ativação dos aminoácidos para formar acil-adenilatos, os quais vão servir de substrato para a formação de um carboxil-tioester, através de uma ligação covalente à subunidade do complexo (ZUBER & MARAHIEL, 1997). De acordo com esse mecanismo, os aminoácidos ligados covalentemente estão organizados na ordem de sua adição à cadeia polipeptídica. Após essa etapa, os aminoácidos são ligados entre si por ligações peptídicas, resultando na formação de um peptídeo linear, que por sua vez sofrerá ciclização ou simples remoção através de uma tioesterase (ZUBER & MARAHIEL, 1997).

Peptídeos não-ribossômicos frequentemente contém aminoácidos não usuais, incluindo aminoácidos não protéicos ou modificados. A habilidade destes complexos multienzimáticos de incluir tais aminoácidos e incorporá-los em ambos os peptídeos cíclicos e lineares resulta em uma grande estrutura natural e diversa nas espécies bacterianas e fúngicas. Os peptídeos não-ribossômicos podem também atuar como esqueleto para a biossíntese de

estruturas mais complexas ou podem ser imcorporados como ácidos graxos ou policetonas. Tais estruturas podem ser biosintetizadas por sistemas de peptídeos sintetase mistos ou híbridos e policetídeo sintase (KLEINKAUF & VON DOHREN, 1996). Portanto, um grande número de estruturas são possíveis devido a diferentes combinações modulares nas etapas de elongação, redução após as reações de condensação e processamento pós-sintético dos produtos, tais como ciclização e glicosilação (MINOWA et al., 2007).

A rota chamada de Peptídeo Sintetase Não-ribossomal (NRPS) é formada por um grande número de proteínas complexas chamadas sintetases. Os complexos multienzimáticos são organizados modularmente e representam ao mesmo tempo o molde e a maquinaria biossintética. As moléculas sintetizadas por NRPS são curtas (dois a quinze monômeros), incluindo uma alta densidade de aminoácidos não protéicos e frequentemente contém aminoácidos ligados a outros peptídeos ou pontes dissulfeto. A diversidade de aminoácidos incorporados aos peptídeos sintetases é grande e por isso chamados de monômeros ao invés de aminoácidos. Portanto, a estrutura primária dos peptídeos não-ribossomais não é sempre linear, podem, também apresentar estruturas cíclicas ou ramificações (CABOCHE et al., 2009). Estes peptídeos possuem importantes propriedades biológicas tais como imunomoduladores, quelantes de ferro ou atividade antimicrobiana, que pode ser ilustrada pelo famoso antibiótico penicilina o qual é sintetizado a partir do precursor tripeptídeo-ACV produzido pela via de NRPS.

Uma outra classe de metabólitos secundários em destaque são os policetídeos (PK), que constituem uma família complexa de produtos naturais

que são construídos a partir de blocos simples de ácido carboxílico. Os policetídeos são produzidos por plantas, fungos e bactérias, sendo principalmente estudados em Actinomicetos, e possuem uma ampla atividade biológica por produzirem uma diversidade extraordinária de estruturas. Os compostos policetídeos apresentam atividades farmacológicas importantes como potentes antibióticos, antitumorais, antifúgicos, agentes imunossupressores e antivirais (JENKE-KODAMA & DITTMANN, 2009).

A biossíntese de policetídeos sintetases (PKSs) ocorre pela ação de no mínimo três domínios por módulo. O módulo principal consiste de um domínio aciltransferase para a transferência e seleção de uma unidade de ácido carboxílico, uma proteína carreadora acila e um domínio betacetossintase para a condensação descaroxilativa (MINOWA et al., 2007).

A principal diferença entre NRPS e PKSs está no fato de que PKSs condensam ácidos carboxílicos a uma cadeia crescente para formar um produto e NRPSs adicionam aminoácidos (MINOWA et al., 2007).

2.3 Peptídeos antimicrobianos produzidos por espécies de *Bacillus*

As espécies de *Bacillus* têm como habitat natural o solo, mas apresentam-se amplamente distribuídas no ambiente. Este gênero constitui um grupo interessante para o estudo de novas substâncias com atividade antimicrobiana, e a produção de peptídeos antimicrobianos com estruturas químicas diferentes tem sido observada (RISOEN et al., 2004).

O gênero *Bacillus* é reconhecido por apresentar muitas espécies que produzem peptídeos com ação bactericida e fungicida. Estes são classificados

como metabólitos secundários, sendo secretados para o meio extracelular, onde exercem a ação antimicrobiana (ZHENG et al., 1999; KENNEY & MORAN, 1991; KATZ & DEMAIN, 1977).

Bacillus subtilis tem sido alvo de estudos bioquímicos durante várias décadas, e é considerado como um marco na pesquisa de bactérias produtoras de peptídeos antimicrobianos que apresentam amplo espectro de atividade (SONENSHEIN et al., 2003; MOSZER et al., 2002).

Algumas espécies de *Bacillus* têm uma história de produção de substâncias utilizadas na indústria alimentícia devido a sua inocuidade, inclusive como aditivos alimentícios (DE BÖER & DIDERICHSEN, 1991; PEDERSEN et al., 2002). Este gênero apresenta uma grande variedade de espécies que produzem bacteriocinas ou substâncias tipo-bacteriocinas (bacteriocin-like substance - BLS), que apresentam atividade antimicrobiana contra microrganismos deteriorantes e patogênicos, tais como: *Listeria monocytogenes, Staphylococcus aureus, Erwinia carotovora* (CLADERA-OLIVEIRA et al., 2006; NAGHMOUCHI et al., 2007). Estes incluem *B. subtilis* (ZHENG et al., 1999), *B. thuringiensis* (KAMOUN et al., 2005), *B. amyloliquefaciens* (LISBOA et al., 2006) e *B. cereus* (BIZANI & BRANDELLI, 2002).

A produção de vários peptídeos antimicrobianos tem sido descrita para este gênero, sendo surfactina, iturina A, subtilina, subtilosina A e fengicina alguns exemplos destas substâncias (KLEIN et al., 1993; LE MARREC et al., 2000; CHEN et al. , 2009).

2.3.1 Surfactina

O peptídeo antimicrobiano surfactina é um heptapeptídeo cíclico produzido por linhagens de *B. subtilis*. A surfactina tem funções biológicas cruciais, incluindo a capacidade de estimular respostas relacionadas ao sistema imunológico em tecidos do hospedeiro, um fenômeno importante para o controle biológico de doenças de plantas (HENRY et al., 2011). Os genes responsáveis pela produção de surfactina estão organizados em um operon em *B. subtilis* e o gene *sfp*, responsável pela produção da enzima 4'-fosfopantateína transferase foi caracterizado como essencial para a produção de surfactina (NAKANO et al., 1992; HSIEH et al., 2004).

2.3.2 Iturina

Grupos de genes envolvidos na síntese de iturina A têm sido intensamente investigados (TSUGE et al., 2001; YAO et al., 2003). O operon de iturina A abrange uma região de mais de 38 kb e é composto por quatro genes: *ituD, ItuA, ituB*, e *ituC*. O gene *ituD* codifica uma malonil coenzima A transacilase putativa, cuja perturbação resulta em uma deficiência específica na produção de iturina A (TSUGE et al., 2001). O gene *lpa-14* codifica a 4'-fosfopantateína transferase necessária para a maturação da enzima modelo de iturina A. Tanto o gene *ituD* quanto o gene *lpa-14* desempenham papéis de liderança na produção desse peptídeo antimicrobiano (TSUGE et al., 2001; HSIEH et al., 2008).

2.3.3 Subtilosina

Subtilosina A é uma bacteriocina isolada originalmente de *B. subtilis* (BABASAKI et al., 1985), mas também foi encontrada em *Bacillus amyloliquefaciens* e *Bacillus atrophaeus* (STEIN et al., 2004; SUTYAK et al., 2008). A produção de subtilosina A madura requer a expressão de oito (*sboA-albABCDEFG*) dos nove genes identificados na cepa produtora de *B. subtilis.* Estes genes agrupados são regulados por um promotor que reside a montante do gene *sboA* e seus produtos estão envolvidos na modificação pós-traducional e processamento de pré-subtilosina, secreção e imunidade (ZHENG et al., 2000; MAQUEDA et al., 2008). Acredita-se que os genes *albABCDEFG* constituem um operon que codifica as proteínas que funcionam no processamento de pré-subtilosina e exportação de subtilosina. O gene *sboA* codifica pré-subtilosina, que é um peptídeo de 43 aminoácidos (ZHENG et al., 1999).

2.3.4 Fengicina

Membros da família fengicina, incluindo os relacionados com a plipastatina, são decapeptideos com um ß-hidroxi ácido-graxo que mostram propriedades incomuns, como a presença de ornitina na porção peptídica. Fengicinas também mostram atividade antifúngica, embora mais específicas para fungos filamentosos (STELLER et al., 1999; VANITTANAKOM et al., 1986). Trabalhos anteriores estabeleceram que fengicina é produzida por síntese não-ribossomal por cinco fengicina sintetases, *fenC, fenD, fenE, fenA* e *fenB* (LIN et al., 1999; STACHELHAUS & MARAHIEL, 1995; WU et al., 2007).

2.4 Aplicações de peptídeos antimicrobianos

2.4.1 Biopreservação e aplicação em alimentos

Muitas bactérias láticas têm papéis importantes na produção de alimentos fermentados, e algumas destas bactérias podem ser capazes de inibir o crescimento de uma grande variedade dos organismos patogênicos que podem estar presentes nos alimentos (RAY et al., 1992; STILLES et al., 1991).

A nisina (Nisaplin®) e a pediocina PA1/AcH (ALTA®) são as únicas bacteriocinas comerciais empregadas atualmente na preservação de alimentos, sendo sintetizadas por bactérias láticas e, normalmente, empregadas na produção de alimentos fermentados (HOLZAPFEL et al., 1995; MONTVILLE & WINKOSWSKI, 1997; ENHAHAR et al., 2000; COTTER et al., 2005; GAUTAM & SHARMA, 2009).

A nisina é produzida especificamente por *Lactococcus lactis* (GROSS et al., 1971). Devido a este tipo de bacteriocina estar naturalmente associada com determinados tipos de alimentos e não produzir nenhum efeito adverso quando ingerido, a agência norte-americana, *Food and Drugs Administration* (FDA), considerou-a como uma substância GRAS (*Generally Regarded as Safe*) (FEDERAL REGISTER, 1998).

Bacteriocinas têm demonstrado potencial na biopreservação de carne, produtos lácteos, alimentos enlatados, peixes, bebidas alcoólicas, saladas, produtos a base de ovos, produtos de padaria e vegetais fermentados. A forma como pode ser inoculada a bactéria depende do tipo de alimento, pode haver a incorporação da cultura bacteriana, da própria bacteriocina de forma direta, da

bacteriocina encapsulada, ou incorporação da bacteriocina na embalagem do produto (CHEN et al. 2003; O'SULLIVAN et al., 2002).

A desvantagem do uso de bacteriocinas como bioconservantes é que estas são geralmente mais sensíveis às variações ambientais, como alterações de pH, temperatura, composição química do alimento, bem como a microbiota normal do produto, que podem inativar ou diminuir a atividade antimicrobiana das mesmas, se comparadas aos conservantes químicos, bem como a necessidade de maiores estudos toxicológicos sobre o seu respectivo uso em alimentos (SCHILLINGER, et al. 1996; MING et al., 1997; UECKERT, et al., 1998; SIRAGUSA et al., 1999; WOGAN et al., 2004; MAURIELLO et al., 2005; SILVÁN et al., 2006; GÁLVEZ et al., 2007; NAGHMOUCHI et al., 2007).

Em geral, para que um peptídeo antimicrobiano possa ser empregado na indústria de alimentos deve cumprir alguns requisitos como: a linhagem produtora deve ter status GRAS; deve apresentar amplo espectro de inibição sobre os principais patógenos de alimentos ou ser altamente específico sobre algum deles; deve ser termoestável; não pode apresentar risco à saúde do consumidor; deve ter efeito benéfico sobre o produto, aumentando sua segurança, sem afetar a qualidade nutricional e sensorial (HOLZAPFEL et al., 1995).

2.4.2 Aplicações clínicas

O entedimento do mecanismo de ação da alguns lantibióticos e sua atividade contra patógenos resistentes a várias drogas torna estes compostos uma opção atraente como possíveis agentes terapêuticos. O amplo espectro de ação dos lantibióticos, teoricamente, poderia permitir seu uso contra

patógenos de humanos ou animais. Por exemplo, a lacticina 3147 tem atividade *in vitro* contra *Staphylococcus aureus*, enterococos, estreptococos, *Clostridium botulinum* e *Propionibacterium acnes* 151. Ensaios *in vivo* com modelos animais têm demonstrado o sucesso de lantibióticos no tratamento de infecções causadas por *S. pneumoniae* 152 e *S. aureus* 153154 e na prevenção de cárie dentária e gengivite (BLACKBURN & GOLDSTEIN, 1995; MCCONVILLE, 1995; HOWELL et al. 1993; RYAN et al., 1999).

Uma cepa que produz a mutacina 1140 passou pela Fase I de ensaios clínicos nos EUA com uma visão à terapia de reposição, e o suplemento dietético BLIS K12, que contém um *Streptococcus salivarius* que produz dois lantibióticos salivaricin A2 e B, é vendido na Nova Zelândia como um inibidor da bactérias responsáveis pelo mau hálito (TAGG, 2004).

2.4.3 Controle biológico

Vírus, bactérias e fungos causam doenças que afetam diversas culturas de plantas, resultando em perdas e diminuindo a qualidade e segurança dos produtos agrícolas. O controle biológico de doenças de plantas por microrganismos é uma alternativa interessante ao uso de produtos químicos prejudiciais ao meio ambiente. Os microrganismos utilizados como agentes de biocontrole frequentemente secretam uma grande variedade de peptídeos antimicrobianos produzidos por síntese ribossomal ou não-ribossomal (MONTESINOS, 2007).

Exemplos de bacteriocinas que inibem bactérias fitopatogênicas têm sido relatados a partir de bactérias associadas com plantas (JABRANE et al., 2002; LAVERMICOCCA et al., 2002; PARRET et al., 2005).

Algumas espécies de *Bacillus* são capazes de formar endósporos, sendo tolerantes ao calor e à dessecação e podendo produzir um amplo espectro de antibióticos (EMMERT & HANDELSMAN, 1999; HANDELSMAN et al., 1990). Lipopeptídeos cíclicos produzidos por *Bacillus amyloliquefaciens* FZB42 demostraram a capacidade de controlar o fungo *Fusarium oxysporum* utilizando caracterizações estruturais e funcionais dos agrupamentos de genes envolvidos na sua síntese e análise de mutantes defeituosos, incapazes de produzir bacilomicina D e fengicina (KOUMOUTSI et al., 2004). Após a combinação de detecção *in situ* e análise de mutantes deficientes, a produção de bacilomicina, fengicina ou iturina por várias cepas de *Bacillus subtilis* foi implicado no mecanismo de controle do oídio em melão, causado por *Podosphaera fusca* (ROMERO et al., 2007).

Algumas cepas de *Bacillus subtilis* têm demostrado grande interesse comercial para biocontrole, sendo que as cepas FZB42 (Abitep, Alemanha), GB03 (Kodiak, EUA), QST713 (Serenade, EUA), e MBI600 (Subtilex, EUA) têm os genes para síntese de fengicina, surfactina, bacilomicina ou iturina (JOSHI & MCSPADDEN GARDENER, 2006).

2.5 Influência das condições ambientais na produção de peptídeos antimicrobianos

Condições ambientais como a composição do meio de cultura, o pH, a temperatura, a aeração e o tempo de incubação influenciam na biossíntese de peptídeos antimicrobianos (ÇALIK et al., 2000; CLADERA-OLIVERA et al., 2004). Guez et al. (2008) destacaram a importância do pH para

microorganismos em geral e *Bacillus subtilis* ATCC 6633, em particular. Em temperaturas mais baixas, as células são confrontadas com vários problemas, incluindo atividades enzimáticas reduzidas, baixa fluidez da membrana e diminuição da iniciação da tradução (FICKERS et al., 2008).

Visando estudar a otimização da produção de antimicrobianos pelo método clássico, o efeito do pH e da temperatura na produção de bacteriocinas foi estudado por Krier et al. (1998) para *Leuconostoc mesenteroides* FR52, por Vignolo et al. (1995) para *Lactobacillus casei* CRL 70, por Motta & Brandelli (2008) para *Brevibacterium linens*, entre outros. A otimização da produção de antimicrobianos pelo método clássico envolve a mudança de uma variável independente (nutriente, pH, temperatura, etc.) fixando todas as outras variáveis. Isto consome muito tempo e é de custo elevado para um grande número de variáveis (ADINARAYARA et al., 2003). Além disso, pode levar a conclusões errôneas (OH et al., 1995).

A metodologia de superfície de reposta (MSR) é um conjunto de técnicas estatísticas para desenhar experimentos, construir modelos, avaliar o efeito de fatores e procurar condições ótimas dos fatores para uma resposta desejada (LI et al., 2002). Tem a vantagem de permitir o estudo de efeitos realizando um número pequeno de experimentos, além de possibilitar o estudo das interações entre estes efeitos, o que o método clássico não permite.

Esta metodologia foi aplicada com sucesso em diversas áreas da biotecnologia como a bioconversão de soro de queijo em micélios de *Ganoderma lucidum* (LEE et al., 2003), a otimização da produção de neomicina por *Streptococcus marinensis* (ADINARAYANA et al., 2003), entre outros. Também tem sido usado para avaliar o efeito dos componentes do meio e das

condições ambientais sobre a produção de atividade antimicrobiana de *Bacillus licheniformis AnBa9* (ANTHONY et al., 2009) e *Bacillus cereus* 8A (DOMINGUEZ et al., 2007).

Alguns fatores ambientais também podem ser trabalhados buscando a indução da produção de peptídeos antimicrobianos. Porém, poucos estudos avaliando a indução da produção de peptídeos antimicrobianos por bactérias do gênero *Bacillus* são reportados na literatura (BENITEZ et al., 2011; VELHO et al., 2011a).

Estudos com sobrenadante de cultura de células-livre da cepa indutora sugerem que o aumento da atividade antimicrobiana não é mediada por um metabólito secretado pela cepa indutora. Benitez et al. (2011), verificaram que cultura de *E. coli* causou um aumento na atividade antimicrobiana de *Bacillus amyloliquefaciens* LBM 5006. No entanto, estudos semelhantes sobre as espécies *Bacillus* são muito escassos, se comparados com os realizados para bactérias ácido lácticas. A produção de kimchicina GJ7 foi aumentada na presença de *Lactobacillus plantarum* KFRI 464, sendo que a fração de restos celulares induziu muito mais a atividade antimicrobiana do que a fração intracelular (CHANG et al., 2007). A máxima indução da produção de bacteriocina por *Leuconostoc citreum* GH7 foi obtida na presença de células termicamente inativadas (121ºC/15 min) de *L. plantarum* KFRI 464, provavelmente ativado pelo contato célula-célula (CHANG et al., 2007). A produção de lactacina B por *Lactobacillus acidophilus* N2 aumentou significativamente quando foi cultivada com células de *Lactobacillus delbrueckii* subsp. *lactis* ATCC 4797 vivas, lavadas ou inativadas, sugerindo que a

substância indutora está associada a célula e pode estar localizada no envelope celular (BAREFOOT et al., 1994).

2.6 Purificação de peptídeos antimicrobianos

A utilização de peptídeos antimicrobianos, tanto para estudo quanto para aplicação, requer geralmente a purificação destas substâncias. Diversas técnicas têm sido utilizadas pelos pesquisadores para conseguir este objetivo. Como são substâncias com diferentes características estruturais e propriedades químicas, não é possível generalizar um método único de purificação. Porém, muitos trabalhos apresentam etapas similares.

Para a purificação de peptidieos antimicrobianos são necessários estudos de produção e grandes volumes de cultivo. A produção pode ser influenciada pelas condições de incubação como pH, temperatura e tempo, além da composição do meio. As condições ótimas de produção devem ser determinadas previamente para cada microrganismo produtor. Em função da grande diversidade de peptídeos antimicrobianos, não existe um método único ou protocolo geral para a purificação (ROJO-BEZARES et al., 2007; MOTTA & BRANDELLI, 2008).

Uma vez liberadas das células produtoras, ou as bacteriocinas permanecem adsorvidas à parede celular, ou ficam livres no meio onde são secretadas. Geralmente o processo inicia-se com a centrifugação da cultura para separar as células do sobrenadante. Este é posteriormente concentrado através da precipitação com sulfato de amônio (outros sais ou solventes podem ser utilizados nesta etapa).

A vantagem desta primeira etapa de purificação é o aumento na concentração da proteína do meio. As proteínas precipitadas são dissolvidas em água deionizada ou tampão e separadas pelo uso de vários métodos, incluindo as cromatografias líquida de alta performance e fase reversa, de gel-filtração, de troca iônica ou, ainda, de interação hidrofóbica, utilizadas de forma isolada ou combinada. Métodos de purificação de peptídeos antimicrobianos que utilizam protocolos complexos e demorados apresentam a desvantagem de obter baixo rendimento de proteínas (DRIDER et al., 2006; GAUTAM & SHARMA, 2009).

Pode ser utilizada também a extração com solventes orgânicos. Apesar destas técnicas levarem a produção de preparações de antimicrobianos altamente puras, o rendimento final é baixo e geralmente inferior a 20% (JACK et al., 1995). Além disso, estes procedimentos funcionam bem para pequenas quantidades de sobrenadante, mas, tornam-se inviáveis para a produção em escala semi-industrial (UTENG et al., 2002).

Os sistemas aquosos bifásicos também podem der utilizados para a purificação de peptídeos antimicrobianos. Sistemas contendo duas ou mais fases líquidas em equilíbrio termodinâmico são muito úteis para aplicações em extração e/ou purificação de compostos presentes em inúmeros processos tecnológicos (PESSOA & KOLIKIAN, 2005). Porém, para aplicação desta técnica de extração líquido-líquido para a obtenção de compostos com importância biotecnológica (proteínas, enzimas, células, vírus, organelas, etc.), faz-se necessária a utilização de sistemas em que as duas fases guardem semelhanças, em termos das propriedades físico-químicas, com o meio aquoso presente nos seres vivos (SELBER et al., 2004). Neste sentido, os sistemas

denominados aquosos bifásicos (SABs ou, em inglês, ATPS) desempenham um papel estratégico, pois as duas fases são formadas predominantemente (60-95%) por água (SILVA & LOH, 2006).

3 OBJETIVOS

3.1 Objetivo Geral

Investigar a produção de peptídeos antimicrobianos sintetizados por *Bacillus* sp.P11.

3.2 Objetivos Específicos

- Obter compostos com atividade antimicrobiana a partir de uma cultura de *Bacillus* sp. P11, isolado da bacia amazônica.

- Avaliar a produção de atividade antimicrobiana utilizando como substrato subprodutos e resíduos de agroindústrias de alimentos.

- Verificar a influência do pH inicial e da temperatura na expressão de genes essenciais para a produção das bacteriocinas subtilosina A e iturina A através da metodologia de qRT-PCR.

- Investigar a produção de atividade antimicrobiana em presença de células inativadas termicamente de bactérias ou fungos e, nestas condições, monitorar a expressão dos genes *sfp, lpa-14, ituD*, *sboA* e *fenA* por qRT-PCR.

- Purificar substâncias antimicrobianas produzidas por *Bacillus* sp. P11, através de métodos cromatográficos.

4 CAPÍTULOS

4.1 Capítulo I

Use of byproducts of food industry for production of antimicrobial activity by *Bacillus* sp. P11

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Abstract

Production of antimicrobial activity by *Bacillus* sp. P11 was tested on different byproducts of food industry, like fish meal, grape waste, an industrial fibrous soybean residue, soybean meal and cheese whey. *Bacillu*s sp. P11 produced the higher amount of antimicrobial activity on soybean meal, followed by fish meal and fibrous soybean residue. Soybean meal was the selected medium to determine the effect of three variables (temperature, initial pH and substrate concentration) on bacteriocin activity by response surface methodology, using a 2^3 factorial design. Statistical analysis showed good adequacy to the model (R^2 of 0.8268). In the range studied, temperature and initial pH of the medium have a significant effect on bacteriocin production and substrate concentration have no significant effect. Response-surface data showed maximum bacteriocin production at initial pH between 7.0 and 8.5 and temperature between 39 and 42ºC. In the optimum conditions (initial pH 7.0 and 42^{o}C), production of bacteriocin activity by *Bacillus* sp. P11 was compared using a commercial medium (BHI broth) and soybean meal. Maximum activity achieved with the soybean meal-based medium was similar to that obtained with BHI, indicating that soybean meal may be a cost-effective substrate for production of antimicrobial activity by *Bacillus* sp. P11.

Keywords: **agroindustrial waste; bacteriocin; *Bacillus*; response surface methodology, soybean meal.**

Introduction

Bacteriocins are antimicrobial peptides widespread produced among bacteria (Cotter et al., 2005). The attractive features of bacteriocins, such as their natural sources, wide range of activities and their proteinaceous nature, which implies a putative degradation in the gastro-intestinal tract of man and animals, have interested researchers seeking to develop new antimicrobial agents (Cleveland et al., 2001; Asaduzzaman & Sonomoto, 2009). Those produced by lactic acid bacteria (LAB) are largely studied with the perspective to search for safe and food-grade preservatives of biological origin (O'Sullivan et al., 2002; Calo-Mata et al., 2008). Despite of the intensive work on bacteriocins produced by LAB, the genus *Bacillus* comprises a variety of industrially important species and has a history of safe use in both food and pharmaceutical industry (Paik et al., 1997; Pedersen et al., 2002; Hong et al., 2008; Vaucher et al., 2010). Indeed, bacteriocins or bacteriocin-like substances (BLS) have been described for *Bacillus* spp., presenting a broad spectrum of antibacterial activity and potential use as biopreservative in food (Bizani et al., 2005; Motta et al., 2007).

Bacteriocin production is often performed in complex media, which promote abundant growth and relatively high bacteriocin levels. The necessity for reduction of pollutants in the environment and the need to maximize returns on raw materials has encouraged the search for new ways of using food industry waste as the basis of culture media. Possible alternatives include byproducts such as milk whey and mussel-processing wastes (Amiali et al., 1998; Guerra & Pastrana, 2002). The use of byproducts will require optimization of bacteriocin production, which is usually dependent on multiple strain-specific factors (Leal-Sánchez et al., 2002; Casarin et al., 2008). Studies on multiple

factors affecting the production of bacteriocins are relatively scarce and it is difficult to optimize them for biotechnological processes. Optimization by a conventional “one-at-a-time-approach” does lead to a substantial increase in bacteriocin yields; however, this approach is not only massive and time consuming, but also has the limitations of ignoring the importance of interaction of various parameters. Response surface methodology (RSM) has been successfully applied in many areas of biotechnology, including reports on bacteriocin production. RSM has been used to evaluate the effect of medium components and environmental conditions on production of antimicrobial activity by *Bacillus licheniformis* AnBa9 (Anthony et al., 2009) and *Bacillus cereus* 8A (Dominguez et al., 2007).

Bacillus sp. P11 is a novel bacteriocin-producing strain isolated from the intestinal contents of the teleost fish Piau-com-pinta (*Leporinus* sp.) of Brazilian Amazon basin (Giongo et al., 2007). The purpose of this work was to select a less expensive medium and study the influence of temperature, pH and concentration of growth substrate on bacteriocin production by *Bacillus* sp. P11. The production of antimicrobial activity in commercial medium was compared with soybean meal-based medium.

Materials and methods

Reagents and media

The following byproducts were individually evaluated. Cheese whey powder from the production of mozzarella cheese (Parmalat, Porto Alegre, Brazil) contains 13% protein, 1% lipids, 77% carbohydrate (lactose) and 9% minerals. Fish meal (Etna, Rio Grande, Brazil), a waste of fish processing, contains 54% protein, 11% lipids, 5% carbohydrate and 30% minerals. Grape waste residue

(30% fiber, 21% carbohydrate, 10% protein, 10% fat) was obtained as described by Silveira et al. (2008). Isolated soybean protein (Bunge, Esteio, Brazil) has 79% protein, 3% lipids, 12% carbohydrates and 5% minerals. Fibrous soybean residue (35% protein, 19% carbohydrates and 46% fibre), is a byproduct from the production of isolated soybean protein. Soybean meal (Cerelus, Ijuí, Brazil) contains 50% protein, 3% lipids, 35% carbohydrate, 5% fibre and 7% minerals. The commercial medium Brain Heart Infusion (BHI) was from Oxoid (Basingsoke, UK).

Bacterial strains

The producer bacterium was the previously characterized strain *Bacillus* sp. P11 (Giongo et al., 2007). The indicator strain was *Listeria monocytogenes* ATCC 7644. BHI medium was used for maintenance of strains with 20% (v/v) glycerol at -20 ºC. The cultivation of strains was performed aerobically.

Selection of the best substrate for bacteriocin production

The inoculum was developed in BHI at 30 ºC under shaking (120 rpm). The absorbance at 600 nm was adjusted ($A_{600\ nm}$ = 2.00) to obtain approximately 10^7 CFU/mL, and 1.0 mL was transferred to 250 mL Erlenmeyer flasks containing 100 mL of medium. Cultures were incubated in a rotary shaker at 120 rpm for 24 h. Aliquots of culture were centrifuged at 10,000g for 15 min. Culture supernatants were boiled at 100 ºC for 5 min, placed in an ice bath and the pH was measured and adjusted to pH 7.0 before being used in the antimicrobial assay. In parallel, bacterial suspensions were diluted to 10^{-8} in 8.75 g/L NaCl

for determination of colony forming units per millilitre (CFU/mL) (Motta & Brandelli, 2002).

The byproducts of the industry were individually tested to select the best substrate for bacteriocin production and its concentration are showed in Table 1 and chosen like elsewhere (Dominguez et al., 2007). Cultivation in BHI broth was developed as control. The pH was adjusted at 7.0 with 0.5 M HCl or 0.5 M NaOH before autoclaving (121ºC, 15 min). Cultivation was developed in 250 mL Erlenmeyer flasks containing 100 mL of each medium. After inoculation with 1 mL culture, incubation was performed at 30ºC under shaking (120 rpm). The food byproduct with best yield (soybean meal) was compared to commercial medium (BHI) to evaluate the growth and bacteriocin production behavior of *Bacillus* sp. P11. Bacteriocin activity was determined after 24 h as described above. The cultivations were performed in duplicate. Two independent activity assays were performed for each cultivation.

Table 1. Growth substrates tested for production of bacteriocin activity

Medium	Concentration (g/L)	Bacteriocin Activity (AU/mL)
BHI	-	1200 ± 565
Cheese whey	70	400 ± 283
Fish meal	10	800 ± 565
Fibrous soybean residue	20	800 ± 0
Soybean meal	20	1600 ± 0
Isolated soybean protein	20	800 ± 565
Grape waste residue	30	0 ± 0

Antimicrobial activity assay

The antimicrobial activity was detected by agar disk diffusion assay (Motta & Brandelli, 2002). Aliquots (20 µL) of culture supernatants were applied to 6mm cellulose paper disks (Whatman n.2) on BHI agar plates previously inoculated with a cell suspension of *L. monocytogenes* ATCC 7644, which corresponded to a 0.5 McFarland turbidity standard solution. Plates were incubated at 37 °C. The antimicrobial activity titre was determined by serial two-fold dilution method. Activity was defined elsewhere as the reciprocal of the dilution after the last serial dilution giving a zone of inhibition and expressed as activity units per milliliter (AU/mL) (Mayr-Harting et al., 1972).

Experimental design of RSM

After selection of the best medium, the next step was to determine the optimal levels of three variables, temperature, initial pH and soy bean meal concentration on bacteriocin production and on bacterial growth. For this purpose, the response surface approach by using a set of experimental design (central composite design with five coded levels) was performed.

A 2^3 full factorial design for three factors with replicates at the centre point and star points was used for optimization of cultivation. The variables used were evaluated each at five coded levels (-a, -1, 0, + 1, +a). The axial distance α was chosen to be 1.68 to make this design orthogonal. A set of 18 experiments was carried out (Table 2). The central values (0 level) chosen for experimental design were: temperature, 30ºC; initial pH, 7.0 and soybean meal concentration, 20 g/L (Dominguez et al., 2007).

Table 2. Experimental design and results of the 2^3 factorial design

Run	Variables[a]			Bacteriocin activity (AU/mL)		Final cell concentration (Log CFU/mL)
	Soybean (g/L) (x_1)	pH (x_2)	Temperature (°C) (x_3)	Observed	Predicted	
1	11 (-1)	5 (-1)	23 (-1)	0±0	169.03	5.22±0.12
2	29 (+1)	5 (-1)	23 (-1)	0±0	169,03	5.91±0.05
3	11 (-1)	9 (+1)	23 (-1)	100±141	169.03	6.15±0.03
4	29 (+1)	9 (+1)	23 (-1)	0±0	169.03	6.78±0.03
5	11 (-1)	5 (-1)	37 (+1)	0±0	660.49	6.78±0
6	29 (+1)	5 (-1)	37 (+1)	0±0	660.49	7.16±0.02
7	11 (-1)	9 (+1)	37 (+1)	800±0	660.49	8.37±0
8	29 (+1)	9 (+1)	37 (+1)	800±0	660.49	8.43±0.03
9	5 (-1.68)	7 (0)	30 (0)	600±283	711.0	8.22±0.01
10	35 (+1.68)	7 (0)	30 (0)	800±0	711.0	8.13±0.06
11	20 (0)	4 (-1.68)	30 (0)	0±0	-125.108	0±0
12	20 (0)	10 (+1.68)	30 (0)	0±0	-125.108	0±0
13	20 (0)	7 (0)	18 (-1.68)	100±141	298.1736	8.67±0
14	20 (0)	7 (0)	42 (+1.68)	1200±566	1123.826	7.97±0.04
15	20 (0)	7 (0)	30 (0)	800±0	711.0	0±0
16	20 (0)	7 (0)	30 (0)	800±0	711.0	7.32±0.03
17	20 (0)	7 (0)	30 (0)	800±0	711.0	6.80±0.03
18	20 (0)	7 (0)	30 (0)	400±0	711.0	8.04±0.02

[a] Coded values are presented in the parenthesis.

Results were analyzed by the Experimental Design Module of the Statistica 7.0 software (Statsoft, OK, USA). The model permitted evaluation of the effects of linear, quadratic and interactive terms of the independent variables on the chosen dependent variables. Three-dimensional surface plots were drawn to illustrate the main and interactive effects of the independent variables on bacteriocin production. The assays were performed in duplicate. The optimum values of the selected variables were obtained by solving the regression equation and also by analyzing the response surface contour plots (Myers & Montgomery, 2002).

Results and discussion

Selection of best substrate for bacteriocin activity

Six different culture media were compared for bacteriocin production by *Bacillus* sp. P11 and the results are shown in Table 1. Higher activity was obtained by cultivation on soybean meal (1600 AU/mL), followed by fish meal, fibrous soybean residue and soybean protein. Antimicrobial activity was not observed on grape waste residue. Similarly, soybean meal has been described as the best substrate to bacteriocin production by other species of *Bacillus* (Dominguez et al., 2007; Motta & Brandelli, 2008). However, Cladera-Olivera et al. (2004) showed that it was not possible to determine a relation between bacteriocin production by *Bacillus licheniformis* P40 and optimal conditions for bacterial growth in cheese whey. Considering the compositions of the byproducts used in this study, soybean meal has elevated quantities of proteins and carbohydrates, N and C sources, respectively. The adequate concentrations of these nutrients in medium can enhance bacterial growth and the high amount of protein and carbohydrate in soybean meal may explain the highest antimicrobial activity by *Bacillus* sp. P11 in this medium.

Bacteriocin activity on soybean meal

The results of central composite design experiments for studying the effects of initial pH, temperature and substrate concentration (independent variables) on bacteriocin activity and bacterial growth are presented in Table 2.

The analysis of variance (ANOVA) was employed for the determination of significant parameters and to estimate the production of bacteriocin as a function of substrate (soybean meal concentration), temperature and pH (Table

3). The computed *F*-value model (4.386) was higher than the *F*-value in statistic tables at 95% of confidence ($Ft_{9,8}$=3.23), which demonstrate significance for the regression model (Myers & Montgomery, 2002). The regression equation obtained indicated the R^2 value of 0.8268 (a value of R^2>0.75 indicates de adequacy of the model), thus 82.68% of the total variation is explained by the model. In addition, there is a good correlation between the experimental and predicted values. The following regression equation was obtained:

$$Y = 711.1 + 45.37x_3 + 296.24x_2^2 \qquad (1)$$

Optimization by a conventional “one-at-a-time-approach” does lead to a substantial increase in bacteriocin yields; however, this approach is not only massive and time consuming, but also has the limitations of ignoring the importance of interaction of various parameters. The significance of coefficients determined *P*-values is shown in Table 3. The larger the magnitude of the *t*-value and smaller the *P*-value, the more significant is the corresponding coefficient. In this study, temperature and second-order pH were the significant variables (P_T < 0.05, $P_{pH\text{-}pH}$ < 0.05), which indicates that they can act as limiting factors and variations in their values will alter bacteriocin activity to a considerable extent. At 90% of confidence, linear pH and the interaction between pH and temperature were significant to the bacteriocin production by *Bacillus* sp. P11. Results showed that neither soybean meal concentration nor interaction between this variable with the other two were not significant (*P*>0.05) for bacteriocin activity, indicating that *Bacillus* sp. P11 produces similar activity

of antimicrobial peptide with high substrate concentration or even with low concentration.

Table 3. Analysis of variance for the model and the regression coefficients estimated to bacteriocin activity

Source	Sum of square	Degree of freedom	Mean square	*F*-value	*t*-value	*P*-value
Soybean meal concentration (L)	4082	1	4082	0.102	7.122	0.770
Soybean meal concentration (Q)	29328	1	29328	0.733	0.319	0.455
pH (L)	211802	1	211802	5.295	-0.856	0.105 [b]
pH (Q)	1106780	1	1106780	27.669	2.301	0.013 [a]
Temperature (L)	821493	1	821493	20.537	-5.260	0.020 [a]
Temperature (Q)	54834	1	54834	1.371	4.532	0.326
Soybean meal (L) by pH (L)	1250	1	1250	0.031	-1.171	0.871
Soybean meal (L) by Temperature (L)	1250	1	1250	0.031	-0.177	0.871
pH (L) by Temperature (L)	281250	1	281250	7.031	0.177	0.077 [b]
Lack of Fit	389106	5	77821	1.946	2.652	0.309
Pure Error	120000	3	40000		7.122	
Total sum of square	2940000	17			0.319	

[a] Statistically significant at 95% of confidence.

[b] Statistically significant at 90% of confidence.

L: linear effect

Q: quadratic effect

The three-dimensional response surface curve of pH versus temperature were then plotted (Fig. 1). Substrate concentration was fixed at central point (20 g/L), because this variable has no significant effect on the response. Maximum bacteriocin activity (1400 AU/mL) was achieved at slightly alkaline pH between 7.0 and 8.5 and temperature between 39 and 42°C (highest value at pH 7.0 and 42^{o}C). Anthony et al. (2009) also found *B. licheniformis* AnBa9 to obtain highest

specific activity at temperature around 42ºC and at alkaline pH (8.0). Bacteriocin activity decreased with decreasing of cultivation temperature and at acid or high alkaline pH. Bacteriocin production by *Bacillus cereus* 8A was also better at alkaline pH (in the range of 6.5 and 9.0) on soybean meal; however that strain produced higher bacteriocin activity at temperature around 30 ºC (Dominguez et al., 2007).

With respect to bacterial growth, the model determined was not significant, because of poor fit of R^2-value (0.6491) and the *F*-test did not showed significance of the regression model (data not shown).

Bacillus growth and bacteriocin production at optimal conditions of temperature and pH

Bacillus sp. P11 was aerobically incubated at optimal conditions obtained with the factorial design (42 °C, initial pH 7.0) on BHI (commercial medium) or soybean meal (alternative medium). Analysis of variance showed that soybean meal concentration was not significant to bacteriocin production, thus it was used at the concentration of central point (20 g/L). *Bacillus* growth was similar in both media and the stationary phase was reached after 12 h of cultivation (Fig. 2A). Cells enter in death phase after 30 h and 36 h in BHI and soybean meal, respectively.

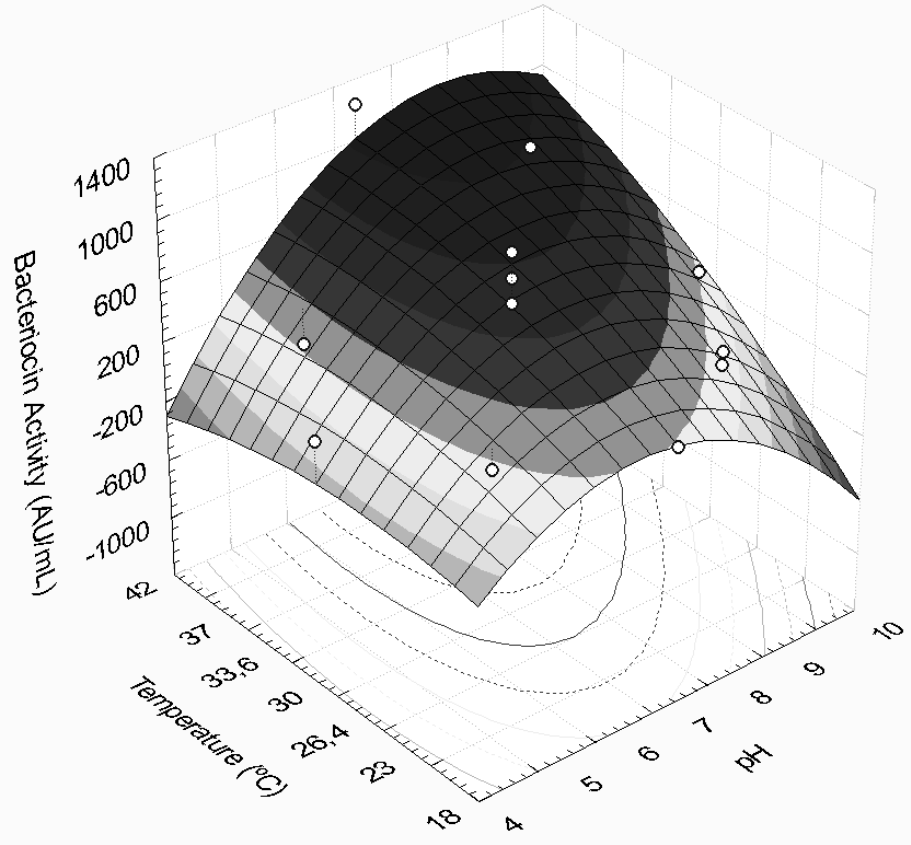

Figure 1: Response surface of bacteriocin activity (AU/mL) by *Bacillus* sp. P11 as a function of temperature (ºC) and initial pH at soybean protein concentration of 20g/L.

Maximum bacteriocin activity was observed at 24 h for soybean meal and this activity was steady until 36 h of cultivation (Fig. 2B). In BHI there was a maximum production of antimicrobial activity at 24 h of cultivation and then an important decrease was observed. Bacteriocin activity was similar in both media at 24 h, with no significant difference (P>0.05), indicating the potential of soybean meal as culture medium for production of bacteriocins by *Bacillus* sp. P11.

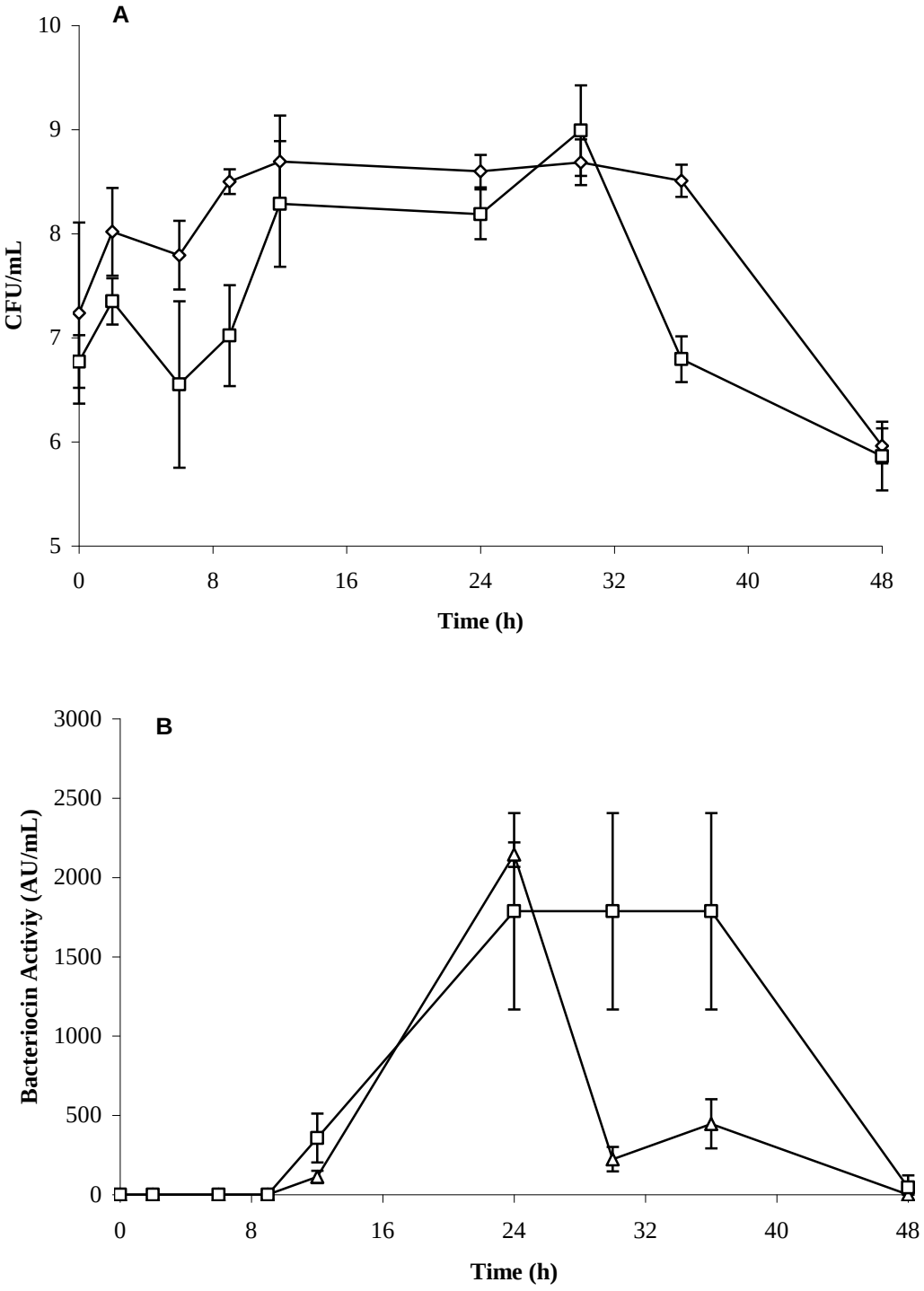

Figure 2: Bacterial growth (A) and bacteriocin activity (B) by *Bacillus* sp. P11 at optimal conditions of temperature (42°C) and initial pH (7.0), and 20 g/L soybean meal concentration (□) or BHI medium (◊). Each point represents the mean ± Standard Error Mean of three independent experiments.

Production of antimicrobial peptides by strains of *Bacillus* is suggested to be under complex genetic regulation (Marahiel et al., 1993; Duitman et al., 1999). Decrease of antimicrobial activity at the late stationary phase could be associated to degradation by extracellular proteases, which are often produced by *Bacillus* spp. (Bizani e Brandelli, 2002). The production of antimicrobial activity started during the exponential growth phase, reaching maximum values at stationary phase. Production of bacteriocin is usually associated with primary metabolite kinetics (Cladera-Olivera et al., 2004; De Vuyst et al., 1996), however, bacteriocin production is recorded as a secondary metabolite in *Lactobacillus plantarum* LPCO10 (Jiménez-Díaz et al., 1993), *B. licheniformis* 26 L-10/ 3RA (Pattnaik et al., 2001), and *Lactobacillus pentosus* B96 (Delgado et al., 2005). In this study, *Bacillus* sp. P11 cultivated in soybean meal, the antimicrobial substances started to be produced during exponential growth phase, suggesting a primary metabolite behavior.

Conclusions

Soybean meal was the best medium to *Bacillus* sp. 11 to produce antimicrobial substances, as good as commercial medium. Maximum bacteriocin activity was achieved at pH between 7.0 and 8.5, temperature between 39ºC and 42ºC. Soybean meal concentration was not significant to bacteriocin activity. *Bacillus* sp. P11 had reached the stationary phase after 12 h of cultivation, and maximum bacteriocin activity was observed from 24 h. Bacteriocin activity was similar in soybean meal and BHI, indicating the waste medium as a potential alternative to produce bacteriocins by *Bacillus* sp. P11.

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4.2 Capítulo II

Influence of pH and temperature on the expression of *sboA* and *ituD* genes in *Bacillus* sp. P11 by qRT-PCR

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Abstract

Temperature and pH are key factors influencing the production of antimicrobial peptides. In this work, qRT-PCR methodology was used to demonstrate the effect of these two variables on *sboA* (subtilosin A) and *ituD* (iturin A) expression in *Bacillus* sp. P11, an isolate from aquatic environment of the Amazon. *Bacillus* sp. P11 was incubated in BHI broth for 36 h at 30^{o}C, 37ºC and 42ºC, and the pH values were 6.0, 7.4 and 8.0. The production of subtilosin A and iturin A, in optimized conditions, was confirmed by mass spectrometry. The *sboA* expression increased 200-fold when the initial pH was 8.0. In contrast, *ituD* expression was maximum at pH 6.0. Increased temperature (42^{o}C) was adverse for both genes, but *ituD* expression increased at 37^{o}C. Expression of *sbo*A and *ituD* was strongly affected by pH and temperature and qRT-PCR proved to be a powerful tool to investigate the potential of *Bacillus* strains to produce subtilosin A and iturin A.

Keywords: *Bacillus*; qRT-PCR; iturin; subtilosin; antimicrobial peptide.

Introduction

Bacteria produce a variety of antimicrobial metabolites such as antibiotics and bacteriocins. Bacteriocins are potent antimicrobial peptides that are often distinguished from traditional antibiotics by their narrow range of activity against closely related bacteria. Strains of the genus *Bacillus* produce an array of antimicrobial peptides that are either synthesized ribosomally or by multienzyme complexes [20,22].

Subtilosin A is a lantibiotic bacteriocin synthetized ribosomally and originally isolated from *Bacillus subtilis* [2], but it was also found in *Bacillus amyloliquefaciens* and *Bacillus atrophaeus* [21,24]. Production of mature subtilosin A requires the expression of eight (*sboA-albABCDEFG*) of the nine genes identified in *B. subtilis* producer strains. These clustered genes are transcribed from a promoter residing upstream of the *sboA* gene and their products are involved in the post-translational modification and processing of presubtilosin, secretion and immunity [16,30]. The *albABCDEFG* genes are believed to constitute an operon that encodes the proteins that function in presubtilosin processing and subtilosin export. The *sboA* gene encodes presubtilosin, which is a 43-aminoacid peptide [29].

Iturin A, a member of iturin group, is a lipopeptide synthetized nonribosomally, depending of a multienzymatic system, and shows a strong antibiotic activity with a broad antifungal spectrum. This peptide has been suggested as an ideal biological control agent with the aim of reducing the use of chemical pesticides in agriculture [3]. Iturin A operon is composed of four open reading frames, *itu*D, *itu*A, *itu*B, and *itu*C. The *itu*D gene encodes a putative malonyl coenzyme A transacylase, whose disruption results in a specific deficiency in iturin

A production [22,25]. Thus, the *ituD* gene has a central role in the production of iturin A.

Environmental conditions such as pH, temperature and oxygen availability influence in the biosynthesis of these peptides [5]. In its natural environment, bacteria are exposed to different conditions that induce modifications in their physiology and metabolism. Guez et al. [10] pointed out the importance of the pH for biosynthesis of lipopeptide antibiotics by *B. subtilis* ATCC 6633. Similarly, temperature influences enzyme activities, membrane fluidity, stability of secondary mRNA structures, and protein folding [8].

Bacillus sp. P11 is a novel bacteriocin-producing strain isolated from the intestinal contents of the teleost fish Piau-com-pinta (*Leporinus* sp.) of Brazilian Amazon basin [18]. The purpose of this work was to employ qRT-PCR to evaluate the effect of two variables, temperature and pH, on the expression of *sboA* and *ituD* genes in *Bacillus* sp. P11.

Materials and Methods

Bacterial strain and growth conditions

The producer bacterium was the previously characterized strain *Bacillus* sp. P11 (GenBank accession DQ3887866). This bacterium was grown in 20 mL of BHI broth (Oxoid, Basingstoke, UK) for 24 h at 30ºC in a rotary shaker at 125 rpm. These cultures were used to inoculate 200 mL of BHI broth with shaking in different conditions. A standard condition (control) was established in BHI pH 7.4, for 36 h at 30ºC. To investigate the effect of temperature, cultivation was performed for 36 h at 37ºC or 42ºC at pH 7.4. The influence of pH was investigated by growing the strain in BHI pH 6 or pH 8 for 36 h at 30ºC. The pHs of cultures were stabilized using

phosphate buffers to pHs 6.0, 7.4 and 8.0. Three cultivations were performed for each condition and samples were removed from these flasks for RNA extraction.

Total RNA extraction and cDNA synthesis

Total RNA was extracted from the cell cultures using 1 mL of TRIzol Reagent (Invitrogen) according to the manufactor's instructions. Cultures corresponding to 1×10^8 CFU/mL were centrifuged and the recovered cells were used. To estimate RNA concentration and quality, each sample was measured in a NanoDrop™ ND-1000 spectrophotometer (NanoDrop Technologies, Rockland, DE) at 260 nm and 280 nm. Samples were eletrophoresed in an agarose gel 1.5% at 75V for 1 h to check integrity. Before application in further reactions, samples of the extracted RNAs were treated with DNAse to eliminate remaining DNA molecules. For this, 1 U of Nuclease-free DNAse (Fermentas Life Science) was used to treat 1 μg of total RNA for 30 min at 37°C.

Specific cDNA for *sboA*, *ituD* and *rpsJ* genes were synthesized by using SuperScript™ One-Step RT-PCR with Platinum® Taq kit (Invitrogen). Thirty nanograms of total RNA was used in a reaction containing 6.25 μL of 2X Reaction Mix, 0.25 μL of RT/ Platinum Taq Mix, 1 μL of forward and reverse primers (5 μM) and DEPC-water to a final volume of 12.5 μL. The thermal cycler conditions were 50ºC for 30 min to first strand synthesis, 94ºC for 2 min to inactivate the RT enzyme and activate Taq polymerase, and 10 cycles of 94ºC for 15 s, 55ºC for 30 s and 72ºC for 1 min. The primers sboA and ituD were described by Velho et al. [27].

Quantitative PCR (Real-time PCR)

The real-time PCR reaction mix contained 1.5 μl of pre-amplified cDNA, 400 nM of primers, 10 μl of Power SYBR® Green PCR Master Mix 2X (Applied Biosystems) and DEPC-water to complete a final volume of 20 μl.

Reactions were performed in triplicate using a StepOnePlus Real-Time PCR system (Applied Biosystems) under the following conditions: 95°C for 10 mim, followed by 40 cycles of 95°C for 15s and 60°C for 1 min with data collection at last stage. Melting curves were included after qPCR to verify the specificity of the fragments generated by each primer pair. The conditions used were: 95ºC for 1 min, 60ºC for 1 min with the temperature raising and data collection each 0.7ºC until it reaches 95ºC.

The *rpsJ* gene (encoding ribosomal protein) was used as reference gene for data normalization. This gene is expressed at a constant level under our conditions and was already used as an internal standard in *Bacillus* [13]. A non-template control also was run with the samples to check for possible mix contamination and the presence of primer-dimer and cross-dimer products. The program LinRegPCR version 11.0 [19] was used to determine the molecular starting concentration (*No*) and the amplification efficiencies (*E*) for each reaction. After normalization to the reference gene *rpsJ*, the expression levels for each strain in the different growth condition were compared with the control condition of each strain, obtaining a ratio for *sboA* and *ituD* expression level.

Isolation of antimicrobial peptides and mass spectrometry

The antimicrobial peptides were isolated from the culture supernatant of *Bacillus* sp. P11, performed in optimized conditions for subtilosin (30ºC and pH 8.0) and iturin

(37ºC and pH 6.0). The extraction of iturin A was performed by acid precipitation as described by Cooper et al. [8]. The isolation of subtilosin A was performed by butanol extraction as described by Kawulka et al. [14]. Samples were concentrated in a vacuum centrifuge (SpeedVac SC100, Savant, USA), dissolved in ethanol and analyzed by mass spectrometry in a MALDI-TOF mass spectrometer (Ettan MALDI-TOF ProSystem, Amersham Biosciences, Sweden) operating in reflecton mode and using a matrix of α-ciano-4-hydroxycinnamic acid.

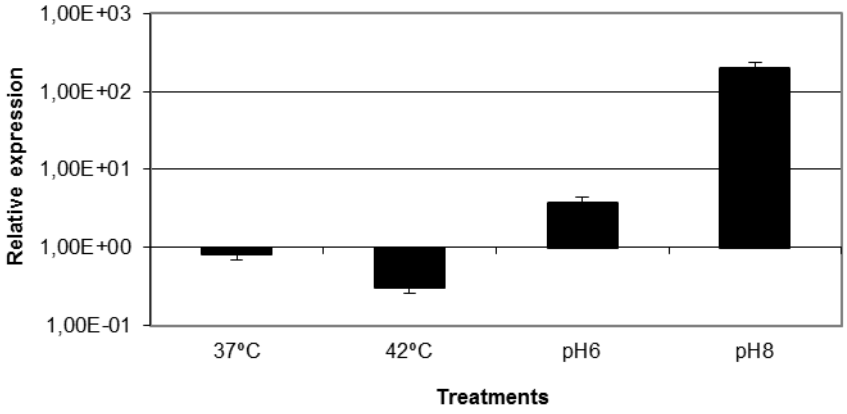
Results and Discussion

Effect of pH and temperature on gene expression

The results for the influence of temperature and pH on the *sboA* expression are presented in Fig. 1A. Growth of the *Bacillus* sp. P11 strain at 37ºC or 42ºC decreased the *sboA* expression compared to growth at 30ºC. However, the expression of *sboA* in *Bacillus* sp. P11 was increased at pH 6 (3.79 times) and especially at pH 8 (198.64-fold increased) compared to growth at pH 7.4.

The effect of growth temperature on the expression of *ituD* is shown in Fig. 1B. The temperature of 37ºC increased the *ituD* expression in *Bacillus* sp. P11 (9.14-fold), compared to growth at 30ºC. The thermal shock caused by the temperature of 42°C caused the decrease of *ituD* expression. The strain was also exposed to different pH conditions, and a 21.29 times increase of *ituD* gene expression was observed in pH 6 (Fig. 1B). However, the stress caused by growth in more alkaline pH (pH 8.0) decreased the expression of *ituD* gene in the strain P11.

(A)



(B)

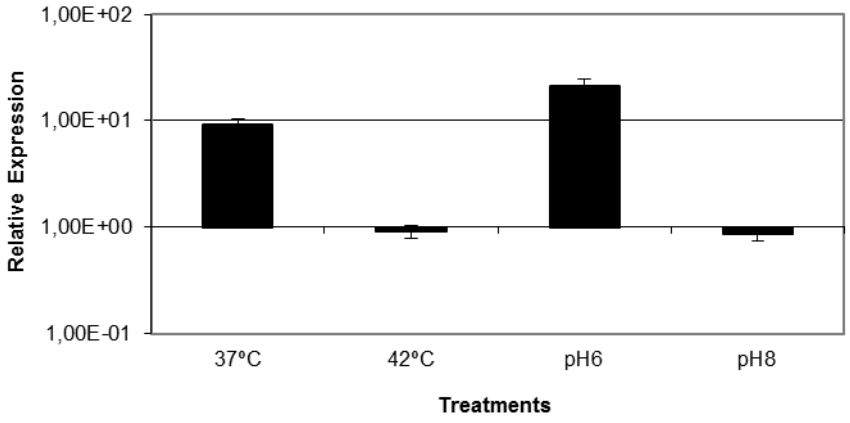


Figure 1. Influence of initial pH and temperature on (A) *sboA* and (B) *ituD* gene expression by *Bacillus* sp. P11. Values are calculated relative to standard condition at pH 7 and 30^{o}C.

Temperature and pH are two variables that strongly influence the production of antimicrobial peptides, and their influence has been reported for several *Lactobacillus* [1] and *Bacillus* strains [4,17]. The production of antimicrobial activity by *Bacillus* sp. P11 growing on agroindustrial byproducts was previously

investigated [15]. Maximum antimicrobial activity was observed at pH 7.0 and temperature ranging 39-42ºC. Using qRT-PCR for transcript quantification, we observed that the optimum pH was pH 8.0 for *sboA* expression and pH 6.0 for *ituD* expression, and the temperature of 37ºC caused increased in *ituD* expression compared to 30ºC. This discrepancy probably is due to the fact that *Bacillus* sp. P11 has genes coding for other bacteriocins [26,27], in addition to those used in this experiment using qRT-PCR. Moreover, the differences may be related to the fact that, using response surface methodology aimed to optimize the production of antimicrobial peptides synthesized by *Bacillus* sp. P11, not the production of subtilosin A and iturin A, specifically [15]. Thus, qRT-PCR may be useful to determine the conditions for selective production of a specific antimicrobial peptide. In addition, this technique has not been used to study the expression of genes involved in the biosynthesis of antimicrobial peptides.

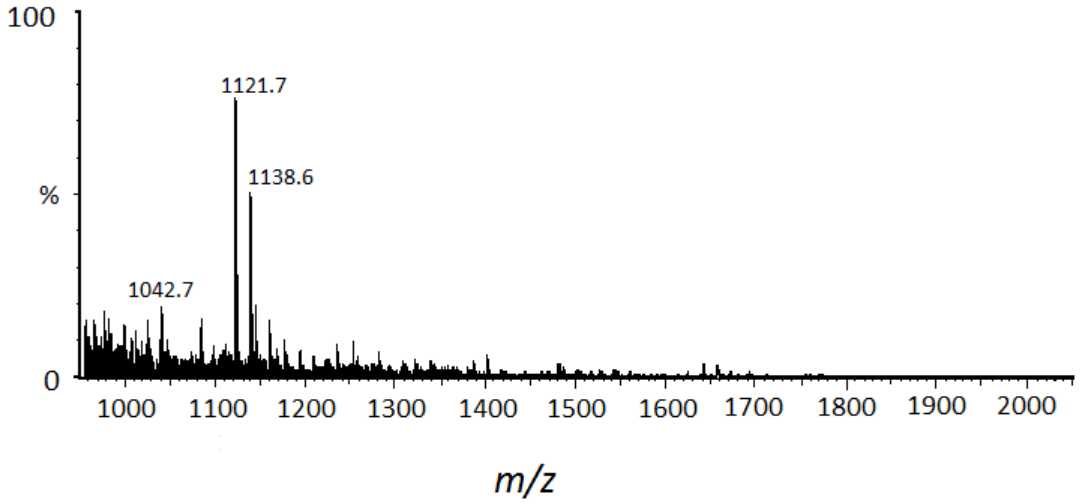
Isolation of antimicrobial peptides and mass spectrometry

The production of subtilosin A and iturin A by *Bacillus* sp. P11 was confirmed by mass spectrometry. The mass spectra of acid extracts revealed major peaks at *m/z* 1000-1100 Da, corresponding to isoforms of iturin lipopeptides (Fig. 2A). The strain showed peaks that were observed at *m/z* 1042 and 1079, and another cluster with peaks between *m/z* 1120 and 1140. The mass spectra of butanol extraction used for subtilosin isolation revealed clusters with peaks above *m/z* 3300 Da (Fig. 2B).

Mass spectrometry has been used as an efficient tool for identification of antimicrobial peptides, including hydrophobic lipopeptides in the range of 1-1.5 kDa and lantibiotic bacteriocins in the mass range 3-5 kDa [23]. The possible assignments for major *m/z* peaks are presented in Table 1. The peaks at *m/z* 1042

and 1079 agree with isomers of iturin A described for *B. amyloliquefaciens* RC-2 [11] and *B. amyloliquefaciens* CCMI 1051 [4]. Also, peaks at *m/z* 1121 and 1136 are associated with bacillomycin F [23]. The major peak at *m/z* 3409 corresponds to the variant A1 of subtilosin A, previously described in *B. subtilis* [12].

(A)



(B)

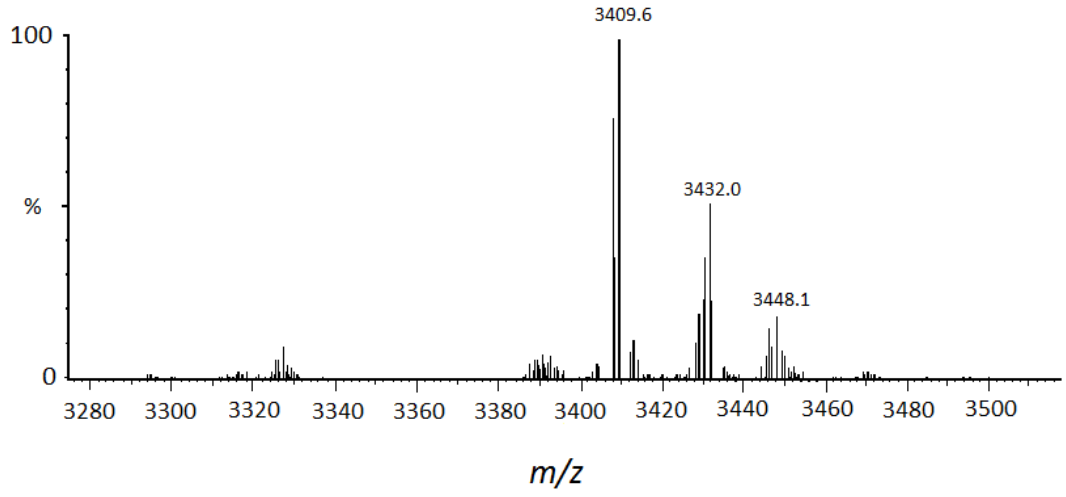


Figure 2. MALDI-TOF spectrum of (A) acid extract and (B) butanol extract from culture supernatants of *Bacillus* sp. P11.

Table 1. Assignments of major *m/z* peaks observed in mass spectra of lipopeptides from *Bacillus* sp. P11.

m/z	Assignment
1042.7	C14 iturin A $[M+H]^+$
1079.8	C15 iturin A $[M+H]^+$
1121.7	Bacillomycin F $[M+Na]^+$
1138.6	Bacillomycin F $[M+K]^+$
3409.6	Subtilosin A1 $[M+H]^+$
3432.0	Subtilosin A1 $[M+Na]^+$
3448.1	Subtilosin A1 $[M+K]^+$

The co-production of different antimicrobial peptides and isoforms by *Bacillus* spp. has been described, and their biosynthetic pathways are under complex regulation [20,22,28]. As a same strain can produce different peptides, it is often difficult to purify, and the huge structural variability makes it complicated to definitively identify different peptides and their homologues [4,6]. Thus, the knowledge on the factors influencing the expression of essential genes may be useful to control selective biosynthesis of these compounds.

In conclusion, our results showed that expression of essential genes for production of subtilosin A and iturin A may be strongly affected by temperature and pH of the culture media and qRT-PCR proved to be a powerful tool to study gene expression related to the production of these antimicrobial peptides by *Bacillus* sp. P11.

Acknowledgments

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4.3 Capítulo III

Expression of essential genes for biosynthesis of antimicrobial peptides of *Bacillus* sp. is modulated by inactivated cells of target microorganisms

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Artigo submetido para publicação no periódico *Biological Control.*

Abstract

Antimicrobial peptide production by *Bacillus* sp. P11 was investigated in presence of thermally inactivated cells of *Escherichia coli*, *Staphylococcus aureus*, *Bacillus cereus*, *Listeria monocytogenes*, *Fusarium oxysporum f. lycopersici, Fusarium graminearum* or *Aspergillus parasiticus*. *Bacillus* sp. P11 exhibited higher antimicrobial activity in presence of inactivated cells of *S. aureus* and *A. parasiticus* as compared to other conditions tested. Both inducing conditions show similar production of antimicrobial activity against *L. monocytogenes*. The expression of essential genes related to the biosynthesis of the antimicrobial peptides surfactin (*sfp*), iturin A (*lpa-14* and *ituD*), subtilosin A (*sboA*) and fengycin (*fenA*) was investigated by quantitative real-time PCR (qRT-PCR) methodology. The genes *lpa-14* and *ituD* were over-expressed in the presence of *S. aureus* (inactivated cells), indicating the induction of iturin A production by *Bacillus* sp. P11. The other inducing condition (inactivated cells of *A. parasiticus*) suppressed the expression of *lpa-14*, but increases the expression of *ituD*. A two-fold increase in *fenA* expression was observed for both conditions, while a strong suppression of *sboA* expression was observed in the presence of inactivated cells of *S. aureus*. These results suggest that the increased antimicrobial activity was related with the production of iturin A, an antimicrobial peptide that shows strong antibiotic activity against a broad spectrum of fungi and can be used in biological control of plants diseases.

Keywords: **antimicrobial peptides; biological control; *Bacillus*; inducing conditions**

Introduction

Viruses, bacteria and fungi cause several diseases that affect plant crops, resulting in losses and decreasing the quality and safety of agricultural products. The biological control of plant diseases by microorganisms is an interesting alternative to the use of environmentally harmful chemicals. The microorganisms used as agents of biocontrol often secrete a wide range of antimicrobial peptides produced through ribosomal or non-ribosomal synthesis (Montesinos, 2007).

The *Bacillus* genus includes several industrially important species and has a historical of safe industrial use, producing antimicrobials, enzymes and amino acids as part of the many commercially available products from *Bacillus* spp. The production of several antimicrobial peptides has been described for this genus, being surfactin, iturin A, subtilin, subtilosin A and fengycin some examples of these substances (Klein et al., 1993; Le Marrec et al., 2000; Chen et al., 2009).

The antimicrobial peptide surfactin is a cyclic heptapeptide produced by strains of *Bacillus subtilis*. The lipopeptide surfactin has crucial biological functions, including the ability to stimulate immune-related responses in host tissues, an important phenomenon for biological control of plant diseases (Henry et al., 2011). The genes responsible for production of surfactin are organized in a large operon in *B. subtilis* and the gene *sfp*, responsible for production of enzyme 4'-phosphopantetheinyltransferase was characterized as essential to surfactin production (Nakano et al., 1992; Hsieh et al., 2004).

Gene clusters involved in iturin A synthesis have been intensively investigated (Tsuge et al., 2001; Yao et al., 2003). The iturin A operon spans a

region of more than 38 kb and is composed of four open reading frames: *ituD, ituA, ituB,* and *ituC*. The *ituD* gene encodes a putative malonyl coenzyme A transacylase, whose disruption results in a specific deficiency in iturin A production (Tsuge et al., 2001). The *lpa-14* gene encodes the 4'-phosphopantheteinyl transferase required for the maturation of the template enzyme of iturin A. Both the *ituD* gene and the *lpa-14* gene play leading roles in the production of this antimicrobial peptide (Tsuge et al., 2001; Hsieh et al., 2008).

The production of mature subtilosin A requires the expression of eight (*sboA-albABCDEFG*) of the nine genes identified in the operon of the *B. subtilis*. These clustered genes are transcribed from a promoter residing upstream of the *sboA* structural gene and their products are involved in the posttranslational modification and processing of presubtilosin, secretion and also immunity (Zheng et al., 2000; Maqueda et al., 2008).

Members of the fengycin family, including the related plipastatin, are decapeptides with a ß-hydroxy fatty acid that show unusual properties, such as the presence of ornithine in the peptide portion. Fengycins also show antifungal activity, although more specific for filamentous fungi (Steller et al., 1999; Vanittanakom et al., 1986). Earlier works have established that fengycin is non-ribosomally synthesized by five fengycin synthetases, *fenC, fenD, fenE, fenA*, and *fenB* (Lin et al., 1999; Stachelhaus and Marahiel, 1995; Wu et al., 2007).

Relatively few studies concerning the induction of antimicrobial peptide production by bacteria of the genus *Bacillus* are reported the literature (Benitez et al., 2011; Leães et al., 2011; Velho et al., 2011a). As many antimicrobial peptides can be produced by *Bacillus* spp., it is often difficult to understand the

production of a specific peptide in response to environmental stimuli. The aim of this study was to investigate the production of antimicrobial activity by *Bacillus* sp. P11 in the presence of thermally inactivated cells of bacteria or fungi. The expression of genes *sfp, lpa-14, ituD*, *sboA* and *fenA* was monitored by quantitative real-time PCR (qRT-PCR).

Materials and methods

Bacterial cultures, media, and growth conditions

The producer strain was *Bacillus* sp. P11, isolated from aquatic environments of the Amazon region, near Manaus, Brazil (Giongo et al., 2007). Bacterium was grown in 20 mL of BHI broth (Oxoid, Basingstoke, UK) for 24 h at 30ºC in a rotary shaker at 125 rpm. This culture was used to inoculate 50 mL of BHI broth with shaking in different induction conditions. The strains *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 25923, *Bacillus cereus* ATCC 9634, *Listeria monocytogenes* ATCC 7644, *Fusarium oxysporum f. lycopersici, Fusarium graminearum* and *Aspergillus parasiticus* were used to induce the production of antimicrobial activity by *Bacillus* sp. P11. *L. monocytogenes* ATCC 7644 was used as indicator strain for detection of antimicrobial activity. All strains were stored at -21ºC in brain–heart infusion (BHI; Oxoid, Basingstoke, UK) containing 20% (v/v) glycerol and propagated in the same medium at 37ºC before use.

Antimicrobial activity assays

The antimicrobial activity was determined by the agar disc diffusion method. An aliquot of 20 µl of filtered supernatant was applied to sterile cellulose discs (6

mm) placed on BHI plates previously inoculated with a suspension of 10^8 cells/ml of the strain of *L. monocytogenes ATCC 7644*, used as indicator microorganism. The plates were incubated at 37ºC for 24 h. The antimicrobial titer was determined by the serial twofold dilution method (Motta and Brandelli, 2002). Activity was defined as the reciprocal of the dilution after the last serial dilution giving a zone of inhibition and expressed as activity units (AU) per milliliter.

Induction of antimicrobial activity

The inducing test for the production of antimicrobial peptides by *Bacillus* sp. P11 was originally performed by cultivation for 24 h with addition of the thermally inactivated cells (autoclaved at 121ºC for 30 min) of *E. coli* ATCC 25922, *S. aureus* ATCC 25923, *B. cereus* ATCC 9634, *L. monocytogenes* ATCC 7644, *F. oxysporum f. Lycopersici, F. graminearum* or *A. parasiticus* and centrifuged at 10,000 x *g* at 4°C for 15 min to obtain a cell pellet of each inductor strain. Each pellet obtained was diluted in BHI until volume of 1.0 ml and then added to a cultive in BHI broth containing 1% pre-inoculum from a producing strain, this mixture was incubated at 30°C for 24h in an orbital shaker at 125 rpm.

For additional tests with *S. aureus* or *A. parasiticus* (autoclaved cells), the best inductors, 24 h cultures were centrifuged at 10,000 x *g*, 4ºC for 15 min, the cell pellets were washed three times and suspended in 5 ml of BHI and added to 50 ml of cultures of *Bacillus* sp. P11. The experiments were incubated at 30ºC and tested for the production of antimicrobial activity at different growth stages (time intervals of 0, 4, 8, 12, 24, 32, 40 and 48 h), under stirring.

For all tests, cell growth was followed by OD_{600} and antimicrobial activity was tested by the agar disc method using *L. monocytogenes* as indicator strain. As control we used the isolated culture of the antimicrobial compounds producer strain. Three independent cultivations were carried out for each condition. Samples were removed from these flasks for RNA extraction.

Real-time reverse transcription (RT)-PCR

To analyze the expression of the target genes *sfp, lpa-14, itu*D, *sbo*A and *fen*A (Table 1) in the *Bacillus* sp. P11, the bacteria was incubated in presence of thermally inactivated cells of *S. aureus* and *A. parasiticus* at 30ºC for 40 h in an orbital shaker at 125 rpm. RNA was extracted using the TRIzol Reagent (Invitrogen) for each condition. Total nucleic acid concentrations and purity were estimated using absorbance readings (260/280 nm) on a NanoDrop™ ND-1000 spectrophotometer (NanoDrop Technologies, Rockland, DE). The samples also were electrophoresed in an agarose gel 1-5% at 75 V for 1 h to check their integrity.

Table 1. Target genes used in this study: function and primer sets

Gene	Function	Forward primer	Reverse primer
sfp	Surfactin synthesis	CATTGGTGCGTTTGATTCAC	CCTTGCCCTCCTGTTTGATA
lpa-14	Iturin synthesis	TATCAAACAGGCCGGAAAAG	TCATCTCAATCCCGTCACAA
ituD	Iturin synthesis	CTCAAGCAGCACATGACGAT	ACCGGCTAAGACATTGTTCG
sboA	Subtilosin synthesis	GGACGGTCCTATCCCTGATT	TGACTATGCCCTTTTGGAGAA
fenA	Fengycin synthesis	ACCATACATCCGGGTAACCA	ACCTCTGGCGACACTTGAAC

*sfp, lpa-14, itu*D, *sbo*A, *fen*A, *tuf*A and *rps*J cDNAs were synthesized from 30 ng of total RNA previously treated with DNAse (Fermentas Life

Sciences) by using SuperScript™ One-Step RT-PCR with Platinum® Taq (Invitrogen). The thermal cycler conditions were 50ºC for 30 min, 94ºC for 2 min and 10 cycles of 94ºC for 15 s, 55ºC for 30 s and 72ºC for 1 min. The target primers were designed for the requirements imposed by real-time quantitative PCR using Primer3 v.4.0 (http://frodo.wi.mit.edu/primer3/).

2.5 Quantitative real-time PCR

The real-time PCR was carried out in a 10 µl reaction containing 2.0 µl of pre-amplified cDNA, 2.500 nM of primers and 5 µl of SensiMix TM SYBR One-Step 2 X (Bioline). PCRs were performed in triplicate and run on the StepOnePlus Real-Time PCR system (Applied Biosystems, Foster City, CA) under the following conditions: 42ºC for 10 min, 95ºC for 10 min, followed by 40 cycles of 95ºC for 15 s, 55ºC for 20 s and 72ºC for 30 s, with data collection at the last stage. To verify the specificity of the fragments, melting curves were determined for each reaction as follow: 95ºC for 15 s, 55ºC for 1 min with the temperature raising and data collection each 0.7ºC until it reaches 95ºC. To normalize the data, was used as reference genes the *rpsJ* gene (encoding ribosomal protein) and the *tufA* gene (elongation factor Tu), expressed at a constant levels under our conditions and already used as internal standard in studies by Jordan et al. (2006) and Savard and Roy (2009). A non-template control also was run with the samples to check for possible mix contamination and primer-dimmers. We used the software LinRegPCR version 11.0 (Ramakers et al., 2003) to determine the molecular starting concentration (N_0) and the amplification efficiencies (E) for each reaction. The expression levels for each growth

condition were also calculated in ratio with the control condition (culture of *Bacillus* sp. P11 alone).

Results

Induction of antimicrobial activity

Seven different culture conditions were compared for antimicrobial peptide production by *Bacillus* sp. P11 and the results are shown in Figure 1. Higher activity was obtained by cultivation with the addition of thermally inactivated cells of *S. aureus* and *A. parasiticus* (1333 AU/mL and 1600 AU/mL, respectively), followed by inactivated cells of *L. monocytogenes* (933 AU/mL), *B. cereus* (800 AU/mL). The values obtained in the presence of inactivated cells of *E. coli* (533 AU/mL), *F. oxysporum* (400 AU/mL) and *F. graminearum* (267 AU/mL) were lower than those observed in the controls. *S. aureus* and *A. parasiticus* were selected for additional experiments.

Bacillus sp. P11 was incubated at 30ºC under two inducing conditions: in presence of heat-inactivated cells of either *S. aureus* or *A. parasiticus*. The antimicrobial activity was monitored at different times of cultivation and the results are showed in Figure 2. *Bacillus* growth and production of antimicrobial activity was similar in both culture conditions. The stationary phase was reached after 12 h of cultivation and the lowest OD values were observed in the presence of thermally inactivated cells of *A. parasiticus* (Fig. 2A). The maximum antimicrobial activity was observed at 40 h for both inducing conditions. In presence of inactivated cells of *S. aureus* this activity was steady until 48 h of cultivation (Fig. 2B). Furthermore, with addition of thermally inactivated cells of

A. parasiticus an important decrease in the antimicrobial activity was observed after 40 h of cultivation.

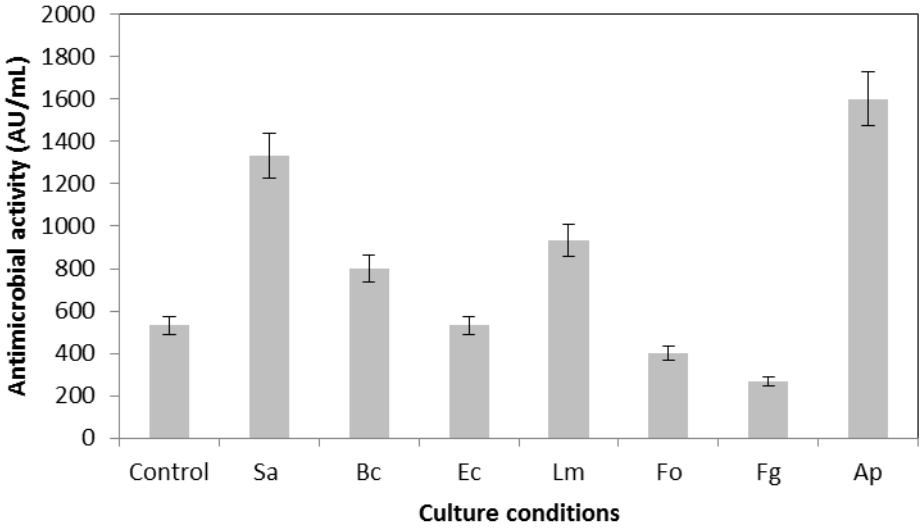

Fig. 1. Production of antimicrobial activity by *Bacillus* sp. P11 in the presence of thermally inactivated cells of *Staphylococcus aureus* (Sa), *Bacillus cereus* (Bc), *Escherichia coli* (Ec) *Listeria monocytogenes* (Lm), *Fusarium oxysporum* (Fo), *Fusarium graminearum* (Fg) or *Aspergillus parasiticus* (Ap). Results are the means of three independent experiments.

Relative expression of genes involved in antimicrobial peptide production

The influence of heat inactivated cells of *S. aureus* and *A. parasiticus* on the expression of essential genes for the synthesis of antimicrobial peptides by *Bacillus* sp. P11 was investigated. The strain P11 was cultivated with thermally inactivated cells at 30ºC during 40 h, and the expression of *sfp* (surfactin), *lpa-14* (iturin A), *itu*D (iturin A), *sbo*A (subtilosin A) and *fen*A (fengycin A) genes was monitored using the qRT-PCR methodology.

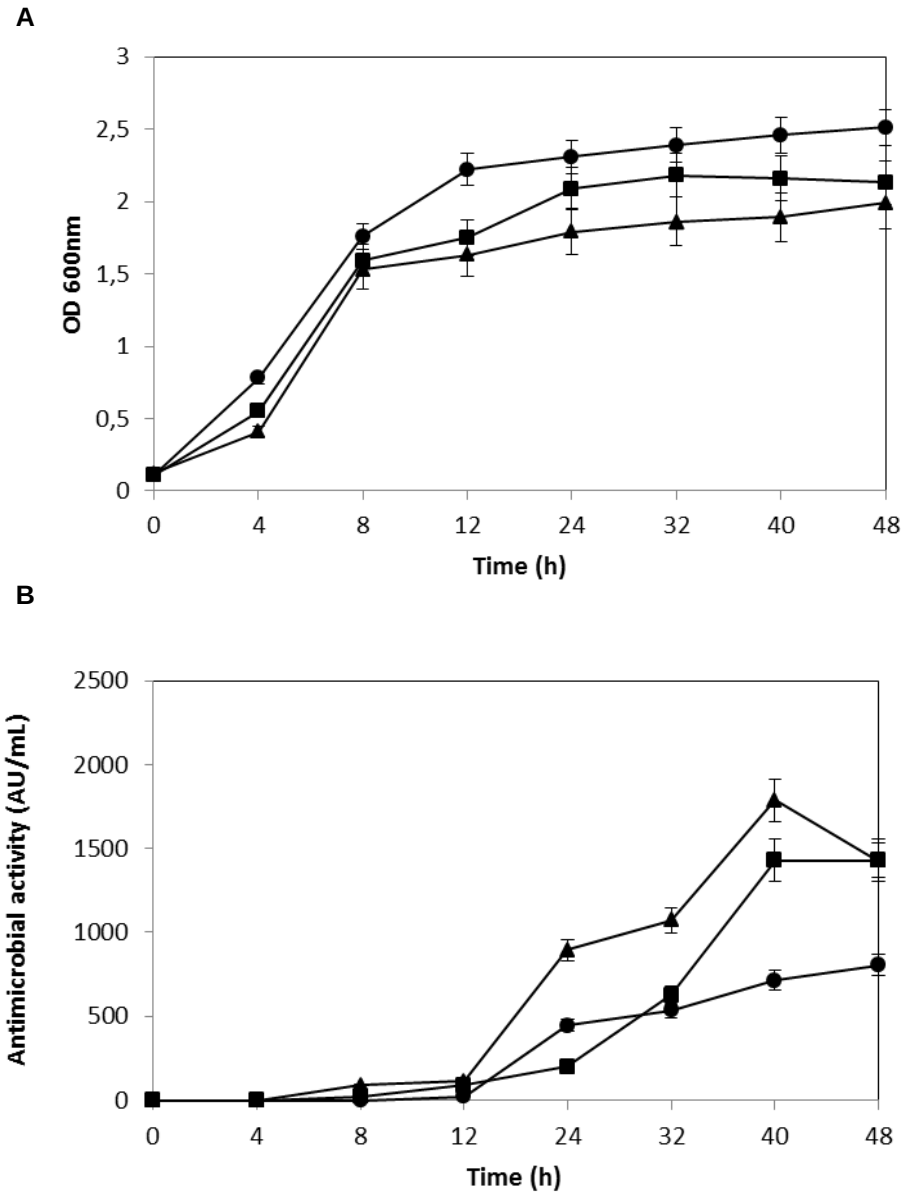

Fig. 2. Growth (A) and antimicrobial production (B) by *Bacillus* sp. P11 in different conditions for 48 h. Cultivation of strain was performed in BHI medium alone (circle control), in the presence of thermally inactivated cells of *Staphylococcus aureus* (square) or in the presence of thermally inactivated cells of *Aspergillus parasiticus* (triangle). Results are the means of three independent experiments.

Figure 3 shows that all genes studied were expressed by *Bacillus* sp. P11 when incubated in the presence of inactivated cells of *S. aureus* or *A.*

parasiticus. The *sfp* gene expression was increased in the first condition, but not significantly when compared with control. As for the second condition, the *sfp* gene expression was decreased significantly (p = 0.001) by 18.85 times. The presence of *S. aureus* stimulated the *lpa-14* expression by 6.87-fold increased (p = 0.001) and the expression was repressed (not significantly) in the presence of *A. parasiticus*. The presence of both inducers represented a significant stimulus (p<0.0025) to *ituD* expression by the *Bacillus*. *S. aureus* increased the expression in 14.60 times and the *A. parasiticus* in 5.00 times. A non-significant decrease of gene expression *sboA* was caused by the presence of both inducers. In the case of gene *fenA*, the expression was not significantly increased in both culture conditions of *Bacillus* sp. P11.

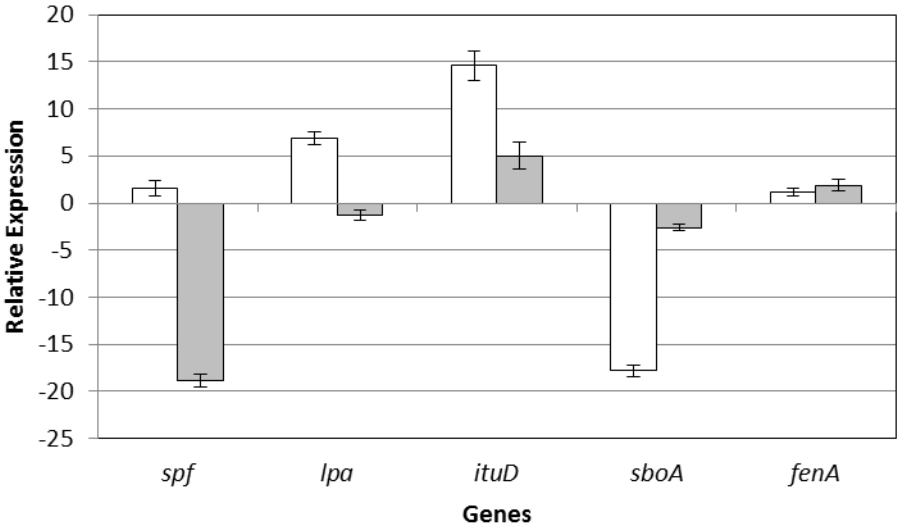

Fig. 3. Relative expression of *sfp, lpa-14, itu*D, *sbo*A and *fen*A genes after growth for 40 h in presence of thermally inactivated cells of *S. aureus* (white bars) or *A. parasiticus* (gray bars). Values are relative to control condition (culture of *Bacillus* sp. P11 alone). Results are the means of three independent experiments.

Discussion

Metabolites produced by some *Bacillus* strains have shown antibacterial and antifungal activity against phytopathogens and food spoilage microorganisms (Bernal et al., 2002; Czaczyk et al., 2000; Földes et al., 2000; McKeen et al., 1986; Milner et al., 1996; Pichard et al., 1995; Shirokov et al., 2002; Touré et al., 2004). *Bacillus* spp. produce a heterogeneous group of antimicrobial proteins or peptides that vary in spectrum of activity, mode of action, molecular mass, biochemical properties and genetic origin (Cotter et al., 2005).

This study provides evidence that the presence of thermally inactivated cells of *S. aureus* or *A. parasiticus* may increase the production of antimicrobial activity by *Bacillus* sp. P11, suggesting that an external stimulus from target microorganisms was able to improve the antimicrobial compounds production.

Studies with cell-free culture supernatant of inducer strains suggesting that the increase of antimicrobial activity was not mediated by a metabolite secreted by the inducer strain. Benitez et al. (2011), verify that *E. coli* culture caused an increase in the antimicrobial activity of *Bacillus amyloliquefaciens* LBM 5006. However, similar studies on *Bacillus* species are very scarce if compared with those performed for lactic acid bacteria. Kimchicin GJ7 production was enhanced in the presence of *Lactobacillus plantarum* KFRI 464, and the cell debris fraction of *L. plantarum* KFRI 464 showed much higher inducing activity than the intracellular fraction (Chang et al., 2007). The maximum enhancement of bacteriocin production by *Leuconostoc citreum* GH7 was obtained in the presence of thermally inactivated cells (121ºC/15 min) of *L. plantarum* KFRI 464, probably activated by cell-to-cell contact (Chang et al., 2007). The production of lactacin B by *Lactobacillus acidophilus* N2 increased

significantly when it was grown with living, washed, or killed cells of *Lactobacillus delbrueckii* subsp. lactis ATCC 4797, suggesting that the inducer substance is cell associated and could be located on the cell envelope (Barefoot et al., 1994).

The interaction of thermally inactivated cells of inducer strain with *Bacillus* sp. P11 is unknown. However, it has been demonstrated in this study that the presence of thermally inactivated cells of *S. aureus* or *A. parasiticus* in the same environment enhances the production of antimicrobial activity. During heating, some cell components that may act as inducers could be exposed and/or solubilized, then causing an increased inducible effect. The enhanced synthesis of antimicrobial peptides in response to other microbial cells may be an efficient strategy for defense against competing microorganisms (Benitez et al., 2011).

In this report, *Bacillus* sp. P11 produced antimicrobial activity and expressed genes related to the synthesis of antimicrobial peptides like surfactin, subtilosin, iturin and fengycin. These antimicrobials have broad inhibitory spectra with high biological activity, good stability and solubility, may have great biopreservative potential (Chang et al., 2007) or can be used as agents to phytopathogen control (Ongena et al., 2005).

Few studies utilize qRT-PCR to investigate the expression of genes involved in the synthesis of antimicrobial peptides (Velho et al., 2011b). In this work *sfp, lpa-14, ituD, sboA* and *fenA* expression by *Bacillus* sp. P11 was checked by qRT-PCR. The expression of these genes was observed in culture with inactivated cells of both *S. aureus* and *A. parasiticus*. In general, under the experimental conditions of this study, the presence of inactivated cells of *S.*

aureus presented a highest induction in the expression of studied genes, when compared whit *A. parasiticus*.

The genes *sfp, lpa-14, ituD, sboA* and *fenA*, related to production of antimicrobial peptides, were identified in different *Bacillus* strains isolated from aquatic environments of Brazilian Amazon basin, including *Bacilus* sp. P11. These bacteria showed antifungal activity against species that causes important economic losses in agriculture. In addition, the inhibition of bacterial pathogens such as *Listeria monocytogenes* and *Bacillus cereus* by these strains was previously described (Motta et al., 2004).

The expression of *sfp* gene was down-regulated approximately 20-fold in presence of inactivated cells of *A. parasiticus*, and showed non-significant regulation in presence of *S. aureus*. The *sfp* gene is responsible for production of enzyme 4'-phosphopantheteinyl transferase that was characterized as essential to production of surfactin (Nakano et al., 1992; Hsieh et al., 2004). Although the use of biosurfactants in the food industries and chemical generally not competitive due to relatively high production costs, the identification of strains showing productivity levels compatible with economic needs is an issue of major importance, which requires the development of simple and reliable methods to select surfactin-producing strains. This is important because biosurfactants have an advantage over synthetic surfactants because most are biodegradable and generally less toxic (Singh and Cameotra, 2004).

Another gene that also encodes the enzyme 4'-phosphopantheteinyl transferase is *lpa-14* gene, that is required for the maturation of the template enzyme of iturin A (Tsuge et al., 2001; Hsieh et al., 2008). In this work, the *lpa-*

14 gene was up-regulated (6.87 fold) when *Bacillus* sp. P11 is incubated with thermally inactivated cells of *S. aureus*.

The *ituD* gene, as well as the *lpa-14* gene is involved in the production of iturin A (Tsuge et al., 2001; Hsieh et al., 2008). This gene encodes a malonyl coenzyme A transacylase, whose disruption results in a specific deficiency in iturin A production. *Bacillus* sp. P11 exhibited higher expression level of *ituD* during growth in presence of inactivated cells of *S. aureus* as compared to *A. parasiticus*.

Both the *lpa-14* gene and the *ituD* gene play leading roles in the production of iturin A and in this work both was up-regulated in presence of inactivated cells of *S. auerus*, suggesting that this condition may induce the production of iturin A by *Bacillus* sp. P11.

Few studies highlighted the regulatory mechanism that controls the synthesis of iturin A in *Bacillus* (Duitman et al., 2007; Koumoutsi et al., 2007; Chen et al., 2009). Some authors (Tsuge et al., 2001; Hofemeister et al., 2004) suggest that the expression of the giant gene clusters governing synthesis of iturin-like compounds in bacilli is control by the same transcriptional regulator of bacillomycin D. The expression of this compound is driven by a stationary-phase induced σ^A promoter, P_{bmy}, and identified three global regulators, DegU, DegQ, ComA and two sigma factors σ^B and σ^H that positively influence the transcriptional activation of P_{bmy} in *B. amyloliquefaciens* FZB42 (Koumoutsi et al., 2007). DegQ is a small pleiotropic regulatory protein that controls the expression of degradative enzymes, intracellular proteases and several other secreted enzymes and seems to control the transcription of peptide antibiotics (Amory et al., 1987; Msadek et al., 1991). ComA is a regulator of late

competence genes and surfactin production (Guillen et al., 1989; Roggiani and Dubnau, 1993; Shimane and Ogura, 2004) that controls *bmy* expression indirectly via DegQ, which serves as an auxiliary factor to DegU.

Velho et al. (2011b) related that Amazon strains of *Bacillus* showed lowest expression levels of *sboA* when compared with *B. subtilis* ATCC 19659. In this work, the gene *sboA* showed non-significant regulation in presence of *S. aureus* or *A. parasiticus* (thermally inactivated cells) by *Bacillus* sp. P11.

The gene *sboA* is related to production of subtilosin. This gene is involved in the post-translational modification and processing of presubtilosin, secretion and also immunity (Zheng et al., 2000; Maqueda et al., 2008). The production of mature subtilosin A requires the expression of eight (sboA-albABCDEFG) of the nine genes identified in the operon of the *B. subtilis.* The sbo-alb operon is known to be under complex control (Nakano et al., 2000; Zheng et al., 2000). This operon is induced in late growth cultures apparently in response to starvation and is also dramatically induced by oxygen limitation (Nakano et al., 2000; Zheng et al., 2000). It is not known how the multiple regulatory factors reflect upon the physiological roles of subtilosin A and the alb genes, but it is known that stress conditions induce the expression of this operon (Nakano et al., 2000; Albano et al., 2005; Strauch et al., 2007).

As well as *sboA*, *Bacillus* sp. P11 also showed no significant regulation of the gene *fenA* under the studied conditions. The gene *fenA* is involved with the synthesis of fengycin, an antifungal lipopeptide antibiotic and biosurfactant (Wei et al., 2010; Vanittanakom et al., 1986). Ongena et al. (2005) provide evidence for the role of fengycins in the protective effect afforded by the *Bacillus subtilis* strain M4 against damping-off of bean seedlings caused by *Pythium ultimum*

and against gray mold of apple in post-harvest disease. Fengycins could also be involved in this systemic resistance-eliciting effect of strain M4, as these molecules may induce the synthesis of plant phenolics involved in or derived from the defense-related phenylpropanoid metabolism. In the same direction, results obtained for Liu et al. (2011) indicated that fengycin played a major role in suppressing mycelial growth of the fungal pathogen *Monilinia fructicola*.

In conclusion, the addition of inactivated cells of bacteria or fungus in culture of *Bacillus* strains can induce the production of antimicrobials compounds. In this work, using qRT-PCR method, we show that the presence of thermally inactivated cells of *S. aureus* or *A. parasiticus* induced the expression of *lpa-14* and *iduD* genes by *Bacillus* sp. P11. These genes were involved with the production of iturin A, an antimicrobial peptide that shows strong antibiotic activity against a broad spectrum of fungi, making it a potential agent for biological control with the aim of reducing the use of chemical pesticides in agriculture.

Acknowledgments

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4.4 Capítulo IV

Dados Complementares: Purificação de compostos antimicrobianos produzidos por *Bacillus* sp. P11

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Dados a serem formatados em artigo para publicação em periódico nacional.

Metodologia e Resultados

Diversas metodologias foram executadas buscando purificar as substâncias antimicrobianas produzidas por *Bacillus* sp. P11. A Figura 1 mostra o esquema de purificação utilizado.

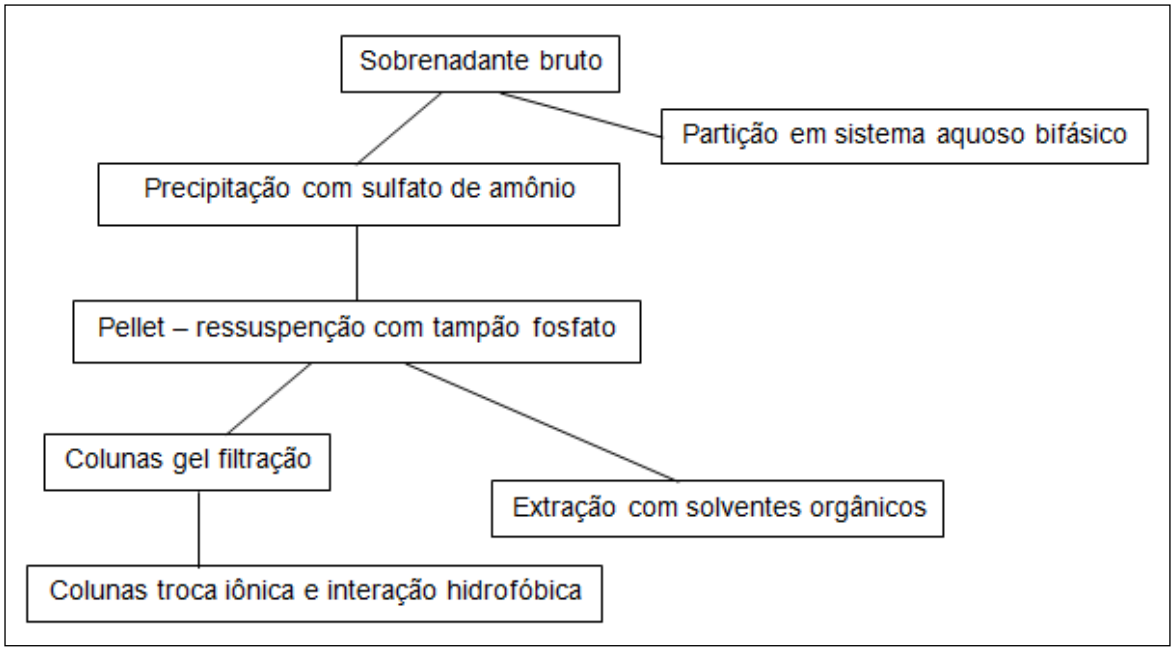

Figura 01: Esquema de purificação utilizado

Obtenção do sobrenadante bruto

Para a obtenção do pré-inóculo de *Bacillus* sp. P11 utilizou-se o meio de cultura BHI por 24 horas a 30°C sob agitação (125 rpm). Após, transferiu-se 1mL de pré-inóculo para frasco de Erlenmeyer contendo o meio de cultura BHI e incubou-se por 24 horas a 30°C sob agitação. Após o meio inoculado foi centrifugado por 20 minutos em rotação 10.000 rpm e 4ºC. Durante as estapas de purificação a atividade antimicrobiana foi detectada pelo método de difusão em ágar com discos (MOTTA & BRANDELLI, 2002) e a concentraçao de proteínas foi determinada pelo método de Lowry et al. (1951).

Precipitação com sulfato de amônio

Na fase inicial da purificação foi feita a definição da concentração de sulfato de amônio necessária para a precipitação dos peptídeos antimicrobianos. A precipitação fracionada foi realizada nos seguintes intervalos: 0-20%; 20-40%, 40-60% e 60-80% de saturação. Para isto, colocaram-se 50mL de sobrenadante em banho de gelo com agitação magnética e foi adicionada aos poucos a quantidade suficiente de sulfato de amônio para atingir a concentração de 20% (ENGLARD & SEIFTER, 1990). Após deixar sob agitação durante 15 minutos, realizou-se a centrifugação a 4ºC, 10.000 g durante 10 minutos. O pellet foi separado do sobrenadante e ressuspendido com 1,0 mL de tampão fosfato 10 mM pH 7. O sobrenadante foi levado ao banho de gelo e procederam-se as precipitações nas demais faixas de saturação, sendo que para cada faixa foi verificada a atividade antimicrobiana dos pellets para estipular a melhor concentração e sulfato de amônio. A Figura 02 mostra que a faixa que apresentou melhor produção de atividade antimicrobiana está entre 0 e 40% de sulfato de amônio.

Colunas de Gel Filtração

Dando sequência à purificação dos compostos antimicrobianos foi realizada cromatografia de gel filtração. Uma alíquota de 1mL do sobrenadante precipitado com sulfato de amônio foi eluído em coluna de gel filtração (0,8 X 30 cm) pré-equilibrada com tampão fosfato 10 mM pH 7. O fluxo utilizado foi de aproximadamente 20 mL/h (gravidade simples). Foram coletadas 20 aliquotas

de 1 mL determinando-se a sua absorbância a 290 nm em espectrofotômetro e a atividade antimicrobiana.

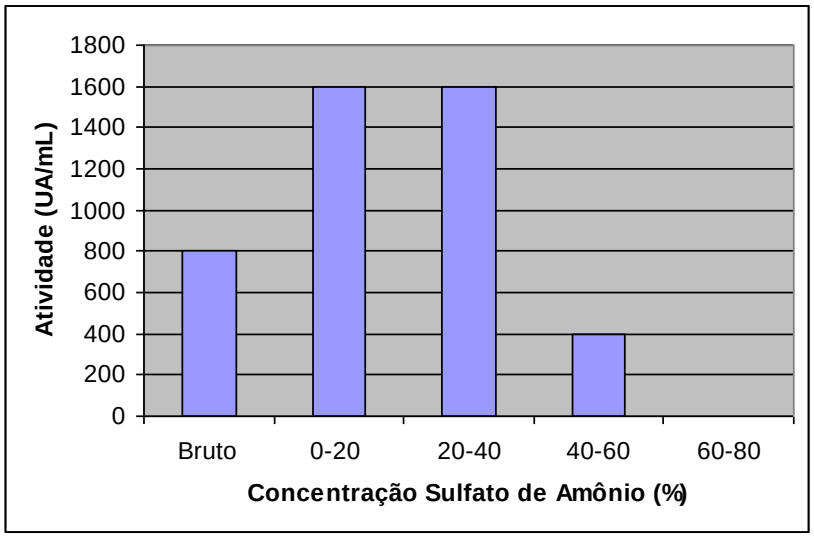

Figura 02: Resultados da precipitação fracionada com sulfato de amônio

Foram testadas as seguintes colunas de gel filtração:

- Sephadex G-50: não foi observada purificação (aparecimento de apenas um pico de proteína).

- Sephadex G-100: não foi observada purificação (aparecimento de apenas um pico de proteína).

- Biogel P6DG: foram observados dois picos de proteína, indicando certo grau de purificação (Figura 03).

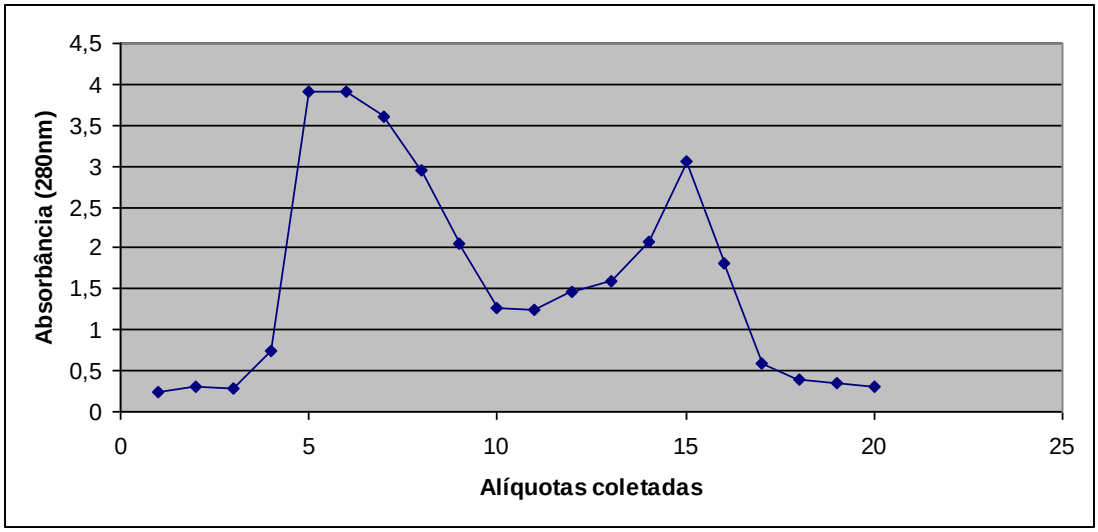

Figura 03: Absorbância (280 nm) das alíquotas coletadas na eluição em coluna Biogel P6DG

Foi determinada a atividade antimicrobiana das frações coletadas, observando-se atividade antimicrobiana nas frações de 5 a 9 (Figura 04), coincidindo com o primeiro pico de proteína. Este resultado indica que os compostos antimicrobianos produzidos, que têm origem protéica, estão no *pool* formado pelas alíquotas acima citadas.

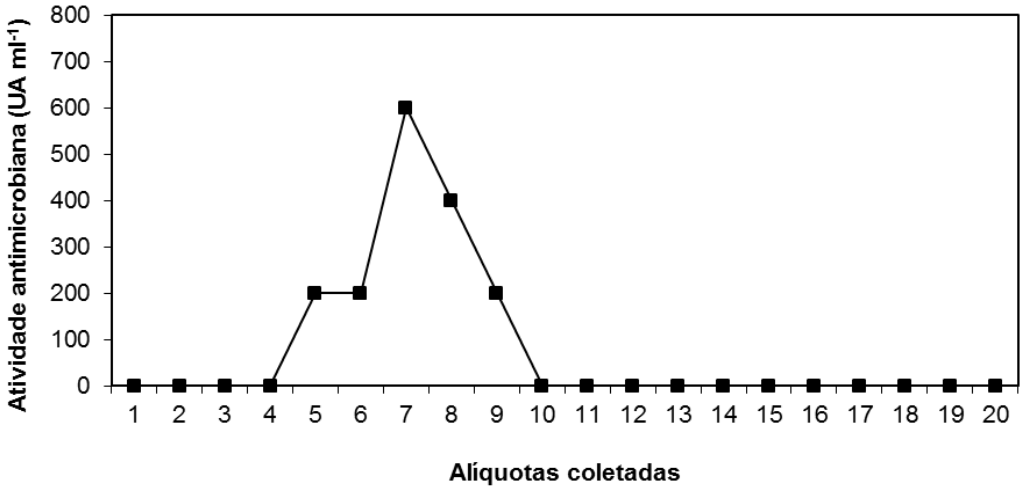

Figura 04: Atividade antimicrobiana (UA/mL) das alíquotas coletadas na eluição em coluna Biogel P6DG

Colunas cromatográficas

A partir da obtenção deste *pool* com atividade antimicrobiana, procedeu-se a purificação utilizando-se cromatografia de troca iônica. Foram utilizadas as seguintes resinas de troca iônica: Q-Sepharose, DEAE-Sepharose e SP-Sepharose. Também foram utilizadas outras resinas: Sephacryl S-200 (gel filtração) e Fenil Sepharose (interação hidrofóbica).

Não foi possível a obtenção de bons resultados com as resinas citadas, sendo que não foram obtidas alíquotas indicando a presença de proteína e atividade antimicrobiana. A não detecção de proteína indica que a quantidade de proteína das frações coletadas estava abaixo da capacidade de detecção do espectrofotômetro usado. A ausência de atividade antimicrobiana indica que os compostos antimicrobianos produzidos por *Bacillus* sp. P11 não se fixaram nas resinas indicadas, sendo que a atividade pode ter sido perdida devido à diluição.

Extração com solventes orgânicos

Ainda buscando uma alternativa para a purificação dos peptídeos antimicrobianos, procedeu-se a extração com solventes orgânicos. Em tubo de ensaio foram misturados 1mL de amostra ressuspendida e 1mL de solvente orgânico (álcool butílico, álcool metílico, acetona e clorofórmio). Após foram coletadas as fases superior e inferior formadas, e acondicionadas em becker até evaporação completa do solvente. Depois de evaporada completamente, a amostra foi dissolvida em 1mL de tampão fosfato pH 7,0.

As amostras foram colocadas em sacos de diálise e imersas em tampão fosfato pH 7,0 com agitação constante por 24h, sob refrigeração.

A tabela 01 apresenta os resultados obtidos nas diversas fases de purificação e com os diferentes solventes orgânicos, sendo que a extração com álcool butílico demostrou melhores resultados. Desta forma, deu-se prosseguimento às análises (eletroforese em gel de poliacrilamida) utilizando-se as amostras de sobrenadante bruto, precipitado e fase superior da extração com álcool butílico.

Tabela 01

Tabela de purificação mostrando os resultados das diferentes fases da purificação com solventes orgânicos

Fase da Purificação	Atividade (UA/mL)	Proteína (mg/mL)	Atividade Específica (UA/mg)	Fator de Purificação
Sobrenadante Bruto	200	0,9778	204,54	1,0
Precipitado	1600	3,6808	434,69	2,12
Fase Superior – Extração Álcool Butílico	200	1,3386	149,41	0,73
Fase Superior – Extração Álcool Metílico	0	0,5571	0	0
Fase Superior – Extração Acetona	0	0,4065	0	0
Fase Superior – Extração Clorofórmio	0	0,4399	0	0

Eletroforese em gel de poliacrilamida

Após a determinação da quantidade de proteína nas amostras, as mesmas foram preparadas com TCA (ácido tricloroacético) 20% e deixadas 24h à 5ºC. Após as amostras foram centrifugadas com rotação de 10.000 rpm

por 15 minutos, o sobrenadante foi descartado e lavado o eppendorf com acetona por três vezes. Foram adicionados 40µL de tampão beta-mercaptoetanol e fervidas por 5 minutos.

Utilizou-se uma cuba de eletroforese adicionada de tampão de corrida e conectada a voltagem de 100V e miliamperagem de 20A. Os reagentes foram colocados entre os vidros especiais para eletroforese afastados por separadores de plástico. Após a polimerização completa dos reagentes *running* e *stacking*, respectivamente, foram colocadas as amostras previamente preparadas com tampão beta-mercaptoetanol.

Após as amostras percorrerem o gel, sem interrupções, o mesmo foi corado com coloração de prata (SWITZER et al., 1979).

A Figura 05 mostra a fotografia de um gel de poliacrilamida corado com nitrato de prata. As bandas indicam o perfil das proteínas, separadas por diferentes pesos moleculares. Na última etapa da extração (A) percebe-se apenas uma banda, bem clara, indicando a purificação do sobrenadante.

Observou-se que o(s) peptídeo(s) antimicrobianos produzidos por *Bacillus sp*. P11 pode(m) ser parcialmente purificado(s) utilizando álcool butílico e que este método apresenta resultados satisfatórios para a verificação da atividade antimicrobiana contra *Listeria monocytogenes*. A eletroforese em gel de poliacrilamida mostra a presença de uma única banda de proteína na fase superior (orgânica) da extração com álcool butílico.

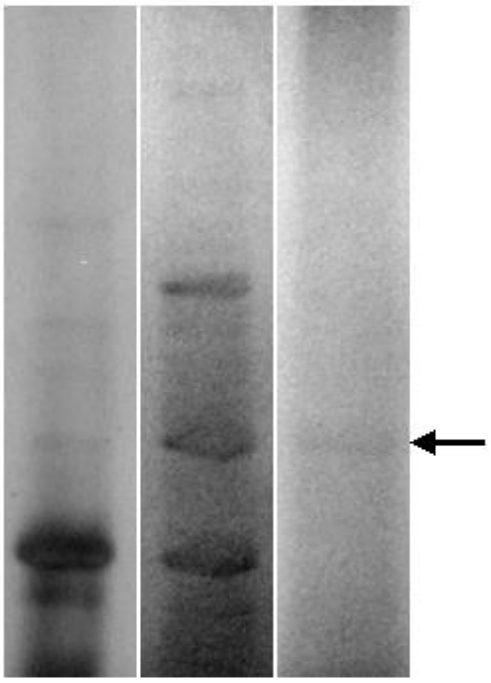

Figura 05: Gel de poliacrilamida corado com nitrato de prata. As bandas indicam o perfil das proteínas, separadas por diferentes pesos moleculares. A: Sobrenadante Bruto. B: Precipitado. C: Fase Superior da Extração com Álcool Butílico.

Partição em Sistema Aquoso Bifásico

Foram feitos testes de partição com 3 sobrenadantes diferentes (cultivos de 3 erlenmeyers diferentes). Foi testado o sistema com PEG (polietilenoglicol) e diferentes sais: sulfato de zinco, sulfato de magnésio, sulfato de amônio, sulfato de sódio, fosfato de potássio e citrato de sódio. Inicialmente foram preparadas soluções estoque em concentração 50% (w/v) do PEG e de cada sal. Foram misturadas as soluções PEG/sal/sobrenadante buscando-se uma concentração final de PEG e sal de 20%. Foi feita a centrifugação a 3000 x g a 4ºC por 10 min. Foi feita a extração das diferentes

fases formadas e medidas a atividade antimicrobiana e a concentração de proteína nas duas fases.

O coeficiente de partição foi definido como a concentração de proteína (K_p) ou atividade antimicrobiana (K_b) na fase superior dividida pelo valor correspondente na fase inferior, conforme mostram as equações a seguir:

$K_p = C_s/C_i$

$K_b = A_s/A_i$

Onde C_s e C_i são a concentração total de proteína em mg/mL das frações superiores e inferiores, respectivamente, e A_s e A_i são a atividade antimicrobiana das frações superiores e inferiores, respectivamente. Os resultados obtidos estão apresentados nas Tabelas 02 e 03.

Tabela 02

Resultado médio das análises de Quantidade de Proteína (mg/mL) – Coeficiente de partição

Sal	Fase superior	Fase inferior	K_p
Sulfato de magnésio	3,61	2,23	1,55
Sulfato de zinco	3,21	1,79	1,79
Sulfato de amônio	3,61	2,52	1,43

Tabela 03

Resultado médio das análises de atividade antimicrobiana (UA/mL) – Coeficiente de partição

Sal	Fase superior	Fase inferior	K_b
Sulfato de magnésio	200	0	200
Sulfato de zinco	1600	0	1600
Sulfato de amônio	400	0	400

No sistema aquoso bifásico formado por um polímero e um sal inorgânico, os melhores resultados para os coeficientes de partição (K_p e K_b) foram encontrados no sistema formado por sulfato de zinco como sal inorgânico. Os resultados indicaram que o sistema ATPS é influenciado pelo tipo de sal inorgânico.

Em todos os casos testados, o coeficiente de partição foi superior a 1, indicando a afinidade dos peptídeos pela fase superior do sistema. Outros sais foram utilizados, mas eles resultaram em recuperações da atividade antimicrobiana muito baixas.

A Tabela 04 mostra os dados de Atividade Específica e Fator de Purificação obtidos para as diferentes frações dos testes de partição em sistemas aquosos bifásicos.

Tabela 04

Tabela de purificação mostrando os resultados das diferentes fases da purificação – sistema aquoso bifásico

Fase da Purificação	Atividade (UA/mL)	Proteína (mg/mL)	Atividade Específica (UA/mg)	Fator de Purificação
Sobrenadante Bruto	200	0,9778	204,54	1,0
Sulfato de magnésio				
Inferior	0	2,23	0	0
Superior	200	3,61	55,40	0,27
Sulfato de zinco				
Inferior	0	1,79	0	0
Superior	1600	3,21	498,44	2,44
Sulfato de amônio				
Inferior	0	2,52	0	0
Superior	400	3,61	110,80	0,54

5 DISCUSSÃO GERAL

De uma maneira geral, todos os trabalhos visaram estudar a otimização da produção de peptídeos antimicrobianos por *Bacillus* sp. P11, verificando a influência de fatores ambientais como pH, temperatura e substrato para crescimento, bem como de fatores indutivos, como a presença de células inativadas de bactérias ou fungos, na produção de atividade antimicrobiana por esta bactéria isolada do ambiente aquático da Amazônia.

Num primeiro momento, buscou-se estudar as melhores condições ambientais para a produção de peptídeos antimicrobianos, bem como selecionar um meio de cultivo alternativo mais viável para a produção destes compostos. Posteriormente, utilizando-se a metodologia de qRT-PCR, foi avaliada a influência do pH e da temperatura na expressão e genes relacionados com a produção dos peptídeos antimicrobianos subtilosina A e iturina A. Também foi estudada a ação da adição de células inativadas de fungos e bactérias, como agentes indutores da produção de peptídeos antimicrobianos. Finalmente, buscou-se a purificação parcial dos compostos antimicrobianos produzidos por *Bacillus* sp. P11.

Metabólitos produzidos por algumas cepas de *Bacillus* mostraram atividade antibacteriana e antifúngica contra fitopatógenos e microorganismos deteriorantes de alimentos (BERNAL et al., 2002; CZACZYK et al., 2000; FOLDES et al., 2000; MCKEEN et al., 1986; MILNER et al., 1996; PICHARD et al., 1995; SHIROKOV et al., 2002; TOURÉ et al., 2004). *Bacillus* spp. produz um grupo heterogêneo de proteínas ou peptídeos antimicrobianos que variam

em espectro de atividade, modo de ação, massa molecular, propriedades bioquímicas e origem genética (COTTER et al., 2005).

Visando selecionar o melhor substrato para a produção de atividade antimicrobiana por *Bacillus* sp. P11, foram testados seis diferentes meios de cultura: farelo de soja, farinha de peixe, resíduo de soja, proteína de soja e resíduo de uva. A maior atividade antimibrobiana foi obtida por cultivo em farelo de soja (1600 UA/mL), seguida pela farinha de peixe, resíduo de soja e proteína de soja. Não foi observada atividade antimicrobiana em cultivo com resíduo de uva. Da mesma forma, farelo de soja tem sido descrito como o melhor substrato para produção de bacteriocina por outras espécies de *Bacillus* (DOMINGUEZ et al., 2007; MOTTA & BRANDELLI, 2008).

Considerando o farelo de soja o melhor substrato para a produção de atividade antimicrobiana, buscou-se avaliar os efeitos do pH inicial, da temperatura e concentração do substrato (farelo de soja) sobre a produção de peptídeos antimicrobianos e sobre crescimento de *Bacillus* sp. P11. Para isso utilizou-se a metodologia de superfície de resposta (MSR).

A análise de variância (ANOVA) foi utilizada para a determinação de parâmetros significativos e para estimar a produção de atividade antimicrobiana em função do substrato (concentração farelo de soja), temperatura e pH. A 90% de confiança, pH linear e interação entre pH e temperatura foram significativas para a produção de atividade antimicrobiana por *Bacillus* sp. P11. Os resultados mostraram que a concentração de farelo de soja e a interação entre esta variável e as outras duas não foram significativas ($P > 0,05$) para a atividade antimicrobiana, indicando que *Bacillus* sp. P11 produz atividade

antimicrobiana semelhante em alta concentração de substrato e em baixa concentração.

As três dimensões da superfície de resposta da curva de pH em função da temperatura foram então plotados e a concentração do substrato foi fixada no ponto central (20 g/L), porque essa variável não tem efeito significativo sobre a resposta. Máxima atividade antimicrobiana (1400 AU/mL) foi alcançada em pH ligeiramente alcalino, entre 7,0 e 8,5, e temperatura entre 39 e 42°C (maior valor em pH 7,0 e 42^{o}C). Anthony et al. (2009) também observaram maior atividade específica à temperatura em torno de 42ºC e em pH alcalino (8,0) para *B. licheniformis* AnBa9. A produção de bacteriocina por *Bacillus cereus* 8A também foi melhor em pH alcalino (no intervalo de 6,5 e 9,0) em farelo de soja, no entanto a bactéria produziu maior atividade antimicrobiana em temperatura em torno de 30ºC (DOMINGUEZ et al., 2007).

Com base nesses resultados, avaliou-se o crescimento bacteriano e a produção de atividade antimicrobiana em condições ideais de temperatura e pH. Para isso, *Bacillus* sp. P11 foi incubado aerobicamente nas condições ótimas obtidas com o esquema fatorial (42°C e pH inicial 7,0) em BHI (meio comercial) ou farelo de soja (meio alternativo). Análise de variância mostrou que a concentração de farelo de soja não foi significativa para a produção de atividade antimicrobiana, assim, ele foi usado na concentração do ponto central (20 g/L). O crescimento bacteriano e a produção de atividade antimicrobiana foi similar em ambos os meios, indicando o potencial do farelo de soja como meio de cultura para a produção de peptídeos antimicrobianos por *Bacillus* sp. P11.

Ainda visando avaliar a influência das condições ambientais na produção de peptídeos antimicrobianos por *Bacillus* sp. P11, utilizou-se a metodologia de qRT-PCR para verificar a expressão de determinados genes relacionados a produção destes compostos. Destaca-se que esta técnica tem sido pouco empregada para estudar a expressão de genes envolvidos na síntese de peptídeos antimicrobianos.

O efeito do pH e da temperatura são muito importantes para a produção de peptídeos antimicrobianos e tem sido relatado para vários compostos produzidos por *Lactobacillus* (AASEN et al., 2000; ZÁLAN et al., 2005) e *Bacillus* (CLADERA-OLIVEIRA et al., 2004; MOTTA & BRANDELLI, 2008; ANTHONY et al., 2009).

Foram verificadas as expressões dos genes relacionados à produção de subtilosina (*sboA*) e iturina (*ituD*). Com relação à expressão do gene *sboA*, o estresse causado pelas temperaturas de 37ºC e 42ºC suprimiu a expressão do referido gene. No entanto, a expressão de *sboA* foi ativada em pH 6 (3,79 vezes) e, especialmente, em pH 8 (198,64 vezes maior).

Já os resultados para a expressão do gene *ituD* mostraram que a temperatura de 37ºC estimulou a expressão *ituD* em *Bacillus* sp. P11 (9,14 vezes maior). O choque térmico causado pela temperatura de 42°C representou a repressão da expressão gênica. O pH 6 induziu a expressão do gene responsável pela síntese de iturin A em *Bacillus* sp. P11 (21,29 vezes). Mas o estresse causado pelo pH alcalino (pH 8) suprimiu a expressão do gene *ituD* por esta cepa.

Para confirmar a produção de subtilosina A e iturina A e determinar com precisão a massa molecular dos peptídeos antimicrobianos, foi realizada

análise de espectroscopia de massa. Para isso, os peptídeos antimicrobianos foram isoladas do sobrenadante de cultura livre de células de *Bacillus* sp. P11, sendo que os espectros de massa revelaram *clusters* com picos principais em m/z 1000-1100 Da, correspondente a isoformas do lipopeptídeo iturina. A cepa mostrou uma ampla distribuição, incluindo um conjunto de picos que foram observados entre m/z 1042 e 1080, e outro *cluster* com picos entre m/z 1120 e 1140. Os espectros de massa também revelaram *clusters* com picos acima de m/z 3.000 Da, correspondentes a subtilosina A, subtilina e isoformas destes peptídeos.

MALDI-TOF tem sido usada como uma ferramenta eficiente para identificação de peptídeos antimicrobianos, incluindo lipopeptídeos hidrofóbicos na faixa de 1-1,5 kDa (STEIN, 2008), bacteriocinas e bactérias produtoras de lantibioticos na faixa de massa de 3-5 kDa (STEIN, 2008). Os picos em m/z 1042-1095 concordam com isômeros de iturina A descrito para *B. amyloliquefaciens* RC-2 (HIRADATE et al., 2002) e *B. amyloliquefaciens* CCMI 1051 (CALDEIRA et al., 2008). A cepa P11 mostrou uma ampla gama de picos, indicando que esta bactéria produz uma mistura complexa de peptídeos antimicrobianos. Estes resultados sugerem que a atividade antimicrobiana dessa cepa está associada com lipopeptídeos iturina e/ou surfactina. Picos com valores similares para ericina A e ericina S também foram identificadas, sugerindo que a atividade antimicrobiana dessas cepas (MOTTA et al., 2004) pode estar associada à produção de subtilosina A e/ou subtilina. Estes resultados indicam que a técnica é valiosa para identificar peptídeos antimicrobianos produzidas por *Bacillus* sp. P11.

Também buscando otimizar a produção de peptídeos antimicrobianos por *Bacillus* sp. P11, realizou-se estudo de indução no qual a cepa produtora foi incubada em presença de células inativadas de bactérias ou fungos. Este estudo forneceu evidências de que a presença de células inativadas termicamente de *S. aureus* ou *A. parasiticus* pode aumentar a produção de atividade antimicrobiana por *Bacillus* sp. P11, sugerindo que um estímulo externo a partir de microrganismos alvo foi capaz de melhorar a produção de peptídeos antimicrobianos.

Estudos com sobrenadante de cultura de células-livre da cepa indutora sugerem que o aumento da atividade antimicrobiana não é mediada por um metabólito secretado pela cepa indutora. Benitez et al. (2011), verificaram que cultura de *E. coli* causou um aumento na atividade antimicrobiana de *Bacillus amyloliquefaciens* LBM 5006. No entanto, estudos semelhantes sobre as espécies *Bacillus* são muito escassos, se comparados com os realizados para bactérias ácido lácticas.

A interação de células inativadas termicamente da cepa indutora com *Bacillus* sp. P11 é desconhecida. No entanto, foi demonstrado neste estudo que a presença de células inativadas termicamente de *S. aureus* ou *A. parasiticus* em um mesmo ambiente aumenta a produção de atividade antimicrobiana. Durante o aquecimento, alguns componentes celulares que podem atuar como indutores podem ser expostos e/ou solubilizados, causando um efeito indutor positivo. A síntese aumentada de peptídeos antimicrobianos em resposta a outras células microbianas pode ser uma estratégia eficiente para defesa contra microorganismos concorrentes (BENITEZ et al., 2011).

Além da indução da produção e atividade antimicrobiana, avaliou-se a expressão de genes relacionados à síntese de peptídeos antimicrobianos como surfactina (*sfp*), subtilosina (*sboA* e *lpa-14*), iturina (*ituD*) e fengicina (*fenA*) por *Bacillus* sp. P11, utilizando qRT-PCR. Esses antimicrobianos têm amplos espectros inibitórios com alta atividade biológica, boa estabilidade e solubilidade, podendo ter um grande potencial biopreservativo (CHANG et al., 2007) ou podendo ser usados como agentes de controle de fitopatógenos (ONGENA et al., 2005).

A expressão dos genes foi observada em cultura com células inativadas de *S. aureus* ou *A. parasiticus*, selecionados após testes com células inativadas de várias cepas de bactérias e fungos. Em geral, nas condições experimentais do estudo, a presença de células inativadas de *S. aureus* apresentou uma maior indução na expressão de genes estudados, quando comparado com *A. parasiticus*.

Os genes *sfp, lpa-14, ituD, sboA* e *fenA*, relacionados com a produção de peptídeos antimicrobianos, foram identificados em diferentes cepas de *Bacillus* isolados de ambientes aquáticos da bacia amazônica brasileira, incluindo *Bacilus* sp. P11. Estas bactérias demonstraram atividade antifúngica contra espécies que causam importantes perdas econômicas na agricultura. Além disso, a inibição de patógenos bacterianos como *Listeria monocytogenes* e *Bacillus cereus* por estas cepas foi descrito anteriormente (MOTTA et al., 2004).

A expressão do gene *sfp* foi reprimido cerca de 20 vezes na presença de células inativadas de *A. parasiticus*, e mostrou regulação não significativa na presença de *S. aureus*. O gene *sfp* é responsável pela produção da enzima 4'-

fosfopantateína transferase que foi caracterizada como essencial para a produção de surfactina (NAKANO et al., 1992; HSIEH et al., 2004).

Outro gene que também codifica a enzima 4'-fosfopantateína transferase é o gene *lpa-14*, que é necessário para a maturação da enzima modelo de iturina A (TSUGE et al., 2001; HSIEH et al., 2008). Neste estudo, o gene *lpa-14* foi reprimido (6,87 vezes) quando *Bacillus* sp. P11 foi incubado com células inativadas termicamente de *S. aureus*.

O gene *ituD*, assim como o gene *lpa-14*, está envolvido na produção de iturina A (TSUGE et al., 2001; HSIEH et al., 2008). Este gene codifica uma malonil coenzima A transacilase, cuja perturbação resulta em uma deficiência específica na produção de iturin A. *Bacillus* sp. P11 apresentou maior nível de expressão de *ituD* durante o crescimento na presença de células inativadas de *S. aureus*, em comparação com *A. parasiticus*.

Poucos estudos destacam o mecanismo de regulação que controla a síntese de iturina A em *Bacillus* (DUITMAN et al., 2007; KOUMOUTSI et al., 2007; CHEN et al., 2009). Alguns autores (TSUGE et al., 2001; HOFEMEISTER et al., 2004) sugerem que a expressão dos grupos de genes que regem a síntese de compostos semelhantes a iturina em *Bacillus* é controlada pelo mesmo regulador transcricional de bacilomicina D. A expressão deste composto é impulsionada por uma promotor σA induzido pela fase estacionária, P_{bmy}, e identificou três reguladores globais, Degu, DegQ, ComA e dois fatores sigma σ^B e σ^H que influenciam positivamente a ativação transcricional de P_{bmy} em *B. amyloliquefaciens* FZB42 (KOUMOUTSI et al. , 2007). DegQ é uma pequena proteína pleiotrópica reguladora que controla a expressão de enzimas de degradação, proteases intracelulares e várias outras

enzimas segregadas e parece controlar a transcrição de antibióticos peptídicos (AMORY et al., 1987; MSADEK et al., 1991). ComA é um regulador de genes de competência e produção final de surfactina (GUILLEN et al., 1989; ROGGIANI & DUBNAU, 1993; SHIMANE & OGURA, 2004) que controla a expressão BMY indiretamente através DegQ, que serve como um fator auxiliar para DegU.

Velho et al. (2011b) relatou que cepas de *Bacillus* amazônicos apresentaram níveis mais baixos de expressão de *sboA* quando comparadas com *B. subtilis* ATCC 19659. Neste estudo, o gene *sboA* mostrou regulação não significativa na presença de *S. aureus* ou *A. parasiticus* (células inativadas termicamente) por *Bacillus* sp. P11. O gene *sboA* está relacionado à produção de subtilosina. Este gene está envolvido na modificação pós-traducional e processamento de pré-subtilosina, secreção e também imunidade (ZHENG et al., 2000; MAQUEDA et al., 2008). A produção de subtilosina A madura requer a expressão de oito (*sboA-albABCDEFG*) dos nove genes identificados no operon de *B. subtilis*. O operon sbo-alb é conhecido por estar sob controle complexo (NAKANO et al., 2000; ZHENG et al., 2000). Este operon é induzido em culturas de crescimento tardio, aparentemente em resposta à falta de nutrientes e também é drasticamente induzida pela limitação de oxigênio (NAKANO et al., 2000; ZHENG et al., 2000).

Assim como para *sboA*, *Bacillus* sp. P11 também não mostrou nenhuma regulação significativa para o gene *fenA* nas condições estudadas. O gene *fenA* está envolvido com a síntese de fengicina, um lipopeptídeo antibiótico, antifúngico e biossurfactante (WEI et al., 2010; VANITTANAKOM et al., 1986).

No que diz respeito à purificação parcial dos peptídeos antimicrobianos, os resultados obtidos não foram satisfatórios. A metodologia que apresentou melhor fator de purificação foi a partição em sistema aquoso bifásico com o sal sulfato de zinco (2,44). Utilizando-se a extração com solventes orgânicos o melhor fator de purificação foi calculado em 0,73. Já a purificação com colunas cromatográficas não apresentou bons resultados de atividade antimicrobiana. Todos esses resultados indicam que é necessária a continuidade dos testes bucando melhores metodologias para purificação de peptídeos antimicrobianos produzidos por *Bacillus* sp. P11, pois Lima et al. (2002) estudaram a purificação de enzimas pectinolíticas usando apenas uma etapa de extração líquido-líquido usando o sistema PEG/fosfato e obtiveram fator de purificação de 14,27. Lappe et al. (2011) obtiveram fator de purificação de 13 para cereina 8A, purificada por uma sequência de precipitação, extração e cromatografia de troca iônica, com um adicional de cromatografia de gel filtração.

Atualmente é alta a demanda por métodos de extração e purificação de biopartículas (proteínas, enzimas, células, etc.) que sejam eficientes e economicamente viáveis. Nessa linha, os sistemas aquosos bifásicos constituem um potencial. Entretanto, é notório que estes sistemas, apesar da intensa aplicação em escala laboratorial para purificação de enzimas e proteínas, não alcançaram ampla aplicação comercial, tendo talvez como possíveis causas o custo dos polímeros formadores das fases e/ou o comportamento complexo (inúmeras variáveis interferindo de forma cinergética ou antagônica) de partição apresentados nestes sistemas bifásicos. Além disso, para sua aplicação sistemática, é necessária maior compreensão dos

fatores que determinam sua formação e a partição de solutos específicos. Contudo, a urgente demanda por parte da indústria biotecnológica, com objetivo de trazer para o mercado consumidor produtos que possuam alto valor agregado, com ampla aplicação médica, certamente motivará a aplicação em escala industrial dos SABs (SILVA & LOH, 2006).

6 CONCLUSÕES

A otimização da produção de atividade antimicrobiana por *Bacillus* sp. P11, isolado do ambiente aquático da Amazônia, é influenciada por fatores ambientais como pH, temperatura e substrato para crescimento, bem como por fatores indutivos, como a presença de células inativadas de bactérias ou fungos.

O farelo de soja apresentou melhores resultados para a produção de substâncias antimicrobianas por *Bacillus* sp. P11, quando comparado a outros subprodutos e resíduos de agroindústrias de alimentos usados como substrato para o crescimento microbiano. Utilizando este meio de cultivo, verificou-se que o pH e a temperatura podem afetar a produção de atividade antimicrobiana, sendo que o cultivo em pH levemente alcalino (entre 7 e 8,5) e temperaturas mais elevadas (entre 39 e 42ºC) demostrou melhores resultados. Além disso, a atividade antimicrobiana foi semelhante em farelo de soja e BHI, indicando que o primeiro pode ser um meio de cultura alternativo, mais viável financeiramente, quando comparado com meio de cultura comercial.

A produção de subtilosina A e iturina A pode ser fortemente afetada por fatores ambientais, sendo que condições de estresse por pH e choque térmico influenciaram na expressão de genes relacionados à produção destes compostos. Além disso, qRT-PCR provou ser uma poderosa ferramenta para otimizar a produção destes peptídeos antimicrobianos *por Bacillus* sp. P11.

A adição de células inativadas de bactérias ou fungos em culturas de cepas de *Bacillus* pode induzir a produção de compostos antimicrobianos. Usando a metodologia qRT-PCR, verificou-se que a presença de células

termicamente inativadas de *S. aureus* ou *A. parasiticus* induziram a expressão dos genes *lpa-14* e *ituD* por *Bacillus* sp. P11. Esses genes estão envolvidos com a produção de iturina A, um peptídeo antimicrobiano que mostra forte atividade antibiótica contra um amplo espectro de fungos, o que torna um potencial agente de controle biológico com o objetivo de reduzir o uso de pesticidas químicos na agricultura.

A purificação dos peptídeos antimicrobianos produzidos por *Bacillus* sp. P11 não foi satisfatória, pois os fatores de purificação obtidos foram muito baixos, tornando as metodologias testadas inviáveis para serem utilizadas em escalas de laboratório e industrial. Assim, torna-se necessária a continuidade da busca por metodologia adequada para a purificação parcial dos compostos estudados.

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APÊNDICE

Artigo publicado no periódico *Food and Bioprocess Technology:*

Use of byproducts of food industry for production of antimicrobial activity by *Bacillus* sp. P11

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COMMUNICATION

Use of Byproducts of Food Industry for Production of Antimicrobial Activity by *Bacillus* sp. P11

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Abstract Production of antimicrobial activity by *Bacillus* sp. P11 was tested on different byproducts of food industry, like fish meal, grape waste, an industrial fibrous soybean residue, soybean meal, and cheese whey. *Bacillus* sp. P11 produced the higher amount of antimicrobial activity on soybean meal, followed by fish meal and fibrous soybean residue. Soybean meal was the selected medium to determine the effect of three variables (temperature, initial pH, and substrate concentration) on bacteriocin activity by response surface methodology, using a 2^3 factorial design. Statistical analysis showed good adequacy to the model (R^2 of 0.8268). In the range studied, temperature and initial pH of the medium have a significant effect on bacteriocin production, and substrate concentration has no significant effect. Response surface data showed maximum bacteriocin production at initial pH between 7.0 and 8.5 and temperature between 39 and 42 °C. In the optimum conditions (initial pH 7.0 and 42 °C), production of bacteriocin activity by *Bacillus* sp. P11 was compared using a commercial medium (BHI broth) and soybean meal. Maximum activity achieved with the soybean meal-based medium was similar to that obtained with BHI, indicating that soybean meal may be a cost-effective substrate for production of antimicrobial activity by *Bacillus* sp. P11.

Keywords Agroindustrial waste · Bacteriocin · *Bacillus* · Response surface methodology · Soybean meal

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Introduction

Bacteriocins are antimicrobial peptides widespread produced among bacteria (Cotter et al. 2005). The attractive features of bacteriocins, such as their natural sources, wide range of activities, and their proteinaceous nature, which implies a putative degradation in the gastrointestinal tract of man and animals, have interested researchers seeking to develop new antimicrobial agents (Cleveland et al. 2001; Asaduzzaman and Sonomoto 2009). Those produced by lactic acid bacteria (LAB) are largely studied with the perspective to search for safe and food-grade preservatives of biological origin (O'Sullivan et al. 2002; Calo-Mata et al. 2008). Despite the intensive work on bacteriocins produced by LAB, the genus *Bacillus* comprises a variety of industrially important species and has a history of safe use in both food and pharmaceutical industry (Paik et al. 1997; Pedersen et al. 2002; Hong et al. 2008; Vaucher et al. 2010). Indeed, bacteriocins or bacteriocin-like substances have been described for *Bacillus* spp., presenting a broad spectrum of antibacterial activity and potential use as biopreservative in food (Bizani et al. 2005; Motta et al. 2007).

Bacteriocin production is often performed in complex media, which promote abundant growth and relatively high bacteriocin levels. The necessity for reduction of pollutants in the environment and the need to maximize returns on raw materials have encouraged the search for new ways of using food industry waste as the basis of culture media.

Possible alternatives include byproducts such as milk whey and mussel-processing wastes (Amiali et al. 1998; Guerra and Pastrana 2002). The use of byproducts will require optimization of bacteriocin production, which is usually dependent on multiple strain-specific factors (Leal-Sánchez et al. 2002; Casarin et al. 2008). Studies on multiple factors affecting the production of bacteriocins are relatively scarce, and it is difficult to optimize them for biotechnological processes. Optimization by a conventional "one-at-a-time-approach" does lead to a substantial increase in bacteriocin yields; however, this approach is not only massive and time consuming but also has the limitations of ignoring the importance of interaction of various parameters. Response surface methodology (RSM) has been successfully applied in many areas of biotechnology, including reports on bacteriocin production. RSM has been used to evaluate the effect of medium components and environmental conditions on production of antimicrobial activity by *Bacillus licheniformis* AnBa9 (Anthony et al. 2009) and *Bacillus cereus* 8A (Dominguez et al. 2007).

Bacillus sp. P11 is a novel bacteriocin-producing strain isolated from the intestinal contents of the teleost fish Piau-com-pinta (*Leporinus* sp.) of Brazilian Amazon basin (Giongo et al. 2007). The purpose of this work was to select a less expensive medium and study the influence of temperature, pH, and concentration of growth substrate on bacteriocin production by *Bacillus* sp. P11. The production of antimicrobial activity in commercial medium was compared with soybean meal-based medium.

Materials and Methods

Reagents and Media

The following byproducts were individually evaluated. Cheese whey powder from the production of mozzarella cheese (Parmalat, Porto Alegre, Brazil) contains 13% protein, 1% lipids, 77% carbohydrate (lactose), and 9% minerals. Fish meal (Etna, Rio Grande, Brazil), a waste of fish processing, contains 54% protein, 11% lipids, 5% carbohydrate, and 30% minerals. Grape waste residue (30% fiber, 21% carbohydrate, 10% protein, 10% fat) was obtained as described by Silveira et al. (2008). Isolated soybean protein (Bunge, Esteio, Brazil) has 79% protein, 3% lipids, 12% carbohydrates, and 5% minerals. Fibrous soybean residue (35% protein, 19% carbohydrates, and 46% fiber) is a byproduct from the production of isolated soybean protein. Soybean meal (Cerelus, Ijuí, Brazil) contains 50% protein, 3% lipids, 35% carbohydrate, 5% fiber, and 7% minerals. The commercial medium Brain Heart Infusion (BHI) was from Oxoid (Basingsoke, UK).

Bacterial Strains

The producer bacterium was the previously characterized strain *Bacillus* sp. P11 (Giongo et al. 2007). The indicator strain was *Listeria monocytogenes* ATCC 7644. BHI medium was used for maintenance of strains with 20% (*v*/*v*) glycerol at −20 °C. The cultivation of strains was performed aerobically.

Selection of the Best Substrate for Bacteriocin Production

The inoculum was developed in BHI at 30 °C under shaking (120 rpm). The absorbance at 600 nm was adjusted ($A_{600\ nm}$=2.00) to obtain approximately 10^7 CFU/mL, and 1.0 mL was transferred to 250 mL Erlenmeyer flasks containing 100 mL of medium. Cultures were incubated in a rotary shaker at 120 rpm for 24 h. Aliquots of culture were centrifuged at 10,000×*g* for 15 min. Culture supernatants were boiled at 100 °C for 5 min, placed in an ice bath, and the pH was measured and adjusted to pH 7.0 before being used in the antimicrobial assay. In parallel, bacterial suspensions were diluted to 10^{-8} in 8.75 g/L NaCl for determination of colony forming units per milliliter (CFU/mL; Motta and Brandelli 2002).

The byproducts of the industry were individually tested to select the best substrate for bacteriocin production, and its concentration are showed in Table 1 and chosen like elsewhere (Dominguez et al. 2007). Cultivation in BHI broth was developed as control. The pH was adjusted at 7.0 with 0.5 M HCl or 0.5 M NaOH before autoclaving (121 °C, 15 min). Cultivation was developed in 250 mL Erlenmeyer flasks containing 100 mL of each medium. After inoculation with 1-mL culture, incubation was performed at 30 °C under shaking (120 rpm). The food byproduct with best yield (soybean meal) was compared to commercial medium (BHI) to evaluate the growth and bacteriocin production behavior of *Bacillus* sp. P11. Bacteriocin activity was determined after 24 h as described above. The cultivations were

Table 1 Growth substrates tested for production of bacteriocin activity

Medium	Concentration (g/L)	Bacteriocin activity (AU/mL)
BHI	–	1,200±565
Cheese whey	70	400±283
Fish meal	10	800±565
Fibrous soybean residue	20	800±0
Soybean meal	20	1,600±0
Isolated soybean protein	20	800±565
Grape waste residue	30	0±0

performed in duplicate. Two independent activity assays were performed for each cultivation.

Antimicrobial Activity Assay

The antimicrobial activity was detected by agar disk diffusion assay (Motta and Brandelli 2002). Aliquots (20 μL) of culture supernatants were applied to 6-mm cellulose paper disks (Whatman no. 2) on BHI agar plates previously inoculated with a cell suspension of *L. monocytogenes* ATCC 7644, which corresponded to a 0.5 McFarland turbidity standard solution. Plates were incubated at 37 °C. The antimicrobial activity titre was determined by serial twofold dilution method. Activity was defined elsewhere as the reciprocal of the dilution after the last serial dilution giving a zone of inhibition and expressed as activity units per milliliter (AU/mL; Mayr-Harting et al. 1972).

Experimental Design of RSM

After selection of the best medium, the next step was to determine the optimal levels of three variables, temperature, initial pH, and soy bean meal concentration on bacteriocin production and on bacterial growth. For this purpose, the response surface approach by using a set of experimental designs (central composite design with five coded levels) was performed.

A 2^3 full factorial design for three factors with replicates at the center point and star points was used for optimization of cultivation. The variables used were evaluated each at five coded levels ($-\alpha$, −1, 0, +1, $+\alpha$). The axial distance α was chosen to be 1.68 to make this design orthogonal. A set of 18 experiments was carried out (Table 2). The central values (0 level) chosen for experimental design were temperature, 30 °C; initial pH, 7.0; and soybean meal concentration, 20 g/L (Dominguez et al. 2007).

Results were analyzed by the Experimental Design Module of the Statistica 7.0 software (Statsoft, OK, USA). The model permitted evaluation of the effects of linear, quadratic, and interactive terms of the independent variables on the chosen dependent variables. Three-dimensional surface plots were drawn to illustrate the main and interactive effects of the independent variables on bacteriocin production. The assays were performed in duplicate. The optimum values of the selected variables were obtained by solving the regression equation and also by analyzing the response surface contour plots (Myers and Montgomery 2002).

Table 2 Experimental design and results of the 2^3 factorial design

Run	Variables[a]			Bacteriocin activity (AU/mL)		Final cell concentration (Log CFU/mL)
	Soybean (g/L) (x_1)	pH (x_2)	Temperature (°C) (x_3)	Observed	Predicted	
1	11 (−1)	5 (−1)	23 (−1)	0±0	169.03	5.22±0.12
2	29 (+1)	5 (−1)	23 (−1)	0±0	169.03	5.91±0.05
3	11 (−1)	9 (+1)	23 (−1)	100±141	169.03	6.15±0.03
4	29 (+1)	9 (+1)	23 (−1)	0±0	169.03	6.78±0.03
5	11 (−1)	5 (−1)	37 (+1)	0±0	660.49	6.78±0
6	29 (+1)	5 (−1)	37 (+1)	0±0	660.49	7.16±0.02
7	11 (−1)	9 (+1)	37 (+1)	800±0	660.49	8.37±0
8	29 (+1)	9 (+1)	37 (+1)	800±0	660.49	8.43±0.03
9	5 (−1.68)	7 (0)	30 (0)	600±283	711.0	8.22±0.01
10	35 (+1.68)	7 (0)	30 (0)	800±0	711.0	8.13±0.06
11	20 (0)	4 (−1.68)	30 (0)	0±0	−125.108	0±0
12	20 (0)	10 (+1.68)	30 (0)	0±0	−125.108	0±0
13	20 (0)	7 (0)	18 (−1.68)	100±141	298.1736	8.67±0
14	20 (0)	7 (0)	42 (+1.68)	1,200±566	1,123.826	7.97±0.04
15	20 (0)	7 (0)	30 (0)	800±0	711.0	0±0
16	20 (0)	7 (0)	30 (0)	800±0	711.0	7.32±0.03
17	20 (0)	7 (0)	30 (0)	800±0	711.0	6.80±0.03
18	20 (0)	7 (0)	30 (0)	400±0	711.0	8.04±0.02

[a] Coded values are presented in the parenthesis

Results and Discussion

Selection of Best Substrate for Bacteriocin Activity

Six different culture media were compared for bacteriocin production by *Bacillus* sp. P11, and the results are shown in Table 1. Higher activity was obtained by cultivation on soybean meal (1,600 AU/mL), followed by fish meal, fibrous soybean residue, and soybean protein. Antimicrobial activity was not observed on grape waste residue. Similarly, soybean meal has been described as the best substrate to bacteriocin production by other species of *Bacillus* (Dominguez et al. 2007; Motta and Brandelli 2008). However, Cladera-Olivera et al. (2004) showed that it was not possible to determine a relation between bacteriocin production by *B. licheniformis* P40 and optimal conditions for bacterial growth in cheese whey. Considering the compositions of the byproducts used in this study, soybean meal has elevated quantities of proteins and carbohydrates, N, and C sources, respectively. The adequate concentrations of these nutrients in medium can enhance bacterial growth, and the high amount of protein and carbohydrate in soybean meal may explain the highest antimicrobial activity by *Bacillus* sp. P11 in this medium.

Bacteriocin Activity on Soybean Meal

The results of central composite design experiments for studying the effects of initial pH, temperature, and substrate concentration (independent variables) on bacteriocin activity and bacterial growth are presented in Table 2.

The analysis of variance was employed for the determination of significant parameters and to estimate the production of bacteriocin as a function of substrate (soybean meal concentration), temperature, and pH (Table 3). The computed F value model (4.386) was higher than the F value in statistic tables at 95% of confidence ($Ft_{9,8}$=3.23), which demonstrate significance for the regression model (Myers and Montgomery 2002). The regression equation obtained indicated the R^2 value of 0.8268 (a value of R^2>0.75 indicates the adequacy of the model); thus, 82.68% of the total variation is explained by the model. In addition, there is a good correlation between the experimental and predicted values. The following regression equation was obtained:

$$Y = 711.1 + 45.37x_3 + 296.24x_2^2 \quad (1)$$

Optimization by a conventional "one-at-a-time-approach" does lead to a substantial increase in bacteriocin yields; however, this approach is not only massive and time consuming but also has the limitations of ignoring the importance of interaction of various parameters. The significance of coefficients determined by P values is shown in Table 3. The larger the magnitude of the t value and smaller the P value, the more significant is the corresponding coefficient. In this study, temperature and second-order pH were the significant variables (P_T<0.05, P_{pH-pH}<0.05), which indicates that they can act as limiting factors and variations in their values will alter bacteriocin activity to a considerable extent. At 90% of confidence, linear pH and the interaction between pH and temperature were significant to the bacteriocin production by *Bacillus* sp. P11. Results showed that neither soybean meal concentration nor interaction between this variable with the other two were not significant (P>0.05) for bacteriocin activity, indicating that *Bacillus* sp. P11 produces similar

Table 3 Analysis of variance for the model and the regression coefficients estimated to bacteriocin activity

Source	Sum of square	Degree of freedom	Mean square	F value	t value	P value
Soybean meal concentration (L)	4,082	1	4,082	0.102	7.122	0.770
Soybean meal concentration (Q)	29,328	1	29,328	0.733	0.319	0.455
pH (L)	211,802	1	211,802	5.295	−0.856	0.105[b]
pH (Q)	1,106,780	1	1,106,780	27.669	2.301	0.013[a]
Temperature (L)	821,493	1	821,493	20.537	−5.260	0.020[a]
Temperature (Q)	54,834	1	54,834	1.371	4.532	0.326
Soybean meal (L) by pH (L)	1,250	1	1,250	0.031	−1.171	0.871
Soybean meal (L) by Temperature (L)	1,250	1	1,250	0.031	−0.177	0.871
pH (L) by temperature (L)	281,250	1	281,250	7.031	0.177	0.077[b]
Lack of fit	389,106	5	77,821	1.946	2.652	0.309
Pure error	120,000	3	40,000		7.122	
Total sum of square	2,940,000	17			0.319	

L linear effect, *Q* quadratic effect

[a] Statistically significant at 95% of confidence

[b] Statistically significant at 90% of confidence

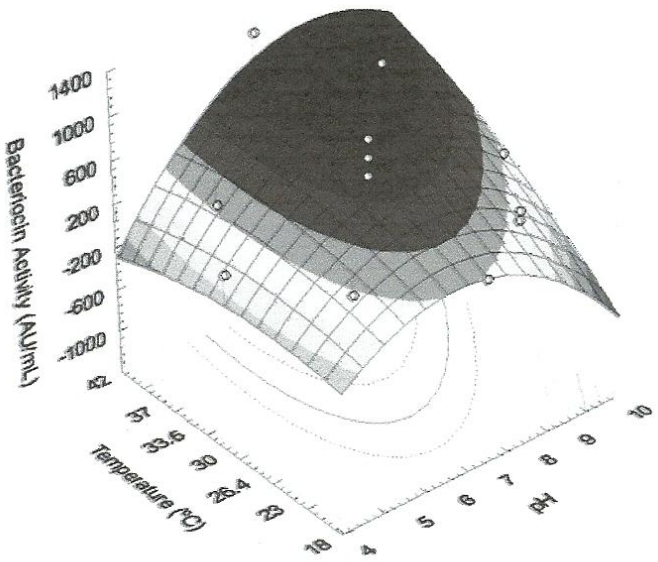

Fig. 1 Response surface of bacteriocin activity (AU/mL) by *Bacillus* sp. P11 as a function of temperature (°C) and initial pH at soybean protein concentration of 20 g/L

activity of antimicrobial peptide with high substrate concentration or even with low concentration.

The three-dimensional response surface curve of pH versus temperature was then plotted (Fig. 1). Substrate concentration was fixed at central point (20 g/L), because this variable has no significant effect on the response. Maximum bacteriocin activity (1,400 AU/mL) was achieved at slightly alkaline pH between 7.0 and 8.5 and temperature between 39 and 42 °C (highest value at pH 7.0 and 42 °C). Anthony et al. (2009) also found *B. licheniformis* AnBa9 to obtain highest specific activity at temperature around 42 °C and at alkaline pH (8.0). Bacteriocin activity decreased with decreasing of cultivation temperature and at acid or high alkaline pH. Bacteriocin production by *B. cereus* 8A was also better at alkaline pH (in the range of 6.5 and 9.0) on soybean meal; however, that strain produced higher bacteriocin activity at temperature around 30 °C (Dominguez et al. 2007).

With respect to bacterial growth, the model determined was not significant, because of poor fit of R^2 value (0.6491) and the F test did not show significance of the regression model (data not shown).

Bacillus Growth and Bacteriocin Production at Optimal Conditions of Temperature and pH

Bacillus sp. P11 was aerobically incubated at optimal conditions obtained with the factorial design (42 °C, initial pH 7.0) on BHI (commercial medium) or soybean meal (alternative medium). Analysis of variance showed that soybean meal concentration was not significant to bacteriocin production; thus, it was used at the concentration of central point (20 g/L). *Bacillus* growth was similar in both media, and the stationary phase was reached after 12 h of cultivation (Fig. 2a). Cells enter in death phase after 30 and 36 h in BHI and soybean meal, respectively. Maximum bacteriocin activity was observed at 24 h for soybean meal, and this activity was steady until 36 h of cultivation (Fig. 2b). In BHI there was a maximum production of antimicrobial activity at 24 h of cultivation, and then an important decrease was observed. Bacteriocin activity was similar in both media at 24 h, with no significant difference ($P>0.05$), indicating the potential of soybean meal as culture medium for production of bacteriocins by *Bacillus* sp. P11.

Production of antimicrobial peptides by strains of *Bacillus* is suggested to be under complex genetic regulation (Marahiel et al. 1993; Duitman et al. 1999). Decrease of antimicrobial activity at the late stationary phase could be associated to degradation by extracellular proteases, which are often produced by *Bacillus* spp. (Bizani and Brandelli 2002). The production of antimicrobial activity started during the exponential growth phase, reaching

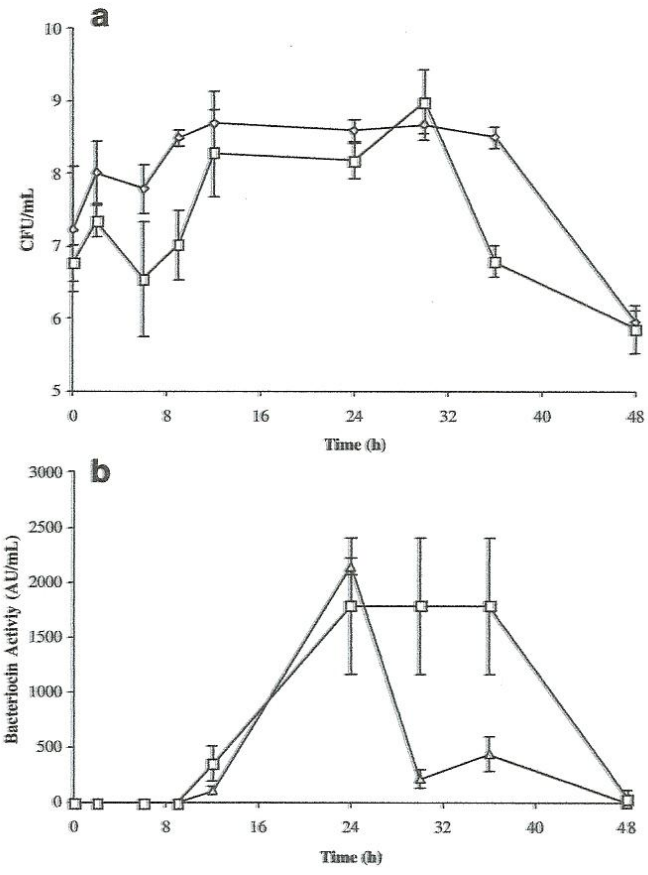

Fig. 2 Bacterial growth (**a**) and bacteriocin activity (**b**) by *Bacillus* sp. P11 at optimal conditions of temperature (42 °C) and initial pH (7.0) and 20 g/L soybean meal concentration (*square*) or BHI medium (*diamond*). Each point represents the mean±standard error mean of three independent experiments

maximum values at stationary phase. Production of bacteriocin is usually associated with primary metabolite kinetics (Cladera-Olivera et al. 2004; De Vuyst et al. 1996); however, bacteriocin production is recorded as a secondary metabolite in *Lactobacillus plantarum* LPCO10 (Jiménez-Díaz et al. 1993), *B. licheniformis* 26L-10/3RA (Pattnaik et al. 2001), and *Lactobacillus pentosus* B96 (Delgado et al. 2005). In this study, when *Bacillus* sp. P11 was cultivated in soybean meal, the antimicrobial substances started to be produced during exponential growth phase, suggesting a primary metabolite behavior.

Conclusions

Soybean meal was the best medium to *Bacillus* sp. 11 to produce antimicrobial substances, as good as commercial medium. Maximum bacteriocin activity was achieved at pH between 7.0 and 8.5, temperature between 39 and 42 °C. Soybean meal concentration was not significant to bacteriocin activity. *Bacillus* sp. P11 had reached the stationary phase after 12 h of cultivation, and maximum bacteriocin activity was observed from 24 h. Bacteriocin activity was similar in soybean meal and BHI, indicating the waste medium as a potential alternative to produce bacteriocins by *Bacillus* sp. P11.

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