KELLY CAMPOS GUERRA PINHEIRO DE GOES

CARACTERIZAÇÃO DE MICRORGANISMOS ISOLADOS DO XISTO PIROBETUMINOSO DA ÁREA DE MINERAÇÃO EM SÃO MATEUS DO SUL, PARANÁ

Londrina
2016

Universidade Estadual de Londrina

Instituto Agronômico do Paraná

Empresa Brasileira de Pesquisa Agropecuária

KELLY CAMPOS GUERRA PINHEIRO DE GOES

CARACTERIZAÇÃO DE MICRORGANISMOS ISOLADOS DO XISTO PIROBETUMINOSO DA ÁREA DE MINERAÇÃO EM SÃO MATEUS DO SUL, PARANÁ

Londrina
2016

KELLY CAMPOS GUERRA PINHEIRO DE GOES

CARACTERIZAÇÃO DE MICRORGANISMOS ISOLADOS DO XISTO PIROBETUMINOSO DA ÁREA DE MINERAÇÃO EM SÃO MATEUS DO SUL, PARANÁ

Tese de Doutorado apresentada ao Programa de Pós-Graduação em Genética e Biologia Molecular da Universidade Estadual de Londrina, como requisito para à obtenção do título de Doutor (a).

Orientadora: Prof[a]. Dra. Maria Helena Pelegrinelli Fungaro

Co-orientadora: Dra. Diva de Souza Andrade

Londrina
2016

Ficha de identificação da obra elaborada pelo autor, através do Programa de Geração Automática do Sistema de Bibliotecas da UEL

Goes, Kelly Campos Guerra Pinheiro de.
Caracterização de microrganismos isolados do xisto pirobetuminoso da área de mineração em São Mateus do Sul, Paraná / Kelly Campos Guerra Pinheiro de Goes. - Londrina, 2016.
111 f. : il.

Orientador: Maria Helena Pelegrinelli Fungaro. Coorientador: Diva Souza Andrade.
Tese (Doutorado em Genética e Biologia Molecular) - Universidade Estadual de Londrina, Centro de Ciências Biológicas, Programa de Pós-Graduação em Genética e Biologia Molecular, 2016.
Inclui bibliografia.

1. Fino de xisto - Teses. 2. Xisto retortado - Teses. 3. Identificação molecular - Teses. 4. BPCP - Teses. I. Fungaro, Maria Helena Pelegrinelli . II. Andrade, Diva Souza. III. Universidade Estadual de Londrina. Centro de Ciências Biológicas. Programa de Pós-Graduação em Genética e Biologia Molecular. IV. Título

KELLY CAMPOS GUERRA PINHEIRO DE GOES

CARACTERIZAÇÃO DE MICRORGANISMOS ISOLADOS DO XISTO PIROBETUMINOSO DA ÁREA DE MINERAÇÃO EM SÃO MATEUS DO SUL, PARANÁ

Tese de Doutorado apresentada ao Programa de Pós-Graduação em Genética e Biologia Molecular da Universidade Estadual de Londrina, como requisito para à obtenção do título de Doutor (a) em Genética e Biologia Molecular.

BANCA EXAMINADORA

Profª. Dra. Maria Helena Pelegrinelli Fungaro
Universidade Estadual de Londrina –- UEL

Dr. Yeshwant Ramchandra Mehta
Instituto Agronômico do Paraná – IAPAR

Dr. Luis Gustavo Morello
Instituto Carlos Chagas – Fiocruz/PR

Prof. Dr. André Luiz Martinez de Oliveira
Universidade Estadual de Londrina – UEL

Prof. Dr. Fernando Gomes Barcellos
Universidade Estadual de Londrina – UEL

Londrina, 29 de abril de 2016.

Aos meus pais, irmãos, sobrinhos e esposo.

Dedico.

AGRADECIMENTOS

À Deus pelo cuidado, constante presença em minha vida e por me sustentar até aqui.

À Prof[a]. Dra. Maria Helena P. Fungaro, pela orientação, oportunidade de aprendizado e conhecimentos que foram imprescindíveis para a realização deste trabalho.

À Dra. Diva Souza Andrade, pela co-orientação, há dez anos tive a oportunidade de iniciar minha carreira ao seu lado, e se cheguei até aqui foi graças ao seu investimento em mim.

À Universidade Estadual de Londrina, ao Programa de Pós Graduação em Genética e Biologia Molecular, especialmente à Profa. Dra. Ana Lúcia Dias, e a todos os professores pelos muitos ensinamentos transmitidos.

Ao Instituto Agronômico do Paraná (IAPAR) e ao Projeto Xisto Agrícola pela disponibilização de dados e estrutura para a realização das atividades.

À Petrobras, Projeto Xisto Agrícola e a Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), pelo apoio financeiro e concessão da bolsa durante o período do curso de Pós-Graduação em Genética e Biologia Molecular.

À minha família, Everaldo, Helzira, Haline, Everaldo Jr, Marcos Henrique, Thiago Hideo, Marcos Hideo, Isaac e Severine, pelo amor, pelas orações e incentivos não apenas neste trabalho, mas em todas as etapas da minha vida.

Ao meu esposo, Luiz Rafael de Almeida Olivieri, pelo amor, cuidados e incentivos que me ajudaram a prosseguir.

As amigas, Gisele Milani Lovato, Nahyara F. P Cezário, Ana Paula Andrade, Maria Aparecida Matos, Fernanda P. Massi, e a Josué J. Silva pelo apoio e ajuda em momentos cruciais desta jornada.

Aos funcionários, colaboradores e estagiários do Laboratório de Microbiologia do Solo do IAPAR, que diretamente ou indiretamente contribuíram para a realização e condução dos projetos.

Finalmente, agradeço a todos que de alguma forma, corroboraram para a concretização deste trabalho.

Muito Obrigada!

GOES, Kelly Campos Guerra Pinheiro de. **Caracterização de microrganismos isolados do xisto pirobetuminoso da área de mineração em São Mateus do Sul, Paraná. 2016**. 111 f. Tese (Doutorado em Genética e Biologia Molecular) - Universidade Estadual de Londrina, Londrina, 2016.

RESUMO

Os finos de xisto (FX) e o xisto retortado (XR) são coprodutos sólidos gerados respectivamente, pela mineração e pela retortagem do xisto pirobetuminoso. Em função da produção de FX e XR em grande quantidade durante o processo de extração do óleo e gás de xisto, há interesse em fazer uso desses coprodutos como, por exemplo, na formulação de fertilizantes e como veículos de inoculantes sólidos. A identificação das comunidades microbianas presentes nos coprodutos do xisto pode fornecer informações sobre os microrganismos a serem incorporados em áreas cultiváveis, além de revelar isolados com potencial biotecnológico. Este trabalho teve como objetivo isolar representantes da comunidade microbiana, identificar e caracterizar por técnicas morfofisiológicas, bioquímicas e moleculares os microrganismos do FX e XR. O plaqueamento dos coprodutos permitiu isolar 40 bactérias, duas leveduras e 56 fungos filamentosos. As bactérias identificadas pertencem aos gêneros, *Arthrobacter, Bacillus*, *Paenibacillus, Ralstonia*, *Serratia, Sphigomonas*, *Terrabacter* e *Xanthobacter*. Dentre os isolados fúngicos foram identificados os gêneros *Acidiella, Aspergillus*, *Cladosporium*, *Penicillium*, *Talaromyces*, *Ochroconis*, *Trichoderma* e *Pseudozyma* pelo sequenciamento da região ITS1-5,8S-ITS2. Algumas estirpes bacterianas isoladas do FX e XR apresentaram potencial como promotoras de crescimento de plantas em *Brachiaria ruziziensis* utilizando os coprodutos como veículos inoculantes. Esse estudo fornece o primeiro panorama sobre a ocorrência de microrganismos cultiváveis presentes nos coprodutos do xisto da Formação Irati, Paraná, Brasil.

Palavras-chave: Fino de xisto. Xisto retortado. Identificação molecular. BPCP.

GOES, Kelly Campos Guerra Pinheiro de. **Characterization of microorganisms isolated from pirobetumen shale from mining area in São Mateus do Sul, Paraná. 2016**. 111 pages. Thesis (Doctoral Degree in Genetics and Molecular Biology) – Universidade Estadual de Londrina, Londrina, 2016.

ABSTRACT

Fine shale (FS) and retorted shale (RS) are solid byproducts generated by mining and retort of oil shale, respectively. FS and RS are generated in large quantities during the process of oil and gas extraction from oil shale, there is interest using these byproducts in the formulation of fertilizers and as a solid vehicle to inoculant. The identification of microbial community present in byproducts can provide information about the microorganisms that can be incorporated into cultivable areas, and to obtain bacterial isolates with biotechnological potential. This work aimed to isolate, identify and characterize by morphophysiology, biochemical and molecular techniques microorganisms from FS and RS. The plating of byproducts allowed isolate 40 bacteria, two yeasts and 56 filamentous fungi. These bacteria were identified as belonging to genera, *Arthrobacter, Bacillus*, *Paenibacillus, Ralstonia*, *Serratia, Sphigomonas*, *Terrabacter* and *Xanthobacter*. Among fungi isolates, were identified the genera *Acidiella, Aspergillus*, *Cladosporium*, *Penicillium*, *Talaromyces*, *Ochroconis*, *Trichoderma* and the yeast *Pseudozyma* by ITS1-5,8S-ITS2 region sequence. Some bacterial strains isolated from FS and RS showed potential as plant-growth promoting in *Brachiaria ruziziensis* using the byproducts as vehicle to inoculant. This study provides the first overview about the occurrence of cultivable microorganisms present in shale byproducts from Irati Formation. The data provided information for future application of these microorganisms.

Key-words: Fine shale. Retorted shale. Molecular identification. PGPB.

LISTA DE FIGURAS

Figura 1 - Imagem da área em processo de mineração na jazida de São Mateus do Sul/PR, SIX-Petrobras. 19

Figura 2 - Amostras de finos do xisto (A) e de xisto retortado (B) coletados na área de mineração na Unidade de Negócios da Industrialização do Xisto.....20

LISTA DE SIGLAS E ABREVIATURAS

ARDRA – *Amplified Ribossomal DNA Restriction Analysis*
BPCP – Bactérias Promotoras do Crescimento de Plantas
CAS – cromoazurol
CTAB – brometo de cetil trimetil amônio
CYA – Czapek *Yeast Autolyzate agar*
Embrapa – mpresa Brasileira de Pesquisa Agropecuária
FX – fino do xisto
GLP – Gás liquefeito de Petróleo
IAPAR – nstituto Agronômico do Paraná
ITAL – Instituto de Tecnologia de Alimentos ITAL
ITS – espaçador interno transcrito (*Internal Transcribed Spacer*)
NCBI – *National Center for Biotechnology Information*
PDA – *potato dextrose agar*
Petrobras – Petróleo Brasileiro S.A.
RDP – *Ribossomal Database Project*
REP – *Repetitive Extragenic Palindromic*
SIX – Unidade de Industrialização do Xisto
UPGMA – *Unweighted Pair-Group Method with Arithmetical Mean*
XR – xisto retortado

SUMÁRIO

1 INTRODUÇÃO 14

2 REVISÃO BIBLIOGRÁFICA 16

2.1 Xisto e coprodutos da mineração 16

2.2 Uso agrícola dos coprodutos do xisto 21

2.3 Microrganismos como promotores de crescimento de plantas 24

2.4 Xisto como fonte de microrganismos com potencial biotecnológico 26

2.5 Identificação molecular de microrganismos do xisto 30

3 OBJETIVOS 34

4 REFERÊNCIAS 35

5 RESULTADOS E DISCUSSÃO 43

5.1 Artigo I: Fungi colonizing Brazilian oil shale byproducts: fine shale and retorted shale 44

5.1.1 Abstract 44

5.1.2 Introduction 45

5.1.3 Material and methods 47

5.1.3.1 Site and sampling description 47

5.1.3.2 Chemical analyses 47

5.1.3.3 Isolation of fungi 48

5.1.3.4 Morphological examination 48

5.1.3.5 Molecular examination 48

5.1.4 Results and Discussion 50

5.1.5 References 56

5.1.7 Figures 64

5.2 Artigo II: Occurrence of bacteria in fine shale and retorted shale byproducts from a commercial oil shale mine in Brazil 69

5.2.1 Abstract 69

5.2.2 Introduction 70

5.2.3 Material and methods 71

5.2.3.1 Site sampling 71

5.2.3.2 Isolation of bacteria 71

5.2.3.3 Molecular characterization 72

5.2.4 Results and discussion 73

5.2.5 References 80

5.2.6 Tables 84

5.2.7 Figure legends 86

5.2.8 Figures 87

5.3 Artigo III: Bacteria isolated from shale byproducts can enhance *Brachiaria ruziziensis* growth 91

5.3.1 Abstract 91

5.3.2 Introduction 92

5.3.3 Material and methods 93

5.3.3.1 Sampling and chemical characterization 93

5.3.3.2 Bacterial isolation 94

5.3.3.3 Biochemical characterization 95

5.3.3.4 Bacterial survival in shale byproducts 96

5.3.3.5 Effects of PGPB strains inoculation in *Brachiaria ruziziensis* 96

5.3.4 Results.....98

5.3.4.1 Chemical characteristics 98

5.3.4.2 Culturable bacteria isolated from shale byproducts 98

5.3.4.3 Biochemical characterization 99

5.3.4.4 Bacterial survival test in shale byproducts.....99

5.3.4.5 Effects of bacteria inoculation on *Brachiaria ruziziensis* growth ..100

5.3.5 Discussion 101

5.3.6 References 102

5.3.7 Tables 107

6 CONSIDERAÇÕES FINAIS 111

1 INTRODUÇÃO

A unidade de Industrialização do Xisto (SIX) pertencente ao sistema Petrobras, está localizada em São Mateus do Sul - PR sobre uma das maiores reservas mundiais de folhelho pirobetuminoso (xisto), a Formação Irati. Os afloramentos de xisto dessa formação cobrem uma área de cerca de 2.000 km^2 de extensão e desde 1972 a Petrobras vem explorando no Paraná essa reserva de onde são extraídos diariamente toneladas de xisto, por meio do processo Petrosix®, para produção de óleo combustível, gás liquefeito, gás de xisto, enxofre entre outros produtos. O método mais utilizado para separar o óleo do xisto é a retortagem ou pirólise, um processo de aquecimento que converte a matéria orgânica do xisto, o querogênio, em óleo e gás. Durante a mineração do xisto e após o processo de pirólise da rocha, são gerados coprodutos sólidos e líquidos, entre eles estão: a água de xisto, o calxisto, o calcário de xisto, as cinzas de xisto, os finos de xisto e o xisto retortado. Alguns desses materiais apresentam potencial para uso na agricultura e despertaram grande interesse para serem utilizados como matéria-prima para a formulação de novos insumos para a agricultura brasileira.

A alta demanda nacional por insumos agrícolas, especialmente os fertilizantes, aliada à elevada disponibilidade de coprodutos do processamento do xisto, induziu o estabelecimento de um termo de cooperação entre o Instituto Agronômico do Paraná (IAPAR) e a Petrobras. Esta parceria teve como objetivo a condução de pesquisas científicas acerca do uso desses coprodutos líquidos e sólidos como matérias-primas na formulação de fertilizantes sólidos, líquidos e como veículos inoculantes de microrganismos. Dentro deste convênio, o Projeto Xisto Agrícola possibilitou o desenvolvimento de pesquisas visando não apenas à obtenção de novos insumos para a agricultura, mas também consistiu em avaliar e garantir a qualidade, a segurança ambiental e alimentar da formulação destes novos produtos à base dos coprodutos do xisto para uso na agricultura. As avaliações dos insumos à base de xisto já resultaram na liberação, pelo Instituto Ambiental do Paraná, da água de xisto na formulação de fertilizantes foliares e do calcário de xisto como corretivo de acidez do solo e como fertilizante agrícola. Dentre os coprodutos sólidos, os finos de xisto e o xisto retortado também podem ser aproveitados por segmentos industriais e agrícolas. Os finos de xisto podem ser utilizados na

agricultura como substrato para produção de mudas, como matéria-prima para formulações de fertilizantes e inoculantes sólidos. O xisto retortado pode ser utilizado para a produção de fertilizantes sólidos, pois é fonte de nutrientes como enxofre, potássio, silício entre outros, podendo servir também como veículo para inoculante microbiano.

A possibilidade de usar os coprodutos do xisto em áreas cultiváveis traz a necessidade de identificar as comunidades microbianas presentes em cada um desses coprodutos, e avaliar os impactos positivos e/ou negativos que estes podem exercer sobre as culturas e solos onde serão incorporados. Além da importância agrícola, a identificação destas comunidades poderá revelar microrganismos de importância biotecnológica. Considerando a inexistência de dados na literatura sobre a comunidade de archaeas, bactérias e fungos presentes nos coprodutos do xisto da Formação Irati, este trabalho teve como proposta realizar o isolamento, identificação e caracterização destes microrganismos em amostras de xisto retortado e finos do xisto. A identificação da comunidade de microrganismos geraram dados pioneiros sobre a microbiota dos coprodutos, sendo possível sugerir como os microrganismos isolados atuam sobre os ciclos biogeoquímicos dos nutrientes presentes no xisto. Os dados ainda contribuem para a melhoria dos coprodutos como insumo agrícola, por exemplo, pela biolixiviação microbiana através da reinoculação de microrganismos isolados, para a disponibilização de macro e micronutrientes complexados no xisto, além da avaliação do potencial de promoção de crescimento de plantas por alguns isolados bacterianos obtidos nesse estudo.

2 REVISÃO BIBLIOGRÁFICA

2.1 Xisto e coprodutos da mineração

Folhelho pirobetuminoso é uma rocha sedimentar, compacta e laminar, com alto teor de matéria orgânica (querogênio), e que, quando submetido a altas temperaturas se decompõe produzindo óleo e gás combustível (BRENDOW, 2003; DYNI, 2006). Comumente conhecido como xisto, essa rocha possui coloração cinza escura, marrom ou preta, e apresenta hidrocarbonetos disseminados em seu meio mineral (NICOLINI et al., 2011; PEREIRA; VITTI; KORNDORFER, 2003). O xisto é um mineral pouco poroso, a água encontrada em sua matriz mineral é superficial e seu teor de umidade é baixo, variando de 3 a 6% (PIMENTEL, 2001; PORTO, 2005).

O querogênio, uma matéria orgânica insolúvel, é a principal forma de carbono orgânico presente no xisto, sendo de extrema complexidade e sólido à temperatura ambiente (AMBLES; BAUDET; JACQUESY, 1993). Resulta da fossilização de matéria orgânica, principalmente algas sedimentadas em ambientes aquáticos, e que com a ação do tempo, temperatura e pressão, foram transformadas em hidrocarbonetos com estruturas macromoleculares complexas (EIS, 2012). Esse composto químico varia em quantidade e qualidade dependendo da formação da rocha, porém é uniforme para as rochas de um mesmo depósito (ALMEIDA, 2005).

Os depósitos de xisto datam de diferentes eras geológicas, foram formados em uma variedade de ambientes e podem ocorrer como acumulações menores de pouco ou nenhum valor econômico, ou como depósitos gigantes que ocupam milhares de quilometros quadrados. As maiores formações de xisto se encontram na Austrália, Brasil, Canadá, China, Estados Unidos, Estônia, Itália, Jordânia, Marrocos, Rússia e Zaire, sendo a Formação Green River no oeste dos Estados Unidos o maior depósito conhecido até o momento (DYNI, 2006). Muitos depósitos não foram suficientemente explorados para obtenção de estimativas precisas e alguns ainda não foram incluídos em levantamentos sobre a quantidade de óleo. Entretanto, estima-se que a quantidade de óleo nesses depósitos, como

reserva potencial, é muitas vezes superior à obtida em poços de petróleo (GOLUBEV, 2003).

O interesse pela potencialidade do xisto como fonte de energia é antigo. Já no final do século XVIII, nos Estados Unidos, cerca de 200 instalações extraíam querosene e óleo desta rocha. Findlay e colaboradores (1974) já relatavam que o xisto betuminoso era fonte de energia de grande potencial, e que as estimativas da abundância de energia fóssil nestas rochas indicavam ser muito maiores do que os depósitos de petróleo dos Estados Unidos.

No Brasil, as reservas de xisto estão bem distribuídas e ao todo foram identificadas 18 reservas no território nacional, das quais 14 podem ser geradoras de petróleo (EIA - U.S. ENERGY, 2015a; SANTOS, 2010). A Formação Irati, a segunda maior reserva brasileira de xisto, está localizada parcialmente na cidade de São Mateus do Sul (PR). Com cerca de 250 milhões de anos, é uma das melhores e mais importantes reservas de xisto brasileira do ponto de vista geológico e econômico, abrangendo os estados de São Paulo, Paraná, Santa Catarina, Rio Grande do Sul, Mato Grosso do Sul e Goiás (NICOLINI et al., 2011). Esta reserva tem potencial para produzir 700 milhões de barris de óleo, 9 milhões de toneladas de GLP, 25 bilhões de m^3 de gás de xisto e 18 milhões de toneladas de enxofre (PIMENTEL et al., 2010).

A Petrobras iniciou em 1954 a exploração de xisto no município de Tremembé no Vale do Paraíba (SP) e em 1959 a empresa construiu a usina de São Mateus do Sul (PR). O processamento da rocha se consolidou com a tecnologia Petrosix®, desenvolvida e completada em dezembro de 1991, quando entrou em operação o primeiro Módulo Industrial da Petrobras em São Mateus do Sul (PEREIRA; VITTI, 2004; TONEL, 2004). Embora a industrialização de xisto seja uma atividade já desenvolvida há décadas, tem sido mantida em um plano secundário devido às vantagens econômicas e tecnológicas apresentadas pela indústria petrolífera (SANTOS; MATAI, 2010).

O processo Petrosix® é mais caro que o de produção de petróleo, mesmo assim, o Brasil investe na exploração, extração e comercialização de óleo e coprodutos do xisto (SANTOS; MATAI, 2010). Em 2014, o volume de xisto bruto

processado na jazida de São Mateus do Sul foi de 1,7 milhão de m^3; a produção de gás somou 8,3 mil toneladas e o volume de óleo combustível obtido a partir do processamento do xisto atingiram 237, 96 mil m^3 (AGÊNCIA NACIONAL DO PETRÓLEO, 2015).

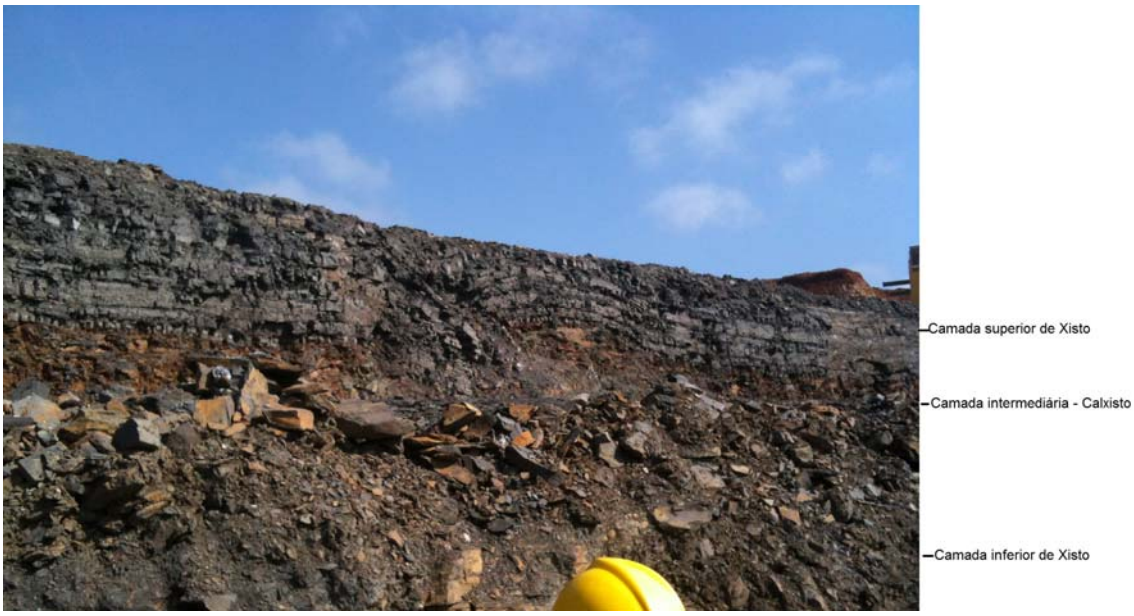
Cinco países no mundo, o Brasil, a Austrália (COOK et al., 2013), a China (JIANG; HAN; CUI, 2007), os Estados Unidos (EIA - U.S. ENERGY, 2015a) e a Estônia (VALGMA et al., 2010) possuem em atividade unidades que fazem o aproveitamento comercial do xisto para obtenção de óleo, gás e produção de energia através de termoelétricas. A produção mundial de derivados de xisto tem aumentado, a exemplo os Estados Unidos, onde sete áreas de produção de gás de xisto contribuíram para um crescimento de 92% da produção nacional de óleo e gás natural de uso doméstico durante os anos de 2011 a 2014, principalmente devido aos avanços nas tecnologias de perfuração (EIA - U.S. ENERGY, 2015b, 2016).

Dentre esses países, o Brasil se destaca como um dos países com tecnologia avançada e de alto rendimento de produção de óleo (PAN et al., 2012). Diversos produtos podem ser obtidos pelo processamento do xisto, tais como: gás combustível industrial, gás liquefeito, nafta e enxofre, além de vários insumos para asfalto e coprodutos que podem ser utilizados nas indústrias cimenteiras, agrícola e de cerâmica (PETROBRAS, 2016; PORTO, 2005). Diariamente no Brasil são extraídas aproximadamente 7.800 toneladas de xisto e em consequência desta atividade, há geração de coprodutos, principalmente o xisto retortado, um rejeito sólido que representa de 80 a 90% da matéria prima usada na produção (SANTOS; MATAI, 2010).

A Petrobras concentra suas operações de mineração e extração de xisto em São Mateus do Sul (PR), onde o minério é encontrado em duas camadas: uma superior e outra inferior, que apresentam teores médios de óleo de 6,4% e 11%, respectivamente (PORTO, 2005). A Figura 01 mostra uma imagem da área de mineração na Petrobras-SIX, onde é possível observar a disposição das camadas de xisto superior e inferior, além da camada intermediária formada por calcário (calxisto). A mineração é feita a céu aberto com lavra em tiras paralelas, removendo-se a vegetação, o solo e a primeira camada de rocha, permitindo a retirada do xisto. O material retirado nas escavações (solo, argila, rocha) é recolocado nas áreas a

serem recuperadas, as quais inicialmente recebem uma camada de xisto retortado, finos do xisto e calcário de xisto para a restauração do relevo e posterior reflorestamento (SANTOS, 2009).

Figura 1 - Imagem da área em processo de mineração na jazida de São Mateus do Sul/PR, SIX-Petrobras.



Fonte: Goes, K. C. G. P (Setembro/2013).

No processo Petrosix® emprega-se uma retorta de leito móvel, no qual a rocha é pirolisada. No preparo da carga para alimentar a retorta, o xisto é britado para reduzir sua granulometria e peneirado para homogeneizar o tamanho das partículas. As partículas padronizadas são transportadas até a retorta onde ocorre o aquecimento (pirólise) a cerca de 500ºC, ocasionando a liberação de óleo, gases de pirólise e vapor de água. Cada fração é tratada separadamente tendo um destino específico para tratamento final.

Os finos do xisto são fragmentos da rocha de xisto, menores que 12 mm, originados em dois estágios dentro da mineração e do processamento do xisto: a) na mina, devido à fragmentação do minério através do desmonte do xisto com explosivos e manuseio com escavadeiras e b) no tratamento do xisto através de britagem e empilhamento do xisto antecedendo a produção do óleo. A quantidade de finos constitui cerca de 20% da carga não utilizável no processo Petrosix®, isso corresponde a 1.200 toneladas de xisto por dia, com teor médio de óleo de 8%. Esse material não é aproveitado por ser considerado pequeno demais para a produção de

óleo, gás e demais derivados, sendo britado em moinho de martelo, para tamanho de fragmentos menores do que 0,84 mm. Posteriormente a esse processo é realizado o peneiramento do material adequando sua natureza física à legislação para uso na agricultura (DOUMER et al., 2011).

O xisto retortado é originado após a extração do óleo e gás da rocha de xisto, constituído de um material rico em micronutrientes, hidrocarbonetos e outros elementos como fósforo, cálcio, magnésio, silício e enxofre (CHAVES; VASCONCELOS, 2006; MEDEIROS, 2013). Apresenta potencial para produção de energia, como adsorvente de íons de metais pesados de águas residuais (PIMENTEL et al., 2010) e para ser aplicado na agricultura (PEREIRA; VITTI, 2004). Após o processo de pirólise, o xisto retortado passa pelo processo de resfriamento. Parte deste material retorna para área minerada, recebendo uma camada de argila e solo vegetal para fins de reabilitação da área.

Figura 2 - Amostras de finos do xisto (A) e de xisto retortado (B) coletados na área de mineração na Unidade de Negócios da Industrialização do Xisto.

Fonte: Goes, K. C. G. P (Junho/2012).

Os coprodutos do xisto apresentam potencial para serem aplicados na agricultura devido à presença de micro e macronutrientes. Entretanto, esses nutrientes complexados precisam ser disponibilizados para as culturas. O processo de biolixiviação para disponibilização de nutrientes é um processo baseado na capacidade dos microrganismos (bactérias e fungos) em transformar compostos sólidos em elementos solúveis e extraíveis, que podem ser recuperados. Ela representa uma “tecnologia limpa” nas indústrias de mineração com baixo custo, em

comparação com os métodos convencionais que são caros para a recuperação de íons metálicos de minérios (DEVASIA; NATARAJAN, 2004).

2.2 Uso agrícola dos coprodutos do xisto

A utilização de resíduos originados de diferentes atividades industriais na agricultura como fonte de nutrientes é uma prática crescente e que pode contribuir para diminuir a dependência externa do Brasil por fertilizantes. O uso dos coprodutos da industrialização do xisto na agricultura como insumo agrícola/fertilizante é antigo e alguns estudos têm gerado informações sobre o efeito dessa prática na produção agrícola. Anjos e Reissmann (1996) realizaram estudos pioneiros sobre o uso dos coprodutos do xisto na agricultura. Os autores avaliaram a influência dos coprodutos, finos do xisto e xisto retortado, no crescimento e no teor foliar de cádmio (Cd), cobalto (Co) e cromo (Cr) em plantas de aveia preta (*Avena strigosa* Schreber). Os resultados indicaram que os finos do xisto contribuíram para o maior crescimento e produção de matéria seca das plantas, e foram observados teores alterados de Cd e Co nas folhas, porém em níveis não causadores de toxidez as plantas.

De acordo com Yoffe et al. (2002), os xistos betuminosos podem ser utilizados como fertilizantes por possuírem minerais e elementos que atuam como macro e micronutrientes de plantas. Pereira e colaboradores (2003) realizaram dois experimentos para avaliar o efeito de diferentes fontes de silício (xisto, escórias de siderurgia e termofosfato - rocha fosfatada com silicato de magnésio) no solo e na cultura do tomateiro. Como resultados foram observados que o tratamento com xisto apresentou aumento de Si nas plantas em contrapartida menores teores de Si no solo, e que as fontes aplicadas forneceram Si para o tomateiro, mas não foram suficientes para aumentar a produtividade.

Pereira e Vitti (2004) avaliaram o efeito do uso do xisto retortado em características químicas do solo e na nutrição do tomateiro em dois experimentos conduzidos em Argissolo Vermelho Amarelo, em casa de vegetação. Foram aplicadas e incorporadas ao solo as doses crescente de 0; 3; 6; 9 e 12 t ha^{-1} de xisto

retortado. Os resultados apresentados demonstraram que a adição de xisto aumentou significativamente os teores de enxofre e silício no solo e nas folhas do tomateiro. Aumentou a disponibilidade de fósforo, não sendo verificadas alterações nos valores de pH do solo. Também não houve diminuição da produtividade do tomateiro em relação a aplicação dos tratamentos. Segundo os autores, os resultados sugerem que o xisto retortado, nas doses utilizadas no referido estudo, apresentam potencial para uso na agricultura.

Chaves e Vasconcelos (2006) avaliaram alterações de atributos químicos do solo e do crescimento de plantas de milho pela aplicação de xisto retortado. Os experimentos foram conduzidos em casa de vegetação, com incorporação de cinco doses crescentes de xisto ao solo (0, 3, 6, 9 e 12 t ha^{-1}). Foram avaliados vários caracteres fenotípicos das plantas (altura, diâmetro do caule, matéria verde e seca) e análises químicas do solo. Os resultados apontaram que o xisto retortado aumentou a soma de bases trocáveis e os teores de enxofre e silício no solo, porém não aumentou os valores de pH do solo. As plantas de milho não responderam significativamente a aplicação de xisto, quanto aos parâmetros de crescimento vegetativo avaliados, apesar de ter havido pequenos acréscimos nos valores de diâmetro do caule e altura da planta.

Doumer et al., (2011) avaliaram o efeito da aplicação de doses crescentes de xisto retortado sobre características biológicas indicadoras da qualidade do solo. Foram realizados experimentos com aplicação de sete doses de xisto no solo em condições de laboratório e quatro doses em campo, combinadas à adubação mineral recomendada e avaliados em dois cultivos de feijão (*Phaseolus vulgaris* L.) em sistema de plantio direto. A aplicação de doses crescentes de xisto melhorou a atividade microbiológica do solo, sem causar variação no carbono da biomassa microbiana (CBM) e sem provocar impactos negativos sobre a atividade enzimática do solo. Os resultados obtidos da atividade de diferentes enzimas em condições de campo, aliados aos de CBM, e do teste ecotoxicológico em condições de laboratório, indicam que o uso do xisto retortado não provoca a degradação biológica do solo.

No Projeto Xisto Agrícola, o calcário de xisto, a água de xisto, finos de xisto e xisto retortado foram caracterizados quimicamente e avaliados como

insumos alternativos para uso na agricultura, quanto à eficiência agronômica, em diferentes culturas, tipos de solo e sistemas de produção. Os resultados do Projeto Xisto possibilitaram a liberação da água de xisto em 2008, para ser utilizada como matéria-prima para elaboração de fertilizantes foliares e o calcário de xisto em 2010, como corretor de acidez do solo e como fertilizante fornecedor de macronutrientes pelo Instituto Ambiental do Paraná – IAP (EMBRAPA, 2010).

Esses coprodutos têm ampla possibilidade de aplicações, por exemplo, podem ser utilizados como veículo na produção de inoculantes de *Rhizobium* para feijão e *Bradyrhizobium* para amendoim e soja, conforme pesquisas conduzidas no Instituto Agronômico do Paraná (IAPAR) em parceria com a Petrobras (ANDRADE, 2013).

Além dos coprodutos sólidos, o processamento do xisto gera a chamada água de xisto. Mehta et al. (2015) verificaram o efeito do tratamento de sementes e a aplicação foliar com água de xisto na indução de resistência sistêmica adquirida de soja a ferrugem da soja em casa de vegetação e no campo. Os resultados do tratamento de sementes com água xisto na casa de vegetação reduziram significativamente a severidade da ferrugem da soja. No campo o tratamento das sementes associado a uma aplicação foliar foi suficiente para induzir a resistência da planta contra a ferrugem da soja.

A microbiota do solo é a principal responsável pela decomposição, ciclagem de nutrientes e pelo fluxo de energia dentro do solo (DEBOSZ et al., 2002). Dados de literatura relacionados à presença de microrganismos nos coprodutos do xisto e os impactos da aplicação no solo sobre a comunidade microbiana são pouco conhecidos, apenas um estudo foi conduzido para avaliar o impacto sobre atributos biológicos do solo (DOUMER et al., 2011). A análise da diversidade de microrganismos cultiváveis nos coprodutos do xisto da Formação Irati leva a uma nova perspectiva microbiológica. O conhecimento da microbiota presente nos coprodutos do xisto permitiu avaliar a ocorrência dos microrganismos, quais gêneros estão presentes, o potencial desses na biolixiviação do xisto para disponibilização de nutrientes e a aplicação em novos processos biotecnológicos e uso agrícola.

2.3 Microrganismos como promotores de crescimento de plantas

As práticas agrícolas tradicionais estão atingindo seus limites de eficácia no aumento da produtividade, mas a biotecnologia microbiana tem oferecido eficientes alternativas que melhoram a sustentabilidade dos sistemas agrícolas existentes (MOSTTAFIZ; RAHMAN; RAHMAN, 2012). Dentre os principais objetivos, destaca-se a substituição de agroquímicos (fertilizantes minerais e defensivos agrícolas) por bioformulações.

Muitas práticas agrícolas afetam a qualidade do solo por alterações nas propriedades físicas, químicas e biológicas e podem levar a uma diminuição da população microbiana do solo e a diminuição da produtividade das culturas (VALARINI et al., 2002). O crescimento e a produtividade das plantas são fortemente influenciados pelas interações entre as raízes das plantas e o solo rizosférico, incluindo as populações microbianas do solo. A rizosfera (região onde o solo e as raízes das plantas se encontram) abriga microrganismos que podem ter efeitos positivos, negativos ou nenhum efeito visível no crescimento das plantas (PEREG; MCMILLAN, 2015). A população microbiana nativa da rizosfera de solos agrícolas é influenciada por práticas agrícolas, por exemplo, o cultivo do solo, a retenção ou queima de palha, a espécie de planta semeada, a cultivar e genótipo, bem como o tipo de solo (BERG; SMALLA, 2009; REEVE et al., 2010).

O fósforo e o nitrogênio são macronutrientes essenciais para o crescimento e desenvolvimento de plantas. Estes nutrientes existem naturalmente no solo, porém em formas não disponíveis para as plantas. Os microrganismos desempenham um papel importante na disponibilização e fornecimento desses nutrientes às culturas. O fungo *Penicillium bilaii*, por exemplo, ajuda a solubilizar o fosfato do solo pela produção de ácidos orgânicos, os quais dissolvem o fosfato de modo que as raízes possam absorvê-lo (MOSTTAFIZ; RAHMAN; RAHMAN, 2012). As bactérias do gênero *Rhizobium* são amplamente utilizadas como inoculante microbiano, e por meio do processo de fixação biológica do nitrogênio contribuem de forma parcial ou total para o fornecimento de nitrogênio às culturas da soja e feijão.

Nos últimos anos, estudos evidenciam as Bactérias Promotoras do Crescimento de Plantas - BPCP (*Plant Growth Promoting Bacteria*) como uma alternativa ao uso de agroquímicos, sendo utilizadas em formulações de inoculantes microbianos para promover o crescimento de plantas. Essas bactérias possuem diferentes maneiras de potencializar o desenvolvimento das plantas em diversas condições ambientais, que envolvem mecanismos relacionados à estrutura e formação do solo, à decomposição da matéria orgânica, à reciclagem de elementos essenciais, à solubilização de nutrientes minerais, à produção de vários reguladores de crescimento, degradação dos poluentes orgânicos, estimulação do crescimento radicular, controle biológico de patógenos de plantas e sementes do solo, entre outros (GUPTA et al., 2015; SIVASAKTHI; USHARANI; SARANRAJ, 2014).

As bioformulações, ou inoculantes, são definidas como produtos biologicamente ativos contendo uma ou mais estirpes microbianas benéficas para as plantas, de fácil uso e formuladas com um veículo econômico, que facilita a adesão das células microbianas às sementes. A maioria das bioformulações é destinada a aplicação no campo, e é essencial que a matéria prima utilizada como veículo seja apropriada para manter a viabilidade das células. Uma formulação de boa qualidade promove a sobrevivência das células mantendo uma população suficiente e disponível para realizar efeitos promotores do crescimento em plantas. Todavia, o sucesso e comercialização de BPCP dependem da articulação entre a comunidade científica e a indústria, além disso, requerem uma ampla demanda de mercado e viabilidade econômica, consistente espectro de ação, segurança e estabilidade, vida útil longa, baixos custos e fácil disponibilidade de matérias primas para produção (GUPTA et al., 2015).

As bioformulações podem ser produzidas a partir dos coprodutos do xisto, que apresentam potencial para serem utilizados como veículo para inoculantes microbianos. Os resultados obtidos no Projeto Xisto permitiram o depósito de uma patente utilizando finos de xisto e xisto retortado como veículos em bioformulações, com estirpes do gênero *Rhizobium*, indicadas para leguminosas como feijão, soja e amendoim. Aliadas a essas informações, a busca por potenciais BPCP entre os isolados obtidos dos coprodutos do xisto apresentam vantagens econômicas, já que esses microrganismos possuem maior capacidade de sobrevivência e de adaptação

às diferentes características químicas e físicas encontradas nos coprodutos, minimizando o tempo de desenvolvimento processual e potencializando a industrialização dos produtos inoculantes.

2.4 Xisto como fonte de microrganismos com potencial biotecnológico

Os microrganismos são essenciais para o funcionamento e sustentabilidade de todos os ecossistemas naturais. Existe em todo o mundo uma crescente procura por microrganismos com potencial biotecnológico, visando sua aplicação para a obtenção de produtos e melhoria de processos. Na agricultura, a aplicação de microrganismos para a diminuição do uso de insumos agrícolas e agroquímicos apresenta crescente interesse e demanda, impulsionando os estudos de bioprospecção.

Na literatura são relatados dados sobre microrganismos isolados de diferentes fontes e tipos de xisto, entretanto, não há informações até esta data sobre a microbiota presente nos coprodutos do xisto da Formação Irati no Brasil. Microrganismos incluindo algas, fungos, protozoários e bactérias, presentes no xisto de diversas partes do mundo, foram descritos, sendo os primeiros relatos sobre a presença de esporos de fungos e fragmentos de hifas no xisto da formação de Green River e ainda fósseis de fungos e algas (BRADLEY, 1931).

Nease e Wolf (1975) apresentaram os primeiros dados sobre fungos presentes em sedimento do xisto Chattanooga (Nashville, Tennessee, USA). Os sedimentos foram observados em microscópio, sendo revelados hifas, esporos e ascocarpos de fungos. Segundo os autores, não foi possível identificar precisamente os fungos fossilizados encontrados no xisto, entretanto as análises das amostras obtidas apontam que possivelmente os fósseis pertencem aos gêneros *Microascus*, *Aspergillus* e *Ustulina*.

Os primeiros relatos sobre microrganismos presentes em resíduos obtidos da exploração do xisto datam da década de 70 (FARRIER et al., 1977). DeVore (1980) isolou quatro leveduras e o fungo filamentoso *Scopulariopsis candida*, que foram capazes de crescer na água de xisto (água de retorta Omega-9). Gauger e Williams (1987) avaliaram o crescimento de bactérias em água de retorta

Omega-9 derivada de experimentos de processamento do xisto em Laramie, Wyoming, USA. A busca por bactérias e fungos nessa água de retorta Omega-9, objetivou o possível uso dos microrganismos para melhorar os parâmetros de qualidade da água como uma estratégia para retirar ou atenuar componentes causadores de riscos ambientais.

Os primeiros estudos sobre a biodegradação do xisto foram realizados por Findlay e colaboradores (1974). O objetivo do trabalho foi determinar se bactérias oxidantes de enxofre, *Thiobacillus thiooxidans* e *T. concretivorous* poderiam liberar o querogênio e o betume do xisto da Formação Green River. Os resultados demonstraram que até 40% do xisto podia ser solubilizado pela ação de bactérias oxidantes de enxofre.

Sztaba e Konopka (1994) apresentaram a possibilidade de aplicação de métodos microbiológicos para recuperação de metais dissipados no xisto, por exemplo, o zinco (Zn), chumbo (Pb), urânio (U), molibdênio (Mo), e vanádio (Va) utilizando uma estirpe de *Thiobacillus ferrooxidans* isolada do xisto. Após 7 meses de incubação, os autores observaram que foram recuperados do substrato xisto: 95% de Zn, 55% de Pb, 75% de U, 60% de Mo e 47% de Va. Aislabie e Atlas (1988) avaliaram a capacidade de microrganismos em remover nitrilos do óleo de xisto, utilizando a metodologia de enriquecimento do óleo de xisto, que foi utilizado como única fonte de nitrogênio em meio de cultura. As análises de cromatografia gasosa mostraram que as populações microbianas degradam nitrilos alifáticos, mas não os hidrocarbonetos alifáticos, hidrocarbonetos aromáticos, ou compostos heterocíclicos de nitrogênio encontrados neste óleo. Foram isoladas destes cultivos *Pseudomonas fluorescens* e *P. aeruginosa*, capazes de utilizar nitrilos como fontes de carbono e nitrogênio.

Dickey (1996) avaliou a origem de microrganismos em 16 amostras de xistos do subsolo e arenitos em Cerro Negro, Novo México. Neste estudo foi realizada a perfuração inclinada com a finalidade de testar a hipótese de que os microrganismos nos estratos do subsolo são nativos do xisto. Os microrganismos totais foram quantificados por contagem direta em microscópio, e os heterotróficos aeróbios cultiváveis foram enumerados por contagem em placa. Nas amostras de xisto, foram encontradas 87 UFC g^{-1} (unidades formadoras de colônia) e 37 UFC g^{-1}

nas rochas de White Water e Clay Mesa, respectivamente. As contagens em placas demostraram que 63% das amostras de xisto apresentaram contagens de UFC abaixo do limite de detecção.

Bactérias com diferentes mecanismos de ação metabólica têm sido isoladas de diversas fontes de xisto. Uma grande variedade de microrganismos que usa matéria orgânica do xisto foi descrita (KRUMHOLZ et al., 1997; PETSCH; EGLINGTON; EDWARDS, 2001; WENGEL et al., 2006). Petsch et al., (2001), demostraram que os microrganismos podem assimilar a matéria orgânica durante o intemperismo do xisto. Durante experimentos de desmineralização do xisto de Aleksinac (Sérvia), Dragutinović e colaboradores (2012) isolaram uma nova estirpe de *Bacillus circulans* (estirpe VD01) que apresentou eficiência na dessilicificação bacteriana do xisto pirobetuminoso.

Petsch e colaboradores (2005) indicaram a ocorrência de diferentes rotas metabólicas de microrganismos que ocorrem nos minérios orgânicos de xisto preto. Nos diferentes tipos de xisto foi verificada a presença de microrganismos heterotróficos aeróbios e anaeróbios tais como bactérias sulfato-redutoras e fermentativas, quimioautótrofos, por exemplo, as bactérias oxidantes de enxofre e ferro, e *archea* metanogênica. Segundo os autores, os microrganismos capazes de utilizar o querogênio do xisto preto (Clay City, Kentucky, EUA), como sua única fonte de carbono e energia, foram identificados e pertenciam a cinco gêneros: *Acinetobacter*, *Pseudomonas*, *Dechloromonas*, *Comamonas* e *Clostridium*.

A degradação da matéria orgânica de xisto preto de Ronnburg (Alemanha) pelo basidiomiceto *Schizophyllum commune* foi estudada por Wengel et al. (2006). Os autores observaram que a degradação do carbono presente no xisto pode ser acelerada pela atividade fúngica. Na França, o projeto “BIOSHALE” teve por objetivo realizar a bioprospecção de minérios de xisto preto da Europa para a seleção de sistemas microbianos eficientes para a biolixiviação de metais nobres dos folhelhos pretos. Os autores buscaram isolar microrganismos com habilidade de degradar a matéria orgânica nos depósitos do xisto. Dados preliminares apontaram que microrganismos heterotróficos, bem como quimiolitotróficos, foram capazes de lixiviar o cobre presente no xisto preto (NMP2-CT-2004-505710 / BIOSHALE, 2007).

Matlakowska e Sklodwska (2009) analisaram a população microbiana da mina de cobre Lubin (Polônia), que é cortada por uma camada de xisto e que apresenta elevado teor de minérios nobres, para elucidar potencial dos microrganismos em processos de lixiviação de metais. Foram isoladas oito bactérias de sedimentos minerais e do xisto preto. As análises filogenéticas baseadas na sequencia de nucleotídeos do gene 16S rRNA demonstraram que as cinco estirpes pertenciam ao filo Proteobacteria, classe ɣ-Proteobacteria (*Pseudomonas*, *Acinetobacter*), uma estirpe pertencia ao filo Firmicutes (*Bacillus*) e duas pertenciam ao filo Actinobacteria (*Microbacterium*). Os autores relatam que o cultivo convencional baseado em técnicas microbiológicas foi um método eficaz de isolamento de bactérias do xisto preto.

Moore e Cripps (2010) avaliaram se as temperaturas extremas (175 ºC) nos poços de fraturamento hidráulico, utilizados para obtenção de gás de xisto, na bacia do Rio Horn no Canadá, eram suficientes para eliminar microrganismos e assim diminuir a preocupação com a corrosão e acidificação microbiológica dos poços. Os autores avaliaram a presença de bactérias viáveis na área de produção de gás e na fonte de água utilizada no processo. Os resultados indicaram que a alta temperatura e as condições de alta pressão nos poços da região não são suficientes para eliminar bactérias introduzidas durante o processo de fratura dos poços e que estas bactérias sobreviventes permaneciam viáveis, se proliferando nos poços. Dentre os microrganismos identificadas nos poços foram encontradas: *Bacillus* gram negativos, cocóides gram positivos, diplococos gram negativos e as possíveis espécies, *Sphingomonas paucimobilis*, *Brevundimonas vesicularis*, *Pseudomonas alcaligenes* e *Aeromonas hydrophila*.

A biolixiviação dos coprodutos do xisto pode ser realizada a partir de microrganismos isolados do xisto, entretanto para tal, são necessários estudos preliminares que permitam responder, por exemplo: (i) quais microrganismos estão presentes nas amostras dos coprodutos do xisto? (ii) qual o papel que esses microrganismos desempenham? (iii) como a comunidade de microrganismos é alterada pelas condições químicas das amostras? (IV) como o metabolismo desses microrganismos atua sobre as características químicas dos coprodutos? Responder

a essas questões permite o entendimento da composição e função de comunidades microbianas do xisto.

2.5 Identificação molecular de microrganismos do xisto

Cada vez mais, abordagens genotípicas vêm sendo incorporadas aos protocolos de identificação microbianas. Dentre os inúmeros procedimentos que permitem a análise genotípica, o “DNA barcode” é um dos que mais tem contribuído para a identificação taxonômica. O “DNA barcode” consiste em utilizar pequenas sequencias DNA suficientemente variáveis entre espécies, porém conservadas entre os indivíduos de uma espécie. A identificação da espécie é feita pela comparação da sequencia de nucleotídeo obtida para o organismo de interesse com as sequencias depositadas em bancos de dados. Essa comparação é feita por meio do uso de uma ferramenta de alinhamento de sequencias, por exemplo, o BLAST - *Basic Local Alignment Search Tool* (ALTSCHUL et al., 1990) ou de um método de agrupamento, especialmente do tipo *Neighbor-joining* (SAITOU; NEI, 1987). Este tipo de análise tem contribuído para a identificação taxonômica de espécies microbianas presentes em diferentes tipos de amostras (CHUN; RAINEY, 2014; SCHOCH et al., 2012; SULTAN et al., 2012; YARZA et al., 2014).

Os genes ribossomais (rDNA) se tornaram padrões na determinação de relações filogenéticas de procariotos e eucariotos. A escolha desses genes decorreu do fato destes apresentarem todas as características necessárias a um marcador molecular ideal, pois são encontrados em todos os organismos procariotos e eucariotos, com estrutura e função conservadas entre os *taxa*, possuem regiões conservadas e variáveis e um tamanho grande o suficiente que permite o aparecimento de divergências na sequência (RASTOGI; SANI, 2011).

Os genes ribossomais de eucariotos são arranjados em clusters que se repetem em tandem. Cada cluster contém genes que codificam RNAs que compõem as subunidades menor (18S, 5,8S) e maior dos ribossomos (28S). Estes genes são separados por regiões denominadas “Internal Transcribed Spacer” (ITS1 e ITS2), as quais são transcritas e depois processadas para dar origem aos RNAs

ribossômicos maduros. Para o reino Fungi a região que compreende ITS1-5,8S-ITS2, frequentemente denominada apenas por ITS, foi formalmente proposta para “barcoding”. A região ITS de fungos é flanqueada por segmentos conservados, é relativamente curta (500 a 800 pares de bases), e aparece em grande número de cópias no genoma. Estas características permitem que a região ITS seja amplificada por meio da reação da polimerase em cadeia (PCR) e sequenciada com facilidade. Como consequência disso, é grande o número de sequências ITS de diferentes fungos que estão atualmente disponíveis nos bancos de dados de sequencias de nucleotídeos.

Para bactérias, o gene 16S rRNA é o mais utilizado como “barcode”. A análise deste gene tem sido particularmente importante para a identificação de bactérias com perfis fenotípicos incomuns, bactérias de crescimento lento e bactérias não cultiváveis. O gene 16S rRNA contêm nove regiões hipervariáveis flanqueadas por regiões relativamente conservadas (NEELAKANTA; SULTANA, 2013). É relativamente curto (1,5 Kb), portanto fácil e rápido de ser sequenciado quando comparado a outros genes bacterianos (WOO et al., 2008). Inúmeros estudos têm usado este marcador como “barcode” para analisar comunidades bacterianas de amostras ambientais (LENOBAH et al., 2014), inclusive do xisto (DRAGUTINOVIC et al., 2012; JIANG et al., 2016; MATLAKOWSKA; SKLODOWSKA, 2009; PETSCH; EDWARDS; EGLINTON, 2005; WUCHTER et al., 2013).

Petsch e colaboradores (2005) estudaram o papel dos microrganismos na utilização, oxidação da matéria orgânica e mineral de amostras de xisto preto expostas ao intemperismo próximos a Clay City nos Estados Unidos, e identificaram a comunidade de microrganismos presentes nesse ambiente. Os autores utilizaram as metodologias de enriquecimento das amostras de xisto, clonagem e análises de sequências de DNA ribossomal para identificação dos microrganismos. Os autores extraíram o DNA de culturas enriquecidas e amplificaram os genes 16S rRNA utilizando os primers universais 27f e 1492r (JIANG et al., 2006). No referido estudo foram identificadas bactérias pertencentes aos gêneros *Acinetobacter*, *Clostridium*, *Comamonas*, *Dechloromonas* e *Pseudomonas*

Matlakowska & Sklodowska (2009) isolaram e caracterizaram microrganismos da mina de cobre Lubin (Polônia) para a utilização destes na recuperação biotecnológica de metais de cobre do xisto preto. Oito estirpes bacterianas foram isoladas de minérios do xisto. Os autores utilizaram a metodologia de PCR de colônia para amplificar o gene 16S rRNA com os mesmos primers universais 27f e 1492r. As análises filogenéticas baseadas nas sequências do gene 16S rRNA mostraram que cinco estirpes pertenciam aos gêneros *Pseudomonas* e *Acinetobacter*, uma estirpe a *Bacillus* e duas estirpes a *Microbacterium*.

Dragutinović et al. (2012) identificaram uma estirpe de *Bacillus* isolada do xisto Aleksinac (Sérvia) e que apresentou uma alta eficiência na desmineralização do xisto. Os autores avaliaram as características bioquímicas, os padrões de proteínas, a composição de ácidos graxos, o perfil genômico e realizaram a amplificação e sequenciamento do gene 16S rRNA. Com base nas comparações com espécies conhecidas do gênero *Bacillus,* a estirpe foi identificada como uma estirpe de *Bacillus circulans* VD01.

A diversidade microbiana e a atividade metanogênica nas águas obtidas de poços recém-fraturados da formação de xisto Antrim, nos Estados Unidos foram estudadas por Wuchter e colaboradores (2013). Os autores sequenciaram a região V4 do gene 16S rDNA, amplificada com os primers universais 519f e 806r (ØVREÅS et al., 1997) utilizando a plataforma de sequenciamento Illumina MiSeq, para analisar a diversidade e a abundância da comunidade de procariotos presentes na água dos três poços fraturados utilizados para produção de gás. Os principais filos bacterianos encontrados foram Bacteroidetes, δ- e ε-Proteobacteria, o filo Firmicutes, e para Archaea o filo Euryarchaeota. As unidades taxonômicas operacionais mais representativas foram: *Marinilabilia salmonicolor*, *Cytophaga* sp., *Halanaerobium hydrogeniformans*, *H. congolense*, *H. cellulosilytica*, *Orenia marismortui* e *O. salinaria*. Os autores concluíram que a composição da comunidade microbiana nos poços podem diferir substancialmente devido a variações locais, às características do reservatório e aos procedimentos de produção de gás. Embora a relevância desses trabalhos deva ser considerada, a identificação da maior parte dos microrganismos ficou restrita a Filo.

Estudos sobre fungos em xistos pirobetuminosos são limitados, entretanto, recentemente o DNA “barcode” também vem sendo utilizado para a identificação de fungos presentes em amostras de xisto.

Jiang e colaboradores (2016) estudaram a diversidade de fungos das três maiores minas de xisto pirobetuminoso da China. Para tanto duas abordagem de análise de sequencias da região ITS foram utilizadas. A primeira utilizou bibliotecas de clonagem direta da região ITS. Dentre os 41 gêneros fúngicos identificados, *Penidiella, Epicoccum* e Cytospora foram os gêneros predominantes nas minas Maoming, Fushun e Huadian, respectivamente. A segunda abordagem foi baseada no isolamento e identificação de fungos cultiváveis nos xistos de cada mina. Os autores identificaram 15 gêneros entre os 150 isolados obtidos das três minas por meio do sequenciamento da região ITS, entre eles os gêneros: *Alternaria*, *Aspergillus*, *Bionectria*, *Candida*, *Cladosporium*, *Coprinopsis*, *Cryptococcus*, *Debaryomyces*, *Eucasphaeria*, *Eupenicillium*, *Nectria*, *Penicillium*, *Penidiella*, *Rhodotorula* e *Sporidiobolus*. *Penicillium* foi o único gênero entre os fungos cultiváveis encontrado nas três minas de xisto.

A identificação taxonômica dos isolados, a caracterização morfológica, bioquímica e molecular, são procedimentos essenciais para a obtenção de um panorama da microbiota dos coprodutos do xisto, para posterior seleção de potenciais microrganismos aplicáveis a estudos de biolixiviação, aplicações biotecnológicas e agronômicas.

3 OBJETIVOS

3.1 Objetivo Geral

O objetivo deste trabalho foi isolar, identificar e caracterizar microrganismos presentes nos finos do xisto e xisto retortado obtidos após a mineração e a pirolise do xisto pirobetuminoso.

3.2 Objetivos Específicos

Analisar as características químicas dos finos de xisto e xisto retortado.

Isolar bactérias, fungos filamentosos e leveduras de amostras dos coprodutos do xisto pirobetuminoso – xisto retortado e finos do xisto.

Identificar as espécies de bactérias, fungos filamentosos e leveduras de amostras dos coprodutos do xisto pirobetuminoso – xisto retortado e finos do xisto.

Avaliar o potencial de bactérias na disponibilização de nutrientes através de técnicas bioquímicas.

Caracterizar a similaridade genética dos isolados de bactérias, através da técnica de ARDRA.

Avaliar o potencial de bactérias isoladas do xisto como promotoras de crescimento de plantas no desenvolvimento vegetal da gramínea *Brachiaria ruziziense*.

4 REFERÊNCIAS

AGÊNCIA NACIONAL DO PETRÓLEO. Anuário estatístico brasileiro do petróleo, gás natural e biocombustíveis: 2015. **ANP 2008**, 2015.

AISLABIE, J.; ATLAS, R. M. Biodegradation of nitriles in shale oil. **Applied and Environmental Microbiology**, v. 54, n. 9, p. 2197–2202, 1988.

ALMEIDA, A. R. F. **Investigação do mecanismo de desvolatização de particulas de xisto**. p. 141. Universidade Estadual de Campinas, 2005.

ALTSCHUL, S. et al. Basic local alignment search tool. **Journal of molecular biology**, v. 215, n. 3, p. 403–410, 1990.

AMBLES, A.; BAUDET, N.; JACQUESY, J. C. Structural study of the kerogen from Brazillian Irati oil shale by selective degradations. **Tetrahedron Letters**, v. 34, n. 11, p. 1783–1786, 1993.

ANDRADE, D. S. **Processo de obtenção de veículo sólidos para inoculantes microbianos de fixadores biológicos de nitrogênio.** Brazil, 2013. Disponível em: <https://gru.inpi.gov.br/pePI/servlet/PatenteServletController?Action=detail&CodPedido=851793&SearchParameter=PI 1100001-5 A2>

ANJOS, A. R. M.; REISSMANN, C. B. Influência dos resíduos da mineração do xisto no crescimento e no teor foliar de Cd, Co e Cr em plantas de aveia preta. **Ciencia Rural**, v. 26, n. 1, p. 75–79, 1996.

BERG, G.; SMALLA, K. Plant species and soil type cooperatively shape the structure and function of microbial communities in the rhizosphere. **FEMS Microbiology Ecology**, v. 68, n. 1, p. 1–13, 2009.

BRADLEY, W. H. Origin and microfossils of the oil shale of the Green River formation of Colorado and Utah. **Geological Survey**, v. 168, p. 1–91, 1931.

BRENDOW, K. Global oil shale issues and perspectives. Synthesis of the Symposium on Oil Shale. 18–19 November, Tallinn. **Oil Shale. A Scientific-Technical Journal**, v. 20, n. 1, p. 81 – 92, 2003.

CHAVES, L. H. G.; VASCONCELOS, A. C. F. Alterações de atributos químicos do

solo e do crescimento de plantas de milho pela aplicação de xisto. **Revista Brasileira de Engenharia Agricola e Ambiental**, v. 10, n. 83, p. 84–88, 2006.

CHUN, J.; RAINEY, F. A. Integrating genomics into the taxonomy and systematics of the *Bacteria* and *Archaea*. **International Journal of Systematic and Evolutionary Microbiology**, v. 64, n. 2, p. 316–324, 2014.

COOK, P. et al. Engineering Energy: Unconventional Gas Production - A study of shale gas in Australia. **Report for the Australian Council of Learned Academics**, p. 252, 2013.

DAVIS, C. A. **Flora of the oil shale of the Green River Formation**. Thesis, 1915.

DEBOSZ, K. et al. Evaluating effects of sewage sludge and household compost on soil physical, chemical and microbiological properties. **Applied Soil Ecology**, v. 19, n. 3, p. 237–248, 2002.

DEVASIA, P.; NATARAJAN, K. Bacterial leaching. **Resonance**, v. 6, n. August, p. 27–34, 2004.

DEVORE, B. **Soil microfungal response to an oil shale retort water**. Thesis, University Wyoming, Laramie, WY, 1980.

DICKEY, C. S. **Origens of microorganisms in deep subsurface shales and sandstones at Cerro Negro, New Mexico**. Thesis. New Mexico Institute of the Mining and Technology, 1996.

DOUMER, M. E. et al. Atividade microbiana e enzimática em solo após a aplicação de xisto retortado. **Pesquisa Agropecuaria Brasileira**, v. 46, n. 11, p. 1538–1546, 2011.

DRAGUTINOVIC, V. et al. Characterisation of new *Bacillus circulans* strain isolated from oil shale. **Food Technology and Biotechnology**, v. 50, n. 1, p. 123–127, 2012.

DYNI, J. R. **Geology and resources of some world oil-shale deposits: U.S. Geological Survey Scientific Investigations Report 2005–5294**. 2006.

EIA - U.S. ENERGY. **Shale gas development in China aided by government investment and decreasing well cost**. 2015a. Disponível em:

<https://www.eia.gov/>. Acesso em: 20 jan. 2016.

EIA - U.S. ENERGY. **Quality Control of Oil Shale Production in Estonian Mines**. 2015b. Disponível em: <https://www.eia.gov/>. Acesso em: 15 fev. 2016.

EIA - U.S. ENERGY. **Drilling Productivity Report - For key tight oil and shale gas regions**. 2016. Disponível em: <https://www.eia.gov/>. Acesso em: 15 fev. 2016.

EIS, O.-O. S. & T. S. P. **About Oil Shale**. 2012. Disponível em: <http://ostseis.anl.gov/guide/oilshale/>. Acesso em: 11 fev. 2016.

EMBRAPA, E. B. DE P. A. Xisto agrícola. **Xisto agrícola**, n. 53, p. 2, 2010.

FARRIER, D. S. et al. Acquisition, processing and storage for environmental research of aqueous effluents derived from in situ oil shale processing. **Proc., 2nd Congress of Chemical Engineering**, p. 1031–1035, 1977.

FINDLEY, J.; APPLEMAN, M. D.; YEN, T. F. Degradation of oil shale by sulfur-oxidizing bacteria. **Applied Microbiology**, v. 28, n. 3, p. 460–464, 1974.

GAUGER, W. K.; WILLIAMS, S. E. Growth of bacteria in an oil shale retort water by indigenous microorganisms. **Bulletin of Environmental Contamination and Toxicology**, v. 38, p. 769–774, 1987.

GOLUBEV, N. Solid heat carrier technology for oil shale retorting. **Oil Shale**, v. 20, n. 3, p. 324–332, 2003.

GUPTA, G. et al. Plant Growth Promoting Rhizobacteria (PGPR): current and future prospects for development of sustainable agriculture. **Journal of Microbial & Biochemical Technology**, v. 07, n. 02, p. 96–102, 2015.

JIANG, H. et al. Microbial diversity in water and sediment of Lake Chaka, an athalassohaline lake in northwestern China. **Applied and Environmental Microbiology**, v. 72, n. 6, p. 3832–3845, 2006.

JIANG, S. et al. Fungal diversity in major oil-shale mines in China. **Journal of Environmental Sciences**, v. 41, p. 81–89, 2016.

JIANG, X. M.; HAN, X. X.; CUI, Z. G. New technology for the comprehensive

utilization of Chinese oil shale resources. **Energy**, v. 32, n. 5, p. 772–777, 2007.

KRUMHOLZ, L. R. et al. Confined subsurface microbial communities in Cretaceous rock. **Letters to Nature**, v. 386, n. 6, p. 64–66, 1997.

LENOBAH, D. E. et al. DNA barcoding on bacteria: A Review. **Advances in Biology**, v. 2014, p. 1–10, 2014.

MATLAKOWSKA, R.; SKLODOWSKA, A. The culturable bacteria isolated from organic-rich black shale potentially useful in biometallurgical procedures. **Journal of Applied Microbiology**, v. 107, n. 3, p. 858–866, 2009.

MEDEIROS, A. G. **Tecnologias de aproveitamento do xisto retortado**. Universidade Federal Rural do Semi-Árido, 2013.

MEHTA, Y. R.; MARANGONI, M. S.; MATOS, J. N.; MANDARINO, J. M. G.; GALBIERI, R. Systemic acquired resistance of soybean to soybean rust induced by shale water. **American Journal of Plant Sciences**, v. 06, n. 14, p. 2249–2256, 2015.

MOORE, S.; CRIPPS, C. **Bacterial survival in fractured shale gas wells of the horn river basin**. Northern Area Western Conference. Calgary, Alberta: 2010

MOSTTAFIZ, S. B.; RAHMAN, M.; RAHMAN, M. Biotechnology: Role of microbes in sustainable agriculture and environmental health. **Internet Journal of Microbiology**, v. 10, n. 1, p. 1–6, 2012.

NEASE, F. R.; WOLF, F. A. Fossilized fungi as evidence of continental drift. **Mycopathologia**, v. 57, n. 1, p. 15–18, 1975.

NEELAKANTA, G.; SULTANA, H. The use of metagenomic approaches to analyze changes in microbial communities. **Microbiology insights**, v. 6, p. 37–48, 2013.

NICOLINI, J. et al. Characterization of Brazilian oil shale byproducts planned for use as soil conditioners for food and agro-energy production. **Journal of Analytical and Applied Pyrolysis**, v. 90, n. 2, p. 112–117, 2011.

NMP2-CT-2004-505710 / BIOSHALE. **Publishable Activity Report of Bioshale Project**. 2007. Disponível em:

<http://bioshale.brgm.fr/Documents/ResultsProducts/Publishable_Activity_Report-Bioshale.pdf>.

ØVREÅS, L. et al. Distribution of bacterioplankton in meromictic lake Saelenvannet, as determined by denaturing gradient gel electrophoresis of PCR-amplified gene fragments coding for 16S rRNA. **Applied and Environmental Microbiology**, v. 63, n. 9, p. 3367–3373, 1997.

PAN, Y. I. et al. A Review on Technologies for oil shale surface retort. **Journal of the Chemical Society of Pakistan**, v. 34, n. 6, p. 1331–1338, 2012.

PEREG, L.; MCMILLAN, M. Scoping the potential uses of beneficial microorganisms for increasing productivity in cotton cropping systems. **Soil Biology and Biochemistry**, v. 80, p. 349–358, 2015.

PEREIRA, H. S.; VITTI, G. C. Efeito do uso do xisto em características químicas do solo e nutrição do tomateiro. **Horticultura Brasileira**, v. 22, n. 2, p. 317–321, 2004.

PEREIRA, H. S.; VITTI, G. C.; KORNDORFER, G. H. Comportamento de diferentes fontes de silício no solo e na cultura do tomateiro. **Revista Brasileira de Ciência do Solo,** v. 27, p. 3–10, 2003.

PETROBRAS. **Unidade de Industrialização do Xisto (SIX).** Disponível em: <http://www.petrobras.com.br/pt/nossas-atividades/principais-operacoes/refinarias/unidade-de-industrializacao-do-xisto-six.htm>. Acesso em: 24 jan. 2016.

PETSCH, S. T.; EDWARDS, K. J.; EGLINTON, T. I. Microbial transformations of organic matter in black shales and implications for global biogeochemical cycles. **Palaeogeography, Palaeoclimatology, Palaeoecology**, v. 219, n. 1-2, p. 157–170, 2005.

PETSCH, S. T.; EGLINGTON, T. I.; EDWARDS, K. J. 14C-dead living biomass: evidence for microbial assimilation of ancient organic carbon during shale weathering. **Science (New York, N.Y.)**, v. 292, n. 5519, p. 1127–1131, 2001.

PIMENTEL, P. M. et al. Caracterização e uso de xisto para adsorção de chumbo (II) em solução. **Cerâmica**, v. 52, p. 194–199, 2006.

PIMENTEL, P. M. et al. Characterization of retorted shale for use in heavy metal removal. **Applied Clay Science**, v. 48, n. 3, p. 375–378, 2010.

PIMENTEL, W. R. O. **Aplicação da cinética de primeira ordem ao processo de desvolatização de xisto**. 181 p. Mestrado em Engenharia Química, Engenharia de Processos. Faculdade de Engenharia Química, Universidade Estadual de Campinas, 2001.

PORTO, P. S. DA S. Investigações sobre a secagem de partículas de xisto. Tese de Doutorado - Faculdade de Engenharia Quimica, p. 198, 2005.

RASTOGI, G.; SANI, R. K. Molecular techniques to assess microbial community structure, function, and dynamics in the environment. In: **Microbes and Microbial Technology: Agricultural and Environmental Applications**. . p. 1–516, 2011.

REEVE, J. R. et al. Effects of soil type and farm management on soil ecological functional genes and microbial activities. **ISME Journal: Multidisciplinary Journal of Microbial Ecology**, v. 4, n. 9, p. 1099–1107, 2010.

SAITOU, N.; NEI, M. The neighbor joining method: A new method for reconstructing phylogenetic trees. **Molecular Biology and Evolution**, v. 4, n. 4, p. 406–425, 1987.

SANTOS, M. M. DOS. **Xisto: um estudo da viabilidade econômica para o Brasil**. Universidade de São Paulo (Escola Politécnica / Faculdade de Economia e Administração / Instituto de Eletrotécnica e Energia / Instituto de Física), 2010.

SANTOS, M. M. DOS; MATAI, P. H. L. D. S. A importância da industrialização do xisto brasileiro frente ao cenário energético mundial. **Revista Escola de Minas**, v. 63, n. 4, p. 673–678, 2010.

SANTOS, J. V. **Caracterização química e espectroscópica de solos de área recuperada após mineração de xisto**. Universidade Federal do Paraná, 2009.

SCHOCH, C. L. et al. Nuclear ribosomal internal transcribed spacer (ITS) region as a universal DNA barcode marker for Fungi. **Proceedings of the National Academy of Sciences of the United States of America**, v. 109, n. 16, p. 6241–6246, 2012.

SIVASAKTHI, S.; USHARANI, G.; SARANRAJ, P. Biocontrol potentiality of plant

growth promoting bacteria (PGPR) - *Pseudomonas fluorescens* and *Bacillus subtilis*: A review. **African Journal of Agricultural Research** , v. 9, n. 16, p. 1265–1277, 2014.

SULTAN, N. S. et al. 16s rDNA based identification of bacteria in the organophosphates treated agricultural soil. **International Journal of Applied Science and Engineering Research**, v. 1, n. 3, p. 212–223, 2012.

SZTABA, K.; KONOPKA, E. Possibilities of recovery of dispersed metals occurring in black shale using microorganisms. **Kwartalnik Geologiczny**, v. 38, n. 4, p. 701–707, 1994.

TONEL, G. **Processamento de xisto**. Universidade Federal de Santa Maria, 2004.

VALARINI, P. J. et al. Integrated evaluation of soil quality after the incorporation of organic matter and microorganisms. **Brazilian Journal of Microbiology**, v. 33, n. 1, p. 35–40, 2002.

VALGMA, I. et al. Quality control of oil shale production in estonian mines. **Oil Shale**, v. 27, n. 3, p. 239–249, 2010.

WENGEL, M. et al. Degradation of organic matter from black shales and charcoal by the wood-rotting fungus *Schizophyllum commune* and release of DOC and heavy metals in the aqueous phase. **Science of the Total Environment**, v. 367, n. 1, p. 383–393, 2006.

WOO, P. C. Y. et al. Then and now: use of 16S rDNA gene sequencing for bacterial identification and discovery of novel bacteria in clinical microbiology laboratories. **Clinical Microbiology and Infection**, v. 14, n. 10, p. 908–934, 2008.

WUCHTER, C. et al. Microbial diversity and methanogenic activity of antrim shale formation waters from recently fractured wells. **Frontiers in Microbiology**, v. 4, p. 1–14, 2013.

YARZA, P. et al. Uniting the classification of cultured and uncultured bacteria and archaea using 16S rRNA gene sequences. **Nature Reviews. Microbiology**, v. 12, n. 9, p. 635–645, 2014.

YOFFE, O. et al. The chemistry and mineralogy of the Negev oil shale ashes. **Fuel**, v. 81, n. 9, p. 1101–1117, 2002.

5 RESULTADOS E DISCUSSÃO

Os resultados e discussão desta tese são apresentados na forma de três artigos científicos que seguem.

5.1 Artigo I: Fungi colonizing Brazilian oil shale byproducts: fine shale and retorted shale

5.1.1 Abstract

Fine shale particles are small fragments of mined shale rock which are not usable for producing oil and gas, while retorted shale are generated by the retorting process. These byproducts are high in silicon and also contain organic matter, micronutrients, hydrocarbons and other elements. The aim of this study was to isolate and identify fungal species present in oil shale byproducts generated by oil shale industrialization in Brazil using *Petrosix® Technology.* We analyzed two samples of fine shale particles and two samples of retorted shale collected at Schist Industrialization Business Unit (Six) – Petrobras, in São Mateus do Sul, State of Paraná, Brazil. A total of eight fungal genera were found, including *Acidiella*, *Aspergillus*, *Cladosporium*, *Ochroconis*, *Penicillium*, *Pseudozyma*, *Talaromyces* and *Trichoderma*. *Acidiella* was the most predominant genus found in the samples of fine shale particles, which is a highly acidic substrate. *Talaromyces* was the most predominant genus in retorted shale samples. *Talaromyces sayulitensis* was the species most frequently found in retorted shale, and *Acidiella bohemica* in fine shale particles.

Keywords: Shale, oil shale, Petrosix, *Talaromyces sayulitensis, Acidiella bohemica*

5.1.2 Introduction

Oil shale is a compact, laminated rock of sedimentary origin, rich in kerogen (insoluble macromolecular organic matter), which is of great value as source of oil and natural gas. There are vast deposits of oil shale around the world, but the most significant deposits are found in the United States, Russia, Brazil, Estonia and China (1). In Brazil, the largest shale deposit is the "Irati Formation" which extends over the states of São Paulo, Paraná, Santa Catarina, Rio Grande do Sul, Mato Grosso do Sul and Goiás. So far, retorting is the main process used to produce oil from shale (2), and the main requirement for successfully utilizing oil shale as a source of liquid and gaseous fuels is a technically and economically feasible retorting process to thermally decompose the kerogen into oil, gas and carbonaceous residue (3).

In 1972, the Petrobras, a Brazilian state oil company, started oil shale processing activities by developing Petrosix® technology for extracting oil from the shale of the Irati Formation in São Mateus do Sul, Paraná. Petrosix® technology is an above-ground retorting process in which mined oil shale is reduced to particles of 10-75 mm and then conveyed to a retorter, where the shale is heated up to around 500 °C, breaking down the kerogen to yield oil vapor and gas (4). Nowadays, the Petrobras Petrosix® plant produces 2.9 thousand barrels per day (http://www.investidorpetrobras.com.br/pt/relatorios-anuais/form-20f) and generates a large volume of waste, mainly consisting of fine shale particles (FS) and retorted shale (RS).

FS consist of small fragments of mined shale rock (particle size < 6.35 mm) which are not usable in oil and gas processing (5). RS is retorting residues with high

silicon content and also containing organic matter, micronutrients, hydrocarbons and other elements such as phosphorus, calcium, magnesium and sulfur (6) [https://books.google.com.br/books?id=z6I7AAAAMAAJ]. At the São Mateus do Sul plant, some 7000 metric tons of shale are removed from the ground daily and the high availability of oil shale waste led to the creation of the "Agricultural Shale Project". This project aims to use shale water, shale limestone, FS and RS as feedstock in the formulation of agricultural inputs. Under this project's scope, our research group has been studied the efficacy and safety of using Brazilian oil shale byproducts as agricultural inputs, and currently our efforts are focused on the mycobiota inhabiting FS and RS.

Some studies on mineral-microbe interactions have concentrated on bacteria and archaea (7, 8), but fungi have, to some extent, been neglected (9). Pfister *et al.* (10), found several fungi living on the surface of oil-shale particles in the deposits of New Albany, US, and more recently Jiang *et al.* (11) reported finding fungi species in major oil shale mines in China.

Against this backdrop, the aim of this study was to isolate and identify fungal species present in FS and RS generated by oil shale industrialization in Brazil using Petrosix® Technology. The results will provide useful knowledge about fungal resources for the bio-utilization of oil shale and bioremediation of the byproducts generated by Petrosix® oil shale industrialization in Brazil.

5.1.3 Material and methods

5.1.3.1 Site and sampling description

Two samples of FS and two samples of RS were collected at the Schist Industrialization Business Unit (Six) – Petrobras, in São Mateus do Sul, Paraná - Brazil (25° 52' 26" S, 50° 22' 58" W) (Fig. 1). The samples were collected at twelve discrete locations in storage piles, and a composite sample (FS or RS) of approximately 1.0 kg was derived after homogenization.

Samples were place in sealed, sterilized polyethylene bags and kept at 27 ± 4 °C ready for chemical and microbial analysis at the Soil Microbiology Laboratory of the Agronomic Institute of Paraná (IAPAR).

5.1.3.2 Chemical analyses

FS and RS samples were oven dried at 55 °C and sieved to <2 mm before chemical analysis using the methods described by Pavan *et al.* (12). Briefly, the pH of the FS or RS was determined potentiometrically: $CaCl_2$ solution (0.01 mol L^{-1}) ratio of 1:2.5 (w/v) after shaking for 15 min and settling for 30 min. Calcium (Ca^{2+}), Magnesium (Mg^{2+}) and Aluminium (Al) were extracted with a non-buffered solution of KCl (1.0 mol L^{-1}) and measured using an atomic absorption spectrophotometer. The total organic carbon concentration was evaluated by the Walkley–Black potassium dichromate–sulphuric acid oxidation procedure (13). Phosphorus and potassium were extracted using the Mehlich-1 method and their concentrations were

determined colorimetrically using a UV–visible spectrophotometer and a flame photometer, respectively.

5.1.3.3 Isolation of fungi

Ten (10) grams of each sample were ressuspended in 90 mL of sterile saline solution (NaCl 0.85%), shaken at 100 rpm for 1 h at 25 ºC, and serially diluted to make six dilutions (10^{-5}) per sample. Aliquots of 100 µL of this dilution series were plated on three media: Sabouraud Agar (14), Martin Medium Agar (15) and DYGS (16). The plates were incubated for 7 d at 28 ºC and 37 ºC. After the incubation period, the colonies were purified by streaking.

5.1.3.4 Morphological examination

The isolates were grown on standard media Czapek Yeast Agar (CYA) and Malt Extract agar (MEA). Macromorphology characteristics such as colony diameter, degree of sporulation, production of sclerotia, colour of mycelia and presence of exudate, were then checked. Microscopic features were also observed in each culture medium. The genus *Penicillium* was identified according to Pitt (17) and Samson *et al.* (18). *Aspergillus* was identified according to Klich and Pitt (19) and Samson *et al.* (18). The other fungi were identified according to Pitt and Hocking (20) and Samson *et al.* (18).

5.1.3.5 Molecular examination

All fungal isolates were subjected to ITS nucleotide sequence analysis. The filamentous fungi were grown on liquid complete medium (21) and the yeast on

Sabouraud medium. After growing, the mycelia or yeast cells were collected, frozen in liquid nitrogen, ground to a fine powder and then a standard phenol:chloroform extraction protocol (22) was used for genomic DNA isolation. The ITS region was amplified using the primers ITS1 and ITS4, adopting a standard amplification protocol of 35 cycles with an annealing temperature of 55 °C (23). The PCR products were cleaned up using ExoProStar® (GE Healthcare, UK) and directly sequenced using the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA) according to the manufacturer's instructions. A volume of Hi-Di formamide (10 µl) was added to the sequencing products, which were then processed in an ABI3500XL Genetic Analyzer (Applied Biosystems, USA).

SeqMan software bundled with the Lasergene package (DNASTAR Inc., Wisconsin, USA) was used to assemble the contigs using the forward and reverse sequences. All ITS sequences were compared using the Basic Local Alignment Search Tool (BLASTn) against the NCBI database to recognize fungal species with similar DNA sequences. In order to avoid BLAST hits to misidentified sequences in the GenBank database, whenever possible we used the BLAST tool with the "sequences from type material" (RefSeq) option. If the identity values generated by BLAST were not sufficient to precisely identify the species, phylogenetic trees were constructed using MEGA 6.0 software based on the Neighbor-Joining (NJ) method and Tamura-Nei Model (24, 25). Nonparametric bootstrap analysis was performed with 1,000 resamplings.

5.1.4 Results and Discussion

In this study we have shown that some fungi species can be found in two types of solid waste (fine shale particles, FS; and retorted shale, RS) generated by the Petrosix® process for oil shale transformation into oil and gas at São Mateus do Sul, in south Paraná, Brazil.

The samples of FS (I and II) and RS (I and II) collected at SIX-Petrobras showed different chemical characteristics (Table 1). Both FS samples showed lower pH values (I= pH 3.6 and II= pH 2.4) than RS samples (I=pH 5.2 and II=pH 6.5). The samples of FS and RS also differed dramatically in Ca and Al content. Aluminum was detected in FS (I=4.63 and II=19.59) but not in RS. Calcium was present in both sample types, but the two FS samples exhibited a higher level (I= 50.47 and II= 49.15) than the two RS samples (I= 35.57 and II 21.95). Phosphorus was present in both sample types, but the two RS samples exhibited a higher level (I= 150.20 and II= 154.10) than the two FS samples (I= 145.30 and II= 134.00). Samples differ in the Magnesium content, FSI and RSI had the major level (FSI= 14.30 and RSI= 11.92) than the FSII and RSII samples (FSI= 7.44 and RSI= 6.70).

Fungi were present in all samples of byproducts studied herein. A total of eight fungal genera were found, including *Acidiella*, *Aspergillus*, *Cladosporium*, *Ochroconis*, *Penicillium*, *Pseudozyma*, *Talaromyces* and *Trichoderma* (Fig. 4). The two samples of the retorted shale harbored the highest fungi population density, the number of fungal colony-forming units (CFU) are 1.3×10^5 to RS I and 3.9×10^5 to RS II samples per gram of byproduct, whereas the fine shale particles had the smallest population density (FS I= 3.8×10^4 and FS II= 4.1×10^4 CFU per gram).

Altogether, 56 filamentous fungi and 2 yeast isolates were obtained from the four samples (30 from FS and 28 from RS - see Table 2). Sequence analysis of the nrDNA internal transcribed spacer (ITS), the official DNA barcode for fungi, were performed on all fungi. ITS sequences were deposited in GeneBank under the following accession numbers: KX363445 to KX363461.

Acidiella was the predominant genus found in FS samples, which is a highly acidic substrate. *Talaromyces* was the most predominant in RS samples.

All isolates recognized as *Acidiella* (*n* = 10) shared 99-100% sequence identity with *Acidiella bohemica* and *Fodinomyces uranophilus*. In Australia, *F. uranophilus* was recently isolated from uranium mine water with typically high acidity (pH 1.7–1.8) and containing high concentrations of total dissolved/colloidal salts (26). In the Czech Republic, *A. bohemica* has been isolated from acid soil in a kaolin quarry (27). More recently, based on the high similarity in rDNA sequences, ecology, physiology and morphology, Kolarik *et al.* (28) proved that these acid-tolerant species are sisters to be placed in a single genus, *Acidiella*, also known under its more recent synonym, *Fodinomyces*. It is important to note, that *A. bohemica* was found preponderantly in FS samples, which had much lower pH values than RS samples.

The genus *Talaromyces* was described as a sexual state of *Penicillium*, but with the recent adoption of the ‘one fungus, one name’ concept in combination with some phylogenetic studies, *Penicillium* subgenus *Biverticillium* was transferred to the *Talaromyces* genus (29, 30). Nowadays an accepted species list is available, endorsed by International Commission of *Penicillium* and *Aspergillus* (ICPA) and providing the accession numbers of ITS barcodes for all ex-type strains of *Penicillium* and *Talaromyces* species (29). Although ITS cannot be used to

differentiate all species, it does provide valuable information on sectional classification and often enough information for identifying a species (29).

Twenty *Talaromyces* ITS sequences were more similar to those of species belonging to *Talaromyces* section *Talaromyces*. The ITS-based neighbor-joining (NJ) tree using the sequences obtained herein and those retrieved from GenBank for each type strain of *Talaromyces* section *Talaromyces*, as suggested by Visagie *et al.* (29), revealed that 19 strains belong to *Talaromyces sayulitensis* species (Fig. 2). *T. sayulitensis* is a recently described species isolated from dust in Mexico (29). Based on the NJ tree, isolate 6054RS was identified as *Talaromyces stolli*. Isolate 6035RS, which does not belong to section *Talaromyces*, has BLAST 100% sequence identity to the type material of *Talaromyces diversus*, which belongs to the section *Trachyspermi*. *Talaromyces* has been considered an important genus for biotechnological purposes, based on its species' ability to produce enzymes and soluble pigments (30). The presence of *T. sayulitensis, T. diversus* and *T. stolli* in oil shale is described herein for the first time.

Regarding *Penicillium* ITS sequences, the BLAST search in NCBI using the "sequences from type material" option revealed that the majority of *Penicillium* sequences (10/11) were more similar to those from the species belonging to *Penicillium* section *Citrina*. Because the identity values generated by BLAST were insufficient to precisely identify the species, we generated an ITS-based neighbor-joining (NJ) tree using the sequences obtained in the present study and those retrieved from GenBank for each type strain of *Penicillium* section *Citrina* included in the updated list (Fig. 3) in Visagie *et al.* (29). Based on the NJ tree, nine isolates were closed related to *Penicillium citrinum*, a species already reported to be present

in shale samples in Pakistan (31). Isolate 6038FS was grouped together with *Penicillium sizovae*, *Penicilium steckii*, *Penicillium tropicalis* and *Penicillium tropicum*, which are not distinguishable by ITS sequences. According to Visagie *et al.* (29), the ITS region is not variable enough for distinguishing all closely related *Penicillium* species. Isolate 6003FS, which does not belong to section *Citrina*, has 100% sequence identity with the type material of *Penicillium parvum*, which belongs to the section *Exilicaulis*. Jiang *et al.* (11) studied three main oil shale production areas in China and observed that the environmental and climatic conditions of each mine significantly affected the preferences of fungal genera, but *Penicillium* was found in all 3 mines. According to the authors, this genus shows potential for the biotransformation of oil shale.

Eight isolates were identified as belonging to the genus *Cladosporium*. This genus has been intensively investigated over the past decade, and according to Bensch *et al.* (32) this has led to the resolution of three major species complexes (*Cladosporium cladosporioides, Cladosporium herbarum* and *Cladosporium sphaerospermum*) based on morphology and DNA phylogeny. Isolates 6051RS, 6053RS and 6057RS were identified as belonging to the *C. cladosporioides complex*. Isolates 6018FS, 6031RS, 6032RS, 6033RS and 6047FS were identified as belonging to the *C. sphaerospermum complex.* The ITS region has limited resolution for many species in *Cladosporium* (33) so our ITS sequence data were insufficient to discriminate the species within each *Cladosporium complex. Cladosporium* is a genus of fungi that has been found on many substrates and regions throughout the world, including oil shale from the Green River Formation, USA (34).

In regard to the genus *Aspergillus*, three isolates were obtained from the samples studied herein. Based on the best BLASTn hits for our sequences against NCBI RefSeq, isolates 6001FS, 6017FS and 6029RS were identified as *Aspergillus flavus* (I=100%), *Aspergillus sydowii* (I=99%) and *Aspergillus niger/ Aspergillus welwitschiae* (I=100), respectively. Anjum *et al.* (31) also reported the presence of *A. niger* and *A. flavus* in black shale samples in Pakistan. *A. niger* and *A. flavus* are predisposed to produce a variety of organic acids (citric and oxalic) for effectively solubilizing rare earth elements (REEs). Amin *et al.* (35) tested the ability of some fungi isolated from the Sinai Peninsula carbonaceous shales to leach REEs from these rocks by microbial means and found that *A. niger* and *A. flavus* were the most efficient organisms.

Two *Trichoderma* isolates (6055RS and 6056RS) were obtained from RS samples. Both were identified, based on the best BLASTn hits against the NCBI RefSeq (I=100%), as *Trichoderma atroviride*. This species has been described as able to solubilize coal (36, 37). Polycyclic aromatic hydrocarbons are common environmental pollutants, and are described by Nicolini *et al.* (38) in Brazilian retorted oil shale samples taken from inside storage piles. The genus *Trichoderma* is also reported as tolerant to a range of recalcitrant pollutants, including heavy metals, pesticides, and polyaromatic hydrocarbons (39).

The ITS sequences from isolate 6042FS was found to be significantly different from all other sequences in NCBI (accessed 18 JAN, 2015). Using the BLAST tool against NCBI, it was found that the ITS sequence of this isolate is more similar to that of *Ochroconis mirabilis*, but with only 87% of sequence identity. The genus *Ochroconis* was reviewed by Samerpitak *et al.* (40), who recognized thirteen species.

After this review, a further eight new species were proposed (41, 42, 43) and the genus *Ochroconis* currently contains 21 species. According Samerpitak *et al.* (42, 44), several species of the genus *Ochroconis* appear to have degrees of molecular heterogeneity that are much higher than observed in most other ascomycetous fungi. Not only ITS, but even nuLSU (usually invariant between closely related ascomycetes) showed significant sequence variation for species recognition (40). The species of the genus *Ochroconis* have a cosmopolitan distribution and can be isolated from various sources, including cave rock (45, 46). *Ochroconis lascauxensis*, for example, was selected on May 22, 2013 by the International Institute for Species Exploration at Arizona State University, as one of the "Top 10 New Species" discovered in 2012. In order to determine whether the 6042FS isolate is or is not a new species of the genus *Ochroconis*, it will be necessary to conduct analysis of morphological characters and extrolite profiling, as well as investigating sequence data from other genes, such as actin, β-tubulin, and translation elongation factor 1-α (TEF-1).

In addition to the filamentous fungi, two basidiomycetous yeasts (6034RS and 6036RS) were obtained from RS samples. Using BLASTn against the NCBI database, it was found that the ITS sequences of these isolates are similar to that of the type strain of *Pseudozyma crassa* (I=98%). The strains belonging to the genus *Pseudozyma* have been described as producers of a glycolipid biosurfactants, known as mannosylerythritol lipids (MEL), which not only exhibit excellent surfactant properties, but also versatile biochemical actions. MEL production has been considered an important taxonomic index for the *Pseudozyma* yeasts (47).

In conclusion, combining morphology and DNA sequence data we have described, for the first time, a snapshot of the diversity of fungi colonizing oil shale byproducts from the Irati Formation in Brazil. *T. sayulitensis* was the species most frequently found in retorted shale, and *A. bohemica* in fine shale particles.

Acknowledgements

This research was supported by Petrobras - *Agricultural Shale Project.* The authors acknowledge the financial support given by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (*CAPES*) and Fundação Araucaria.

5.1.5 References

1. Knaus, E., J. Killen, K. Biglarbigi, and P. Crawford. 2010. An Overview of Oil Shale Resources, p. 3-20. *In* Oil Shale: A Solution to the Liquid Fuel Dilemm. Am. Chem. Soc. press, Washington.
2. Pan, Y., Z. Xiaoming, L. Shouhui, Y. Shuangchun and R. Nan. 2012. A Review on Technologies for Oil Shale Surface Retort. J. Chem. Soc. Pak. 34:1331-1338.
3. Dinneen, G.U. 1976. Chapter 9: Retorting Technology of Oil Shale, p. 181-198. *In*: Developments in Petroleum Science, Elsevier B.V, Pennsylvania.
4. Cogo, S.L., A.M. Brinatti, S.C. Saab, M.L. Simões, L. Martin-Neto, J.A. Rosa, and Y.P. Mascarenhas. 2009. Characterization of oil shale residue and rejects from Irati Formation by electron paramagnetic resonance. Brazilian. J. Phys. 39:31–4.
5. Mangrich, A., L. Tessaro, A. Dos Anjos, F. Wypych, and J.A. Soares. 2001. A slow-release K^+ fertilizer from residues of the Brazilian oil-shale industry: synthesis of kalsilite-type structures. Environ. Geol. 40:1030–6.

6. Energy USD. Oil Shales and Tar Sands: A Bibliography [Internet]. Department of Energy, Technical Information Center; 1977.
7. Dragutinović, V, M.M. Vrvić, I. Swiecicka, O. Cvetković, T. Berić, and S. Stanković. 2012. Characterization of new *Bacillus circulans* strain from oil shale. Food Tech. Biotech, 50:123–127.
8. Matlakowska, R., and A. Sklodowska. 2009. The culturable bacteria isolated from organic-rich black shale potentially useful in biometallurgical procedures. J. Appl. Microbiol. 107:858-866.
9. Burford, E.P., M. Kierans, and G.M. Gadd. 2003. Geomycology: fungi in mineral substrata. Mycologist. 17:98–107.
10. Pfister, R.M., E. Krieger, and J. Ridge. 1991. Microbial presence and potential in eastern oil shale. Resour. Conserv. Recycl. 5:231–43.
11. Jiang, S., W. Wang , X. Xue, C. Cao, and Y. Zhang. 2016. Fungal diversity in major oil-shale mines in China. J. Environ. Sci.
12. Pavan, M.A., F.T. Bingham, and P.F. Pratt. 1982. Toxicity of aluminum to coffee (*Coffea arabica* L.) in ultisols and oxisols amended with $CaCO_3$, $MgCO_3$, and $CaSO_4$-$2H_2O$. Soil. Sci. Soc. Am. J. 46:1201-1207.
13. Nelson D. W., and L. E. Sommers. 1982. Total carbon, organic carbon and organic matter. *In* Page, A. L.; R. H. Miller, D. Keeney. Methods of Soil Analysis, Eds. Am Soc Agron: Madison, 2:539-594.
14. Silva, J.O., S.A. Franceschini, M.A. Lavrador, and R.C. Candido. 2004. Performance of selective and differential media in the primary isolation of yeasts from different biological samples. Mycopathologia 157:29-36.
15. Martin, J.P. 1950. Use of acid, rose bengal and streptomycin in the plate method for estimating soil fungi. Soil Sci. 69:215-232.

16. Eckert, B., O.B. Weber, K. Guldrun, A. Halbritter, M. Storffels, and A. Hartmann. 2001. *Azospirillum doebereinerae* sp. nov., a nitrogen-fixing bacterium associated with the C4-grass *Miscanthus*. Int. J. Syst. Evol. Microbiol. 51:17-26.
17. Pitt, J.I. 1991. A Laboratory Guide to Common *Penicillium* Species. Common wealth Scientific and Industrial Research Organization, Sydney.
18. Samson, R.A., E.S. Hoekstra, U. Thrane, J.C. Frisvad, and B. Andersen. 2010. Food and indoor fungi. CBS Laboratory Manual series 2, Netherlands.
19. Klich, M.A., and J.I. Pitt. 1988. A Laboratory Guide to the Common *Aspergillus* Species and Their Teleomorphs [Internet]. Commonwealth Scientific and Industrial Research Organisation, Division of Food Processing . Available from: http://books.google.com.br/books?id=ntMbNAAACAAJ.
20. Pitt, J.I., and A.D. Hocking. 2009. Fungi and food spoilage. Springer, 3rd edn, New York.
21. Todd, R.B., M.A. Davis, and M.J. Hynes. 2007. Genetic manipulation of *Aspergillus nidulans*: meiotic progeny for genetic analysis and strain construction. Nat. Protoc. 2:811–821.
22. Sambrook, J., and D.W. Russell. 2001. Molecular Cloning: A Laboratory Manual, 3rd ed. Cold Spring Harbor Laboratory Press, New York.
23. White, T., T. Bruns, S. Lee, and J. Taylor. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics, p. 315–322. *In* M. Innis, D. Gelfand, J. Shinsky, and T. White, PCR Protocols: A Guide to Methods and Applications. San Diego: Academic Press, New York.
24. Saitou, N., and M. Nei. 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol. Biol. Evol. 4:406–25.
25. Tamura, K., and M. Nei. 1993. Estimation of the number of nucleotide

substitutions in the control region of mitochondrial DNA in humans and chimpanzees. Mol. Biol. Evol. 10:512–26.

26. Vázquez-Campos, X., A.S. Kinsela, T.D. Waite, R.N. Collins, and B.A. Neilan. 2014. *Fodinomyces uranophilus* gen. nov. sp. nov. and *Coniochaeta fodinicola* sp. nov., two uranium mine-inhabiting Ascomycota fungi from northern Australia. Mycologia 106:1073–89.
27. Hujslová, M., A. Kubátová, M. Kostovčík and M. Kolařík. 2013. *Acidiella bohemica* gen. et sp. nov. and *Acidomyces* spp. (Teratosphaeriaceae), the indigenous inhabitants of extremely acidic soils in Europe. Fungal Divers. 58:33–45.
28. Kolařík, M., M. Hujslová, and X. Vázquez-Campos. 2015. Acidotolerant genus *Fodinomyces* (Ascomycota: Capnodiales) is a synonym of *Acidiella*. Czech Mycol. 67: 37–38.
29. Visagie, C.M., Y. Hirooka, J.B. Tanney, E. Whitfield, K. Mwange, M. Meijer, A.S. Amend, K.A. Seifert, and R.A. Samson. 2014. *Aspergillus*, *Penicillium* and *Talaromyces* isolated from house dust samples collected around the world. Stud. Mycol. 78:63–139.
30. Yilmaz, N., C.M. Visagie, J. Houbraken, K.C. Frisvad and R.A. Samson. 2014. Polyphasic taxonomy of the genus *Talaromyces*. Stud. Mycol. 78:175–341.
31. Anjum, F., S.A. Bukhari, M. Shahid, A. Akcil, A. Tahir, H.Z.E. Jaafar, M. Zia-Ul-Ha and I. Samota. 2015. Bio-mechanical leaching of uranium from low grade black shale. Environ. Eng. Manag. J. 14:297-496.
32. Bensch, K., J.Z. Groenewald, U. Braun, J. Dijksterhuis, M. de Jesús Yáñez-Morales and P.W. Crous. 2015. Common but different: The expanding realm of *Cladosporium*. Stud. Mycol. 82:23–74.

33. Zalar, P., G.S. de Hoog, H-J. Schroers, P.W. Crous, J.Z. Groenewald, and N. Gunde-Cimerman. 2007. Phylogeny and ecology of the ubiquitous saprobe *Cladosporium sphaerospermum*, with descriptions of seven new species from hypersaline environments. Stud. Mycol. 58:157–83.

34. Bradley, W.H. Origin and microfossils of the oil shale of the Green River formation of Colorado and Utah. 1931. U. S. Govt. Print. Off. Available from: https://books.google.com.br/books?id=_IvnAAAAMAAJ.

35. Amin, M.M., I.E. Elaassy, M.G. El-Feky, A.S.M. Sallam, M.S. Talaat and N.A. Kawady. 2014. Effect of mineral constituents in the bioleaching of uranium from uraniferous sedimentary rock samples, Southwestern Sinai, Egypt. J. Environ. Radioact. 134:76–82.

36. Hölker, U., and M. Höfer. 2002. Solid substrate fermentation of lignite by the coal-solubilizing mould, *Trichoderma atroviride*, in a new type of bioreactor. Biotechnol. Lett. 24:1643–1645.

37. Oboirien, B.O., S.G. Burton, D. Cowan, and S.T.L. Harrison. 2008. The effect of the particulate phase on coal biosolubilisation mediated by *Trichoderma atroviride* in a slurry bioreactor. Fuel Process Technol. 89:123–30.

38. Nicolini, J., B.F. Pereira, C.N. Pillon, V.G. Machado, W.A. Lopes, J.B. Andrade, and A.S. Mangrich. 2011. Characterization of Brazilian oil shale byproducts planned for use as soil conditioners for food and agro-energy production. J. Anal. App. Pyrol. 90: 112–117.

39. Tripathi, P., P.C. Singh, A. Mishra, P.S. Chauhan, S. Dwivedi, R.T. Bais, and R.D. Tripath. 2013. *Trichoderma*: a potential bioremediator for environmental clean up. Clean Technol. Envir. Policy 15:541-550**.**

40. Samerpitak, K., E. Linde, H.J. Choi, A.H.G. Gerrits van den Ende, M. Machouart,

C. Gueidan, G.S. de Hoog. 2014. Taxonomy of *Ochroconis*, genus including opportunistic pathogens on humans and animals. Fungal Divers. 65:89–126.

41. Crous, P.W., R.G. Shivas, W. Quaedvlieg, *et al.* 2014. Fungal Planet description sheets: 214-280. Persoonia 32:184–306.
42. Samerpitak, K., A.P.M. Duarte, D. Attili-Angelis, F.C. Pagnocca, G. Heinrichs, A.J.M.M. Rijs, *et al.* 2015. A new species of the oligotrophic genus *Ochroconis* (Sympoventuriaceae). Mycol. Prog. 14:1–10.
43. Samerpitak, K., A.H.G. Gerrits van den Ende, S.B.J. Menken, and G.S. de Hoog. 2015. Three New Species of the Genus *Ochroconis*. Mycopathologia 180:7–17.
44. Samerpitak, K., B.H.G. Gerrits van den Ende, J.B. Stielow, S.B.J. Menken, and G.S. de Hoog. 2016. Barcoding and species recognition of opportunistic pathogens in *Ochroconis* and *Verruconis*. Fungal Biol. 120:219–30.
45. Martin-Sanchez, P.M., A. Nováková, F. Bastian, C. Alabouvette, and C. Saiz-Jimenez. 2012. Two new species of the genus *Ochroconis*, *O. lascauxensis* and *O. anomala* isolated from black stains in Lascaux Cave, France. Fungal Biol. 116:574–89.
46. Nováková, A. 2009. Microscopic fungi isolated from the Domica Cave system (Slovak Karst National Park, Slovakia). A review. Int. J. Speleol. 38:71–82.
47. Morita, T., M. Konishi, T. Fukuoka, T. Imura, H.K. Kitamoto, and D. Kitamoto. 2007. Characterization of the genus *Pseudozyma* by the formation of glycolipid biosurfactants, mannosylerythritol lipids. FEMS Yeast Res. 7:286–92.

5.1.6 Tables

Table 1. Chemical characteristics of two samples of fine shale particles (FS I and FS II) and two samples of retorted shale (RS I and RS II) collected at Schist Industrialization Business Unit (Six) – Petrobras, in São Mateus do Sul, Paraná – Brazil.

Characteristics	FS I	FS II	RS I	RS II
pH	3.60	2.40	5.20	6.50
Calcium ($cmol_c\ kg^{-1}$)	50.47	49.15	35.57	21.95
Aluminium ($cmol_c\ kg^{-1}$)	4.63	19.59	ND	ND
Phosphorus ($mg\ dm^{-3}$)	145.30	134.00	150.20	154.10
Carbon ($g\ dm^{-3}$)	29.22	27.23	31.79	31.63
Magnesium ($cmol_c\ kg^{-1}$)	14.39	7.44	11.92	6.70
Potassium ($cmol_c\ kg^{-1}$)	0.05	0.03	0.14	0.47

pH in $CaCl_2$ 0.01 mol L^{-1}; Mg, Ca and Al by KCl 1 mol L^{-1}; Total Carbon by Walkley e Black; P and K in $Mehlich^{-1}$;ND=not detected.

Table 2. Fungal species isolated from samples of fine shale particles and retorted shale collected at Schist Industrialization Business Unit (Six) – Petrobras, in São Mateus do Sul, Paraná – Brazil.

Species	Isolates from fine shale particles (FS)	Isolates from retorted shale (RS)
Aspergillus flavus	6001FS (KX363445)	-
Aspergillus sydowii	6017FS (KX363451)	-
Aspergillus niger/ welwitschiae	-	6029RS (KX363462)
Acidiella bohemica	6008FS (KX363448), 6014FS, 6015FS (KX363450), 6016FS, 6040FS (KX363456), 6043FS, 6044FS, 6045FS (KX363458), 6046FS	6030RS
Cladosporium cladosporioides complex	-	6051RS, 6053RS, 6057RS (KX363461)
Cladosporium sphaerospermum complex	6018FS (KX363452), 6047FS	6031RS, 6032RS, 6033RS
Ochroconis sp.	6042FS (KX363457)	-
Penicillium citrinum	6000FS, 6002FS (KX363446), 6009FS, 6037FS (KX363454), 6041FS, 6010FS	6025RS, 6048RS, 6049RS
Penicillium parvum	6003FS (KX363447)	-
Penicillium sp.	6038FS (KX363455)	-
Pseudozyma crassa	-	6034RS, 6036RS (KX363463)
Talaromyces diversus	-	6035RS (KX363453)
Talaromyces sayulitensis	6004FS, 6005FS, 6006FS, 6007FS, 6011FS (KX363449), 6012FS, 6013FS, 6039FS	6019RS, 6020RS, 6021RS, 6022RS, 6023RS, 6024RS, 6026RS, 6027RS, 6028RS, 6050RS, 6052RS
Talaromyces stolli	-	6054RS (KX363459)
Trichoderma atroviride	-	6055RS (KX363460), 6056RS

5.1.7 Figures

Fig. 1. Geographic location of the SIX-Petrobras mine in Paraná State, the sampling site for fine shale particles and retorted shale. Source: IBGE, 2014 – adapted by João Henrique Caviglione.

Fig. 2. Neighbor joining tree reconstructed from the ITS sequence of *Talaromyces* section *Talaromyces* isolated from the samples of fine shale particles and retorted shale (bold) aligned with corresponding sequences of *Talaromyces* section *Talaromyces* deposited in public databases. Numbers at branch nodes refer to bootstrap values (1000 replicates). Only values >70% are shown.

Fig. 3. Neighbor joining tree reconstructed from the ITS sequence of *Penicillium* section *Citrina* isolated from the samples of fine shale particles and retorted shale (bold) aligned with corresponding sequences of *Penicillium* section *Citrina* deposited in public databases. Numbers at branch nodes refer to bootstrap values (1000 replicates). Only values >70% are shown.

Fig. 4. Isolates from retorted shale and fine shale byproducts. *Aspergillus sydowii* isolate 6017 (A); *Aspergillus welwitschiae/niger* isolate 6029 (B) - colonies grown on CYA incubated for 7 days at 25°C; *Acidiella bohemica* isolate 6008 (C); *Cladosporium sphaerospermum complex* isolate 6018 (D) - colonies grown on PDA incubated for 7 days at 25°C; *Penicillium citrinum* isolate 6000 (E); *Penicillium parvum* isolate 6003 (F); *Penicillium* sp. isolate 6038 (G); *Talaromyces stolli* isolate 6054 (H) - colonies grown on CYA incubated for 7 days at 25°C, *Pseudozyma crassa* isolate 6034 (I) - colonies grown on PDA incubated for 7 days at 25°C; *Talaromyces sayulitensis* isolate 6019 (J) - colonies grown on CYA incubated for 7 days at 25°C; *Ochroconis* sp. isolate 6042 (K); *Trichoderma atroviride* 6055 (L) - colonies grown on PDA incubated for 7 days at 25°C.

Figure 1.

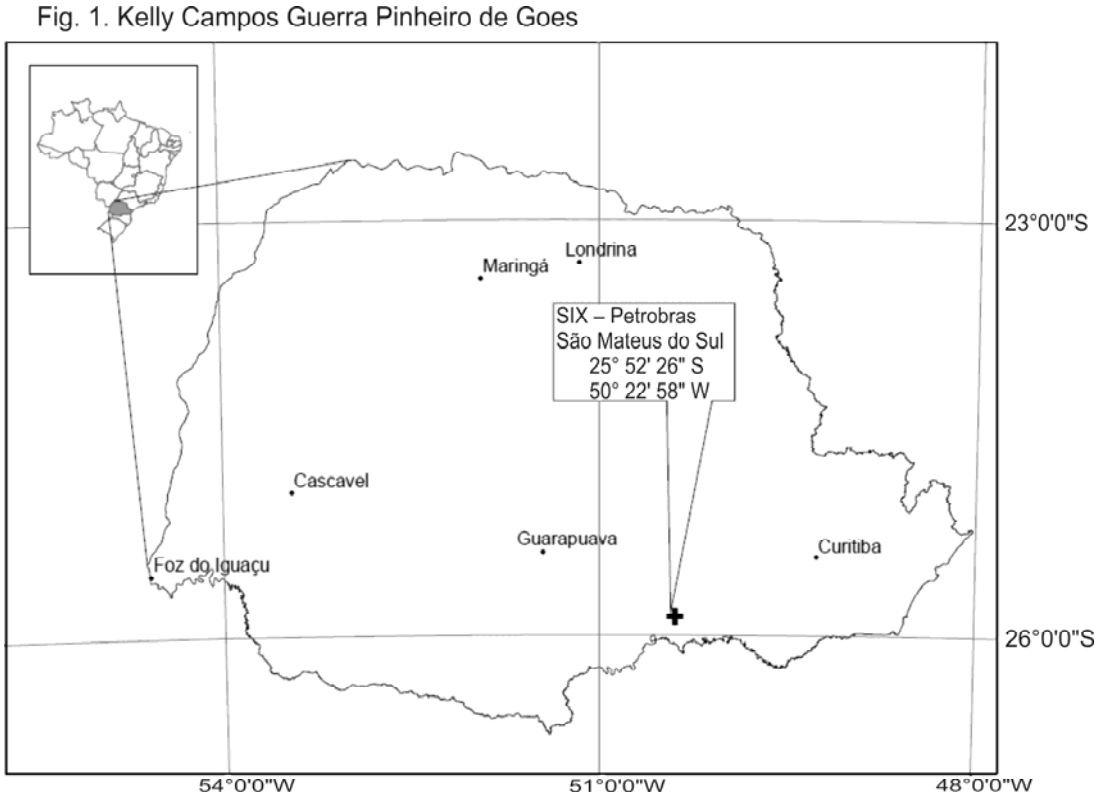


Fig. 1. Kelly Campos Guerra Pinheiro de Goes
Londrina
Maringá
SIX – Petrobras
São Mateus do Sul
25° 52' 26" S
50° 22' 58" W
Cascavel
Guarapuava
Curitiba
Foz do Iguaçu
23°0'0"S
26°0'0"S
54°0'0"W
51°0'0"W
48°0'0"W

Figure 2.

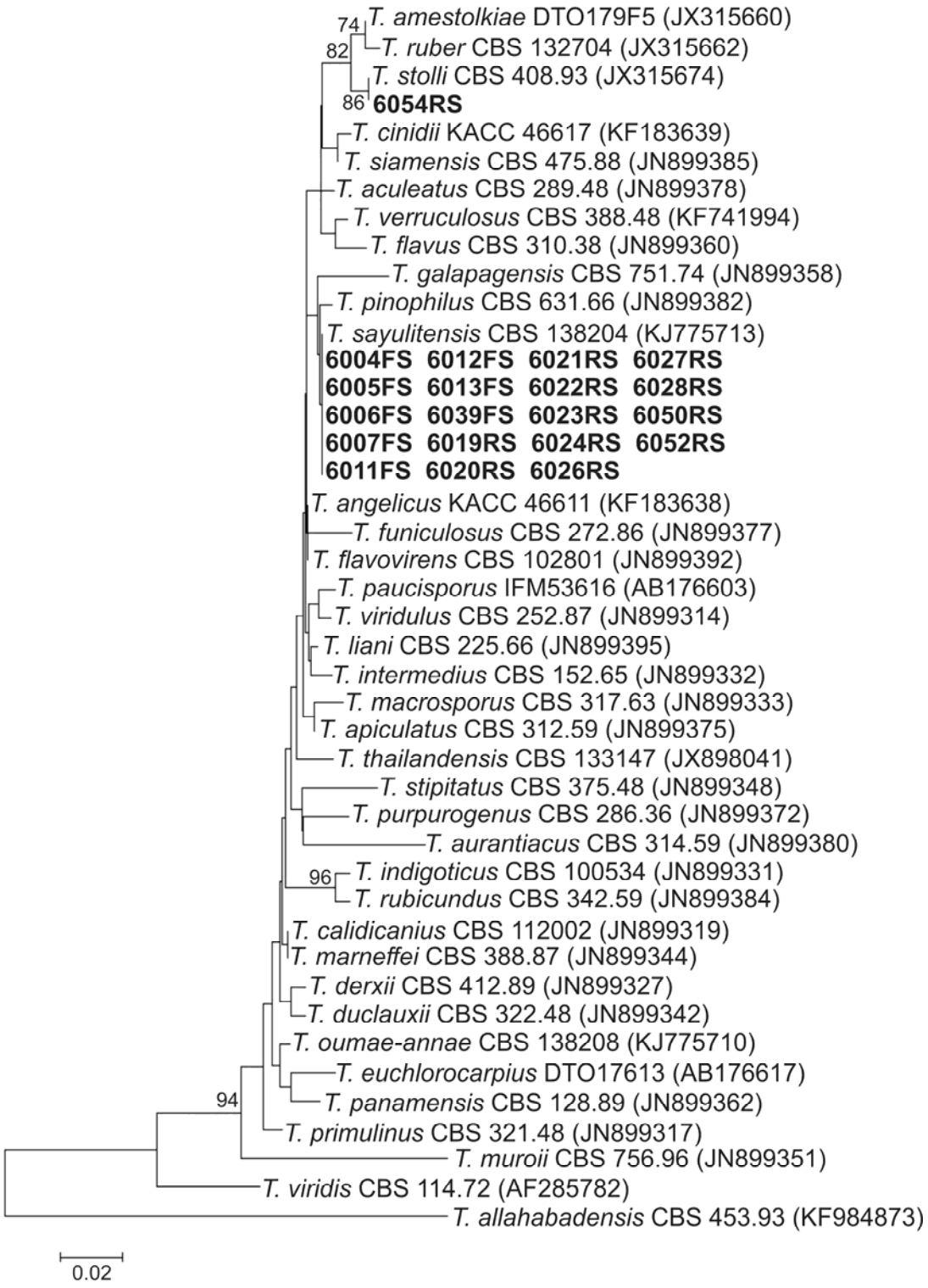

74
82
86
T. amestolkiae DTO179F5 (JX315660)
T. ruber CBS 132704 (JX315662)
T. stolli CBS 408.93 (JX315674)
6054RS
T. cinidii KACC 46617 (KF183639)
T. siamensis CBS 475.88 (JN899385)
T. aculeatus CBS 289.48 (JN899378)
T. verruculosus CBS 388.48 (KF741994)
T. flavus CBS 310.38 (JN899360)
T. galapagensis CBS 751.74 (JN899358)
T. pinophilus CBS 631.66 (JN899382)
T. sayulitensis CBS 138204 (KJ775713)
6004FS 6012FS 6021RS 6027RS
6005FS 6013FS 6022RS 6028RS
6006FS 6039FS 6023RS 6050RS
6007FS 6019RS 6024RS 6052RS
6011FS 6020RS 6026RS
T. angelicus KACC 46611 (KF183638)
T. funiculosus CBS 272.86 (JN899377)
T. flavovirens CBS 102801 (JN899392)
T. paucisporus IFM53616 (AB176603)
T. viridulus CBS 252.87 (JN899314)
T. liani CBS 225.66 (JN899395)
T. intermedius CBS 152.65 (JN899332)
T. macrosporus CBS 317.63 (JN899333)
T. apiculatus CBS 312.59 (JN899375)
T. thailandensis CBS 133147 (JX898041)
T. stipitatus CBS 375.48 (JN899348)
T. purpurogenus CBS 286.36 (JN899372)
T. aurantiacus CBS 314.59 (JN899380)
96
T. indigoticus CBS 100534 (JN899331)
T. rubicundus CBS 342.59 (JN899384)
T. calidicanius CBS 112002 (JN899319)
T. marneffei CBS 388.87 (JN899344)
T. derxii CBS 412.89 (JN899327)
T. duclauxii CBS 322.48 (JN899342)
T. oumae-annae CBS 138208 (KJ775710)
T. euchlorocarpius DTO17613 (AB176617)
94
T. panamensis CBS 128.89 (JN899362)
T. primulinus CBS 321.48 (JN899317)
T. muroii CBS 756.96 (JN899351)
T. viridis CBS 114.72 (AF285782)
T. allahabadensis CBS 453.93 (KF984873)
0.02

Figure 3.

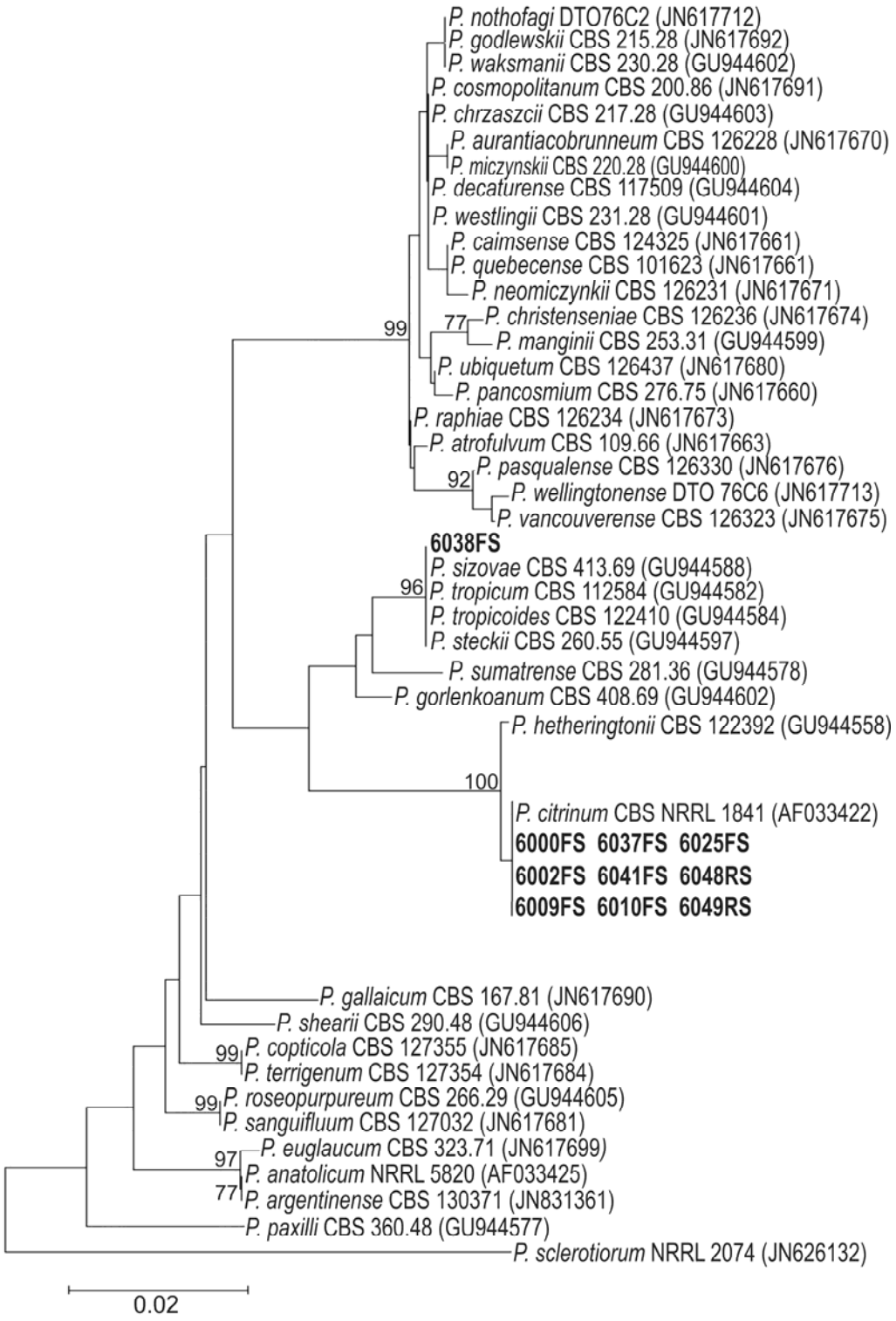
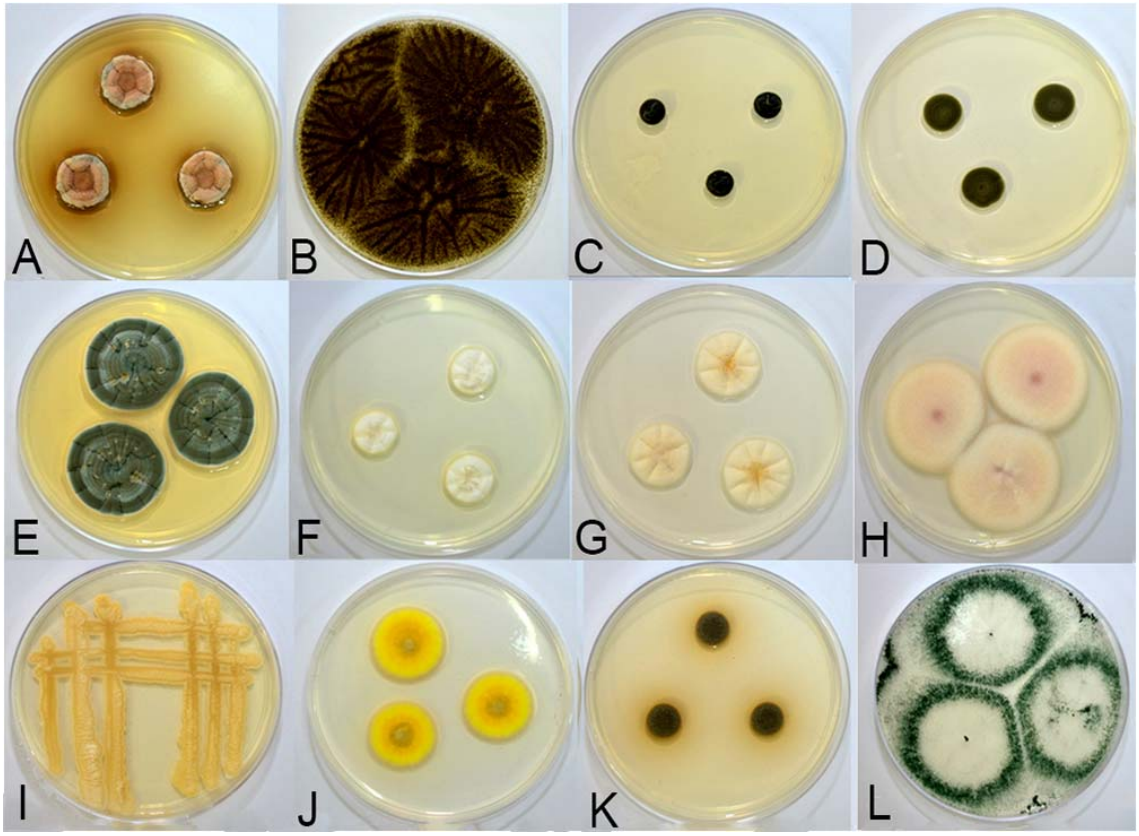

P. nothofagi DTO76C2 (JN617712)
P. godlewskii CBS 215.28 (JN617692)
P. waksmanii CBS 230.28 (GU944602)
P. cosmopolitanum CBS 200.86 (JN617691)
P. chrzaszcii CBS 217.28 (GU944603)
P. aurantiacobrunneum CBS 126228 (JN617670)
P. miczynskii CBS 220.28 (GU944600)
P. decaturense CBS 117509 (GU944604)
P. westlingii CBS 231.28 (GU944601)
P. caimsense CBS 124325 (JN617661)
P. quebecense CBS 101623 (JN617661)
P. neomiczynkii CBS 126231 (JN617671)
P. christenseniae CBS 126236 (JN617674)
P. manginii CBS 253.31 (GU944599)
P. ubiquetum CBS 126437 (JN617680)
P. pancosmium CBS 276.75 (JN617660)
P. raphiae CBS 126234 (JN617673)
P. atrofulvum CBS 109.66 (JN617663)
P. pasqualense CBS 126330 (JN617676)
P. wellingtonense DTO 76C6 (JN617713)
P. vancouverense CBS 126323 (JN617675)
6038FS
P. sizovae CBS 413.69 (GU944588)
P. tropicum CBS 112584 (GU944582)
P. tropicoides CBS 122410 (GU944584)
P. steckii CBS 260.55 (GU944597)
P. sumatrense CBS 281.36 (GU944578)
P. gorlenkoanum CBS 408.69 (GU944602)
P. hetheringtonii CBS 122392 (GU944558)
P. citrinum CBS NRRL 1841 (AF033422)
6000FS 6037FS 6025FS
6002FS 6041FS 6048RS
6009FS 6010FS 6049RS
P. gallaicum CBS 167.81 (JN617690)
P. shearii CBS 290.48 (GU944606)
P. copticola CBS 127355 (JN617685)
P. terrigenum CBS 127354 (JN617684)
P. roseopurpureum CBS 266.29 (GU944605)
P. sanguifluum CBS 127032 (JN617681)
P. euglaucum CBS 323.71 (JN617699)
P. anatolicum NRRL 5820 (AF033425)
P. argentinense CBS 130371 (JN831361)
P. paxilli CBS 360.48 (GU944577)
P. sclerotiorum NRRL 2074 (JN626132)
99
77
92
96
100
99
99
97
77
0.02

Figure 4.



5.2 Artigo II: Occurrence of bacteria in fine shale and retorted shale byproducts from a commercial oil shale mine in Brazil

5.2.1 Abstract

Fine shale are small fragments of mined rock not usable in Petrosix® process, and retorted shale are originated after pyrolysis of oil shale to extract oil and gas. The aims of this study were to isolate, characterize and identify bacterial species present in oil shale byproducts. We also investigated if the temperature of incubation can broaden the diversity of bacterial isolates obtained from fine shale and retorted shale. In total, 40 bacteria were obtained using serial dilution and plating, being 27 isolates at 28°C and 13 at 37°C. A total of eight bacteria genera were found in oil shale byproducts, including *Arthrobacter*, *Bacillus*, *Paenibacillus*, *Ralstonia*, *Serratia, Sphingomonas*, *Terrabacter* and *Xanthobacter*. Molecular characterization carried out using ARDRA fingerprint analyses allowed characterized the isolates and shows that the chemical composition of the two byproducts influenced bacterial diversity indicated by Shannon index. *Bacillu*s and *Paenibacillus* were the most predominant genera found in the fine shale and retorted shale samples. The other six genera were found only in retorted shale samples, which show higher diversity than in the fine shale particles.

Keywords: Shannon index, 16S rRNA, molecular analysis, phylogenetic analysis.

5.2.2 Introduction

Oil shale is a fine-grained sedimentary rock that contains organic matter that is predominantly kerogen, which yield in substantial quantities of oil and combustible gas upon destructive distillation [1]. Oil shale resources are abundant and distributed in several countries around the world [2]. The most important Brazilian oil shale reserve, the Irati Formation located in São Mateus do Sul, Paraná, has the greatest potential for economic development because of its accessibility, grade, and wide-spread distribution [1].

The mining and processing of oil shale which generate solid byproducts occur in two distinct stages, the fine shale particles (FS) and retorted shale (RS). The FS are small fragments of rock measuring less than 6.35 mm with an oil content of approximately 15%, representing approximately 20% of mined rock not usable in oil and gas processing [3, 4]. Retorted shale (RS) is a waste byproduct obtained from oil shale pyrolysis (550°C) to oil and gas extraction [5].

Considering that the oil shale from Irati Formation is not markedly enriched in heavy metals according to Dyni [1], studies have shown uses for its byproducts, for example, as a slow-release potassium fertilizer made from the fine and lime shale as suggested by Mangrich et al. [3] and the potential use of RS in crop production [6, 7]. The high organic matter and silicon contents in the byproducts could explain its positive effects on crops production. These shale byproducts have a wide range of applications, for instance, current data obtained from the Shale Project, which was carried out by the Agronomic Institute of Paraná (IAPAR) in partnership with Petrobras, included a patent on the use of FS and RS as a carrier of *Rhizobium* and *Bradyrhizobium* inoculants for legumes [8].

Studies have revealed that black shale can host intriguing microbial communities in spite of its low porosity and permeability [9, 10]. Recently, the presence of cultivable microorganisms in different sources and types of shale from Germany, Finland, Poland and Serbia were reported [11-13].

Although the interaction of oil shale with the microbial community is an interesting topic, until now, this subject has not been well examined in the shale byproducts from the Irati Formation. Due to this lack of information regarding the occurrence of

microorganisms, we analysed samples of fine shale and retorted shale collected at Schist Industrialization Business Unit, Paraná State, Brazil. In addition, molecular analyses of bacterial isolates associate to these byproducts were carried out aiming to enhance knowledge on the microbial composition, which can generate important data and suggests how bacteria can act on nutrient cycling and degrading chemical compounds. In this context, the aim of this study was to isolate, characterize and examine the bacterial diversity based on ARDRA fingerprinting analyses and identify bacteria species present in shale byproducts. We use the plating technique with incubation at 28°C and 37°C to expand the growth range of some groups of bacteria and to obtain a microbial collection for future studies directed to agricultural applications.

5.2.3 Material and methods

5.2.3.1 Site sampling

Shale byproducts sampling was carried out at Schist Industrialization Business Unit (Six) of Petrobras, in São Mateus, Paraná, Brazil (25° 52' 26" S, 50° 22' 58" W). Samples of each byproduct, Fine Shale (FS) and Retorted Shale (RS), were collected in twelve discrete locations in storage piles, and a composite sample of approximately 1.0 kg of each byproduct was derived after homogenization. Samples were placed in sealed and sterilized polyethylene bags and sent to Soil Microbiology Laboratory of Agronomic Institute of Paraná (IAPAR) where isolation analyses were performed. Chemical analyses were performed in the byproducts samples according to methods described by Pavan et al. [14].

5.2.3.2 Isolation of bacteria

To obtain viable bacteria, serial dilution and the spread plate method were performed for each byproduct. A subsample of approximately 10 g of each byproduct was added to 90 mL of sterile saline solution (NaCl 0.85%) with glass beads and shaken at 100 rpm at 25°C for 30 min and serially diluted. Aliquots of 100 µl (10^{-2} to 10^{-4} dilutions) were seeded in sterilized modified DYGS media with agar (contained

the following masses of compounds in g per litre of demineralized water: 2 of glucose; 1.5 of peptone; 2.0 of yeast extract; 0.5 of KH_2PO_4. $7H_2O$; 0.5 of $MgSO_4$ $7H_2O$; pH 6.0) [14]. Two sets of Petri plates were prepared for incubation at 28°C and 37°C for 7 days.

Colonies of bacteria were purified in culture medium and each strain assigned an IPR designation number (Table 1). All bacterial strains isolated from FS and RS used in this study are kept cryopreserved in glycerol (65% v/v) at -80°C and lyophilized at the Collection of Microorganisms of Interest of Agribusiness of the Laboratory of Soil Microbiology – IAPAR. These strains are also deposited lyophilized at the Diazotrophic and Plant Growth Promoting Bacteria Culture Collection of Embrapa Soja (WFCC Collection # 1213, WDCM Collection # 1054) with the same IPR number designation. The isolates were grown on DYGS medium and the morphology of cells was examined by Gram staining [15].

5.2.3.3 Molecular characterization

The bacteria isolates were grown in DYGS liquid medium for 24 h on a rotatory shaker at 100 rpm and 28 °C. After growing, the cells were collected by centrifugation and total genomic DNA was extracted using a phenol:chloroform modified method [15]. DNA concentration and purity was determined by spectrophotometry on ScanDrop® using the BioMethods software (analytic jena®/ Jena - Germany).

The 16S rRNA gene was amplified using fD1 (5′-AGAGTTTGATCCTGGCTCAG-3′) and rD1 (5′-AAGGAGGTGATCCAGCC-3′) universal primers [16]. Amplicons were cleaned up with PureLink™ PCR Purification kit (Invitrogen™) and direct sequenced using fD1, 362f (5'-CTCCTACGGGAGGCAGCAGTGGGG-3'), 768f (5'-CGAAAGCGTGGGGAGCAAACAGG-3') and 1203f (5'-GAGGTGGGGATGACGTCAAGTCCTC-3'), from Menna et al. [17], and Y2 (5'-CCCACTGCTGCCTCCCGTAGGAGT-3') from Young et al. [18], using the Big Dye® Terminator v3.1 Cycle Sequence Kit (Applied Biosystems®, USA). A Hi-Di formamide (10 µL) was added to sequence products, and sequencing was performed in an ABI 3500XL Genetic Analyzer (Applied Biosystems®, USA).

Sequences of each strain were assemble in contigs using Contig Assembly Program (CAP) according to Huang [19] using the forward and reverse sequences and corrected manually when needed using the BioEdit v7.2.5 [20]. All 16S rRNA

sequences were compared with sequences in NCBI (National Center for Biotechnology Information) [http://www.ncbi.nlm.nih.gov/] with BLASTn (Basic Local Alignment Search Tool) and against the RDP (Ribosomal Database Project) [http://rdp.cme.msu.edu/index.jsp] database with the aid of Classifier tool for taxonomic identification of strains. Phylogenetic analysis was performed using the software CLUSTAL_W 2.0.12 for alignment [21]. Phylogenetic trees were constructed using MEGA 6.06 software on the Neighbor-Joining (NJ) method [22, 23] in the Tamura 3-Parameter model. Bootstrap analyses were made with 1000 replicates [24].

The 16S rRNA sequences obtained in this study were deposited in the GenBank database and assigned to accession numbers KT933198 to KT933230 (Table 1).

Amplified Ribosomal DNA Restriction Analysis (ARDRA) was performed to characterize and cluster genetically similar isolates. The 16S-23S rRNA intergenic spacer region was amplified using fC16S and rD23S primers [25]. PCR-products were restricted using *Rsa* I (GT/AC), *Nde* I (CA/TATG) e *Hae* III (GG/CC) (Invitrogen™) endonucleases. Restricted products were electrophoresed on 1.8% (*w/v*) agarose gel and fragments sizes were estimated by comparison with molecular weight marker 1 Kb Plus DNA Ladder™ (Invitrogen™).

For ARDRA technique, bacterial isolates were clustered by analyses of presence or absence of bands that were performed using BioNumerics program (AppliedMaths). Similarity with established tolerance from 1.0 % was calculated with coefficient of Jaccard and dendrograms were constructed with Unweighted Pair-Group Method with arithmetical Mean algorithm (UPGMA) [27].

Data of ARDRA and the number of isolates of each genus found in the two byproducts samples were subjected to a diversity analysis, using the Past program software [28] and the diversity indexes were subject to t test.

5.2.4 Results and discussion

This study presents the Shannon and Margalef diversity indexes of bacterial strains in association with two different shale byproducts, collected in storage piles in SIX-Petrobras mining area in São Mateus do Sul – Paraná, Brazil. Bacteria were isolated in all samples of byproducts studied and a total of eight genera were

isolated, including *Arthrobacter*, *Bacillus*, *Paenibacillus*, *Ralstonia*, *Serratia*, *Sphingomonas*, *Terrabacter* and *Xanthobacter*. *Bacillus megaterium* and *Paenibacillus taichungensis* were the most predominant species in FS and RS samples.

Based on cultivation analysis, a total of forty bacteria were isolated in all plated dilutions and at 28°C and 37°C, with the most strains obtained from the 10^{-3} dilution. The RS housed the largest number of isolates being twenty two obtained at 28°C and seven at 37°C. From FS samples at 28°C were obtained five isolates, while six were obtained from 37°C. Gram staining of microorganisms showed predominance of Gram positive bacteria in the two byproducts samples (Table 1).

Sequences analyses of the 16S rRNA gene were performed with all IPR strains isolated from FS and RS. Phylogenetic trees reconstructed from partial sequences of 16S rRNA genes from byproducts representative isolates and some type strains allowed the proposed classification at the genus level and for some isolates, strong evidence of species (Fig. 1 and 2).

The IPR isolates identified as *Bacillus* (n = 18) shared 99-100% sequence identity with *Bacillus aryabhattai*, *B. cereus*, *B. megaterium*, *B. muralis* and *B. thuringiensis*. Phylogenetic tree reconstructed with 14 types of strains of *Bacillus* and eighteen IPR strains are shown in Fig. 1. In the first cluster, IPR4885 (KT933201) and IPR4890 (KT933202) from RS at 37°C, IPR4897 (KT933205) from FS at 28°C, IPR4898, IPR4900, IPR4910 (KT933208), IPR4911 and IPR4921 from RS at 28°C and IPR4884 (KT933200) from FS at 37°C were identified as *B. megaterium* grouped with the type strain ATCC 14581 (GU252112). Grouped in this same cluster, isolates IPR4883 from RS at 37°C, IPR4899, IPR4900, and IPR4921 from RS at 28°C, were identified as *B. aryabhattai* closed related to strain B8W22 (NR_115953). Isolate IPR4895 (KT933204) from FS isolated at 28°C was identified as *B. cereus* with 99% identity with type strain ATCC 14579 (NR_074540). In the last cluster isolates IPR4882 (KT933199), IPR4894 from FS isolated at 37°C and IPR4909, IPR4915, IPR4916 (KT933210) and IPR4917 from RS at 28°C, were identified as *B. thuringiensis* closely related to type strain ATCC 10972 (AF290545).

According to Bhandari et al., [29] the genus *Bacillus* is a large heterogeneous group consisting of highly diverse organisms. The genus is comprised of aerobic and anaerobic spore-forming bacteria, and includes diverse types of cells organisms of

great medical, economic and historical importance. Among byproducts isolates we identified a *B. cereus* specie, that is a common cause of food-poisoning [30], *B. thuringiensis*, which is utilized as biocontrol of insects and has a great utility in agricultural processes [31], *B. megaterium*, a commercially specie, important for the biotechnological production of several substances [32] and *B. aryabhattai* related as a zinc solubilizing bacteria and potential bio-inoculant [33]. The genus *Bacillus* has been found in samples of oil shale and black shale from Poland and Serbia [12, 34] that were reported to be very efficient in bioleaching process, desilicification and demineralisation of the shale. In this study, with oil shale byproducts from Irati Formation the most representative specie was *B. megaterium*.

Eleven IPR 16S rRNA sequences were more similar to species belonging to genus *Paenibacillus.* The 16S rRNA-based neighbor-joining (NJ) tree using sequences obtained from isolates and sequences of 27 type strains of *Paenibacillus* retrieved from GenBank, revealed that three strains belongs to *P. chibensis*, four strains belongs to *P. pabuli*, and four to *P. taichungensis* (Table 1). Based on NJ tree, (Fig. 2) in the first cluster, IPR4880 (KT933213) and IPR4893 (KT933216) isolated from FS at 37°C and 28°C, respectively and isolates from RS isolated at 28°C, IPR4901 (KT933218) and IPR4906 (KT933220), were closely related to type strain *P. taichungensis* BCRC 17757 (EU179327). The FS strains, IPR4881 (KT933214) and IPR4896 (KT933217) isolated at 28°C and 37°C, respectively, and IPR4905 and IPR4919 isolated from RS at 28°C, were grouped with *P. pabuli* (AB07391). The RS IPR4887 (KT933215) and FS IPR4886, isolated at 37°C and IPR4905 isolated from RS at 28°C were identified as *P. chibensis* (AB073194) in the NJ tree (Table 1).

In 1993, based on phenotypic and phylogenetic analyses, Ash et al. [35] proposed that members of group 3 within the genus *Bacillus* should be transfer to the genus *Paenibacillus*. This genus include aerobic or facultative anaerobic, endospore forming microorganisms, neutrophilic and usually exhibiting a positive Gram reaction, but with some representatives exhibiting gram-variable or –negative staining [36]. Members of the genus *Paenibacillus* are common saprophytic components of soil, water, plants, food, faeces and diseased insect larvae [37]. Among this genus, species of *P. taichungensis* and *P. pabuli* were related as important enzymes producers [38, 39], and *P. chibensis* described as producer of antimicrobial compounds against bacteria and pathogenic fungi [40]. Our findings show that *P.*

taichungensis and *P. pabuli* are the predominant isolated species from this genus presented in the byproducts samples.

Regarding to the *Ralstonia* 16S rRNA sequences, revealed that three strains isolated from RS at 28°C were more similar to *R. pickettii* and *R. mannitolilytica* species (Table 1). Based on the NJ tree, the isolates IPR4902 (KT933222), IPR4904 (KT933224), IPR4907 (KT933225) were identified as *R. picketti* (AJ270260), and IPR4903 (KT933223) as *R. mannitolilytica* (AY043379) (Data not shown). Species of the genus *Ralstonia* occupy diverse ecological niches, including water, soil, activated sludge and human clinical samples [41]. Berlendis et al. [42] studied the mineralogy, organic geochemistry and the microbial diversity associated with a carbon-rich kerogen rock (Autun shale) and a coal rich in aromatic carbon in France. According to authors, in the Autun shale, the dominant specie observed is *R. picketti*. Some *Ralstonia* species are known as both iron oxidizers [43] and involved in polyaromatic hydrocarbons (PHA) biodegradation [44]. It is important to note, that *R. picketti* was found in this study only in RS samples, may be due the presence of PHAs in this byproduct composition, as described by Nicolini et al. [45].

We identified two isolates from RS as belonging to the genus *Serratia*, where IPR4888 (KT933226) was identified as *S. nematodiphila* and IPR4889 (KT933227) as *S. marcescens*, both with 100% of sequence identity with strains, D2050SBS1 (EU036987) and DSM 4582 (AJ233431), respectively (Table 1). In black shale weathering profiles samples from China, the genus *Serratia* was reported within uncultivated bacteria using pyrosequencing analysis [46].

In regard to *Arthrobacter*, *Sphingomonas*, *Terrabacter* and *Xanthobacter* genera, four isolates were obtained in RS samples studied herein. Based on the best BLASTn hit, isolate IPR4891 (KT933228) were identified as *Sphingomonas* sp. (100% identity with strain IFO 15915 [AB033949]) and IPR4892 (KT933229) as *Terrabacter tumescens* (100% identity with strain AF005023); both IPR were isolated from RS at 37°C. The IPR4920 (KT933230) was identified as *Xanthobacter autotrophicus* with 99% sequence identity with strain c7 (X94201) and IPR4912 (KT933198) as *Arthrobacter oryzae* (100% identity with strain KV-651^{T}) (Table 1 and data not shown) both isolated from RS at 28°C.

The genus *Sphingomonas* was created by Yabuuchi *et al*. [47] to accommodate strictly aerobic, chemoheterotrophic, yellow-pigmented, Gram-negative, rodshaped

bacteria that contain glycosphingolipids as cell envelope components. The classification in the genus *Sphingomonas* has been complicated due the increase in the number of species and in 2001, a phylogenetic analysis based on the 16S rDNA sequences shows that the currently known species of *Sphingomonas* were clearly separated into four clusters [48]. In this study, IPR4891 was identified at genus level, being necessary complementary analysis to allow the specie identification of this isolate.

The genus *Terrabacter*, with the type species is *Terrabacter tumescens* isolated from soil, was created by the transfer of *Pimelobacter tumescens* based on phylogenetic and chemotaxonomic data [49]. Members of the genus *Terrabacter* have been isolated from soil, air and stone [50] and are reported to produce the enzyme urease and precipitate carbonate minerals [51].

Members of the genus *Xanthobacter* are yellow, Gram staining reaction negative; cells are pleomorphic rods that can become highly irregular, multibranched or twisted. Because of their metabolic diversity, *Xanthobacter* species are widespread [52]. *Xanthobacter autotrophicus* is described as the type species of the genus and this species has been demonstrated as suitable for biodegradation of monochloroacetate in culture medium [53]. The genus *Arthrobacter*, first defined by Conn and Dimmick [54], belongs to the class Actinobacteria and includes Gram-stain-positive coryneform bacteria with aerobic metabolism and little or no acid production from glucose. Bacteria of the genus *Arthrobacter* are common inhabitants of the soil environment.

These four genera, *Sphingomonas*, *Terrabacter*, *Xanthobacter* and *Arthrobacter*, were not correlated to oil shale samples in the literature. Is possible that these IPR isolates obtained from RS byproducts samples may be considered as settler microorganisms. To compare the genotypic similarity among IPR strains isolated from oil shale samples and calculate the diversity indexes the ARDRA fingerprint techniques was used.

Thirty one bacteria previous identified as *Bacillus* and *Paenibacillus* were analyzed by ARDRA technique. Eleven *Paenibacillus* strains yielded ten restriction band patterns. A total of 77 fragments were obtained with the three restriction enzymes used. Nineteen *Bacillus* strains yielded fifteen restriction patterns and a total of 120 fragments were obtained. The dendrograms derived from UPGMA cluster analysis

presents the polymorphism among *Paenibacillus* and *Bacillus* strains isolated from RS and FS byproducts (Fig. 3 and 4).

ARDRA results from *Paenibacillus* distinguished eight main groups at 70% similarity, being five of them formed by only one strain. Group I was formed by isolates IPR4880 and IPR4881 with 78% of similarity. Group II and IV comprised isolates IPR4905 (*P. pabulli*) with IPR4906 (*P. taichungensis*) and IPR4896 (*P. pabulli*) with IPR4901 (*P. taichungensis*) in a clonal relationship (Fig. 3).

ARDRA results from *Bacillus* strains distinguished eleven main groups at 70% similarity, being five of them formed by only one strain (Fig. 4). Group I comprised in a clonal relationship bacterial strains *B. thuringiensis* (IPR4882) and *B. cereus* (IPR4894 and IPR4895). Group III comprised the strains IPR4899 and IPR4900 (*B. aryabhattai*) with 72% of similarity. Group IV and V comprised in a clonal relationship bacterial strains identified as *B. megaterium* IPR4897, IPR4898, IPR4884 and IPR4885). Group IX comprised in a clonal relationship bacterial strains *B. megaterium* (IPR4921) and IPR4911 (*B. aryabhattai*). Group XI comprised bacterial strains isolated from RS identified as *B. cereus* (IPR4909 and IPR4915) and *B. thuringiensis* (IPR4916).

According to the two molecular techniques, the highest Shannon and Margalef indexes were found to *Baciilus* strains (Shannon index = 2.306 and Margalef = 3,396) and RS strains, analyzed by 16S rRNA gene sequence (Shannon index = 1.608 and Margalef = 2.079), showing that its community had the most bacterial diversity (Table 2). The different ARDRA patterns indicate the co-occurrence of distinct population of *Bacillus* and *Paenibacillus*, suggesting a great intraspecific diversity.

The Irati Formation is comprised of two distinct beds of oil shale, separated by non-bituminous shale and limestone. For years, the limiting factor for the extraction and processing of shale was the generation of large amounts of byproducts. Currently, the valuation of byproducts has led to the development of new technologies aimed at utilizing them not only for restoration of mined land but also as raw materials for other processes, primarily in agriculture. Taking into account the chemical properties of fine shale, Mangrich and collaborators [3] suggested that this byproduct has a potential to act as slow-release fertilizer mainly if added to acid soils with low levels of potassium. Due to the possible use of shale in agriculture, studies of microbial community composition, biodegradation and/or biotransformation of

byproducts are important, particularly for indigenous and early colonizer microorganisms.

No significant differences in bacterial density were observed among the populations associated to both FS samples. Nevertheless, the diversity of the RS bacterial isolates suggested qualitative differences regarding the populations in association with this byproduct, mainly considering that the two temperatures of incubation allowed the isolation of three different genera (*Serratia*, *Terrabacter* and *Sphingomonas*), comparing to FS samples.

The role of byproduct in the associated bacterial population is unclear, but a low number of bacteria recovery from shale samples has been previously reported [13, 55] and supports our findings.

In previous studies, as well as in our study, bacteria have been reported as indigenous to shale byproducts. At times, relatively limited diversity has been observed, such as in the work performed by using denaturing gradient gel electrophoresis (DGGE) analysis to study bacteria distributed across the shale-sandstone at Cerro Negro, New Mexico [56]. For instance, indigenous bacteria play a prominent role in the weathering of black shale and in the biogeochemical cycles of elements in this rock [55, 57].

This study provides the first data on the occurrence, characterization and diversity of cultivable microorganisms present in oil shale byproducts from Irati Formation, which will contribute to a better understanding, giving a wider representation of bacterial communities. Among the cultivable bacteria, the *Bacillus and Paenibacillus* genera were the most prevalent. The presence of cultivable microorganisms is low, but there are distinct that were recovered by direct plating techniques in the two temperatures of incubation. The retorted shale had a higher number of cultivable microorganisms than fine shale samples. The results have yielded new insights into the microbiology of these shale byproducts. Also, it is important to consider that the data make available information for future agricultural applications.

Compliance with Ethical Standards statement

This manuscript is in compliance with Ethical Standards.

Conflict of interest

The authors declare no competing financial interest.

Acknowledgements

K.C.G.P. Goes and D. S. Andrade acknowledge PhD fellowship from CAPES (National Council for the Improvement of Higher Education) and a grant from Fundação Araucária/Paraná, Brazil, respectively. Institute Agronômico do Paraná (IAPAR) and Petrobras (SIX) had a bilateral collaboration Project of Shale Agricultural that financially supported this study. The authors also thank Andréa Borgatto Appolinario, Edson Marcio de Siqueira, Francisco Skora Neto and Renato Yagi for support during shale byproducts sampling.

5.2.5 References

1. Dyni JR: **Geology and resources of some world oil-shale deposits**. *Oil Shale* 2003, **20**(3):193-252
2. Knaus E, Killen J, Biglarbigi K, Crawford P: **An Overview of Oil Shale Resources**. In: *Oil Shale: A Solution to the Liquid Fuel Dilemma.* vol. 1032: American Chemical Society; 2010: 3-20.
3. Mangrich AS, Tessaro LC, Anjos AD, Wypych F, Soares JF: **A slow-release K+ fertilizer from residues of the Brazilian oil-shale industry: synthesis of kalsilite-type structures**. *Environ Geol* 2001, **40**:1030–1036.
4. Doumer ME, Giacomini SJ, Silveira CAP, Weiler DA, Bastos LM, de Freitas LL: **Atividade microbiana e enzimática em solo após a aplicação de xisto retortado**. *Pesq Agropec Bras* 2011, **46**(11):1538-1546.
5. Pimentel PM, Oliveira RMPB, Melo DMA, Anjos MJ, Melo MAF, Gonzalez G: **Characterization of retorted shale for use in heavy metal removal**. *Appl Clay Sci* 2010, **48**:375-378.
6. Pereira HS, Vitti GC: **Efeito do uso do xisto em características químicas do solo e nutrição do tomateiro**. *Hort Bras* 2004, **22**:317-321.
7. Chaves LHG, Vasconcelos ACF: **Alterações de atributos químicos do solo e do crescimento de plantas de milho pela aplicação de xisto**. *Rev Bras Eng Agric Ambient* 2006, **10**:84-88.
8. Andrade DS: **Processo de Obtenção de Veículos Sólidos para Inoculantes Microbianos de fixadores biológicos de nitrogênio**. In: *Instituto Nacional de Propriedade Industrial (INPI)* Edited by Instituto Agronômico do Paraná PS, vol. PI11000015 A2. Brazil; 2013.
9. Petsch ST, Edwards KJ, Eglinton TI: **Microbial transformations of organic matter in black shales and implications for global biogeochemical cycles**. *Palaeogeogr Palaeocl* 2005, **219** 157 – 170.
10. Cockell CS, Pybus D, Olsson-Francis K, Kelly L, Petley D, Rosser N, Howard K, Mosselmans F, Cockell CS, Pybus D *et al*: **Molecular characterization**

and geological microenvironment of a microbial community inhabiting weathered receding shale cliffs. *Microbial Ecol* 2011, **61**(1):166-181.

11. Johnson DB, Hallberg KB: **Bioprospecting for micro-organisms associated with black shale ores. Publishable Activity Report of Bioshale Project. Bioshale project: A Specific Targeted Research Project co-funded by the Europen Commission (FP6 programme)**. In., vol. 54; 2007.
12. Dragutinović V, Vrvić MM, Swiecicka I, Cvetković O, Berić T, Stanković S: **Characterization of new *Bacillus circulans* strain from oil shale**. *Food Technol Biotech* 2012, **50**(1):123–127.
13. Matlakowska R, Sklodowska A: **The culturable bacteria isolated from organic-rich black shale potentially useful in biometallurgical procedures**. *J Appl Microbiol* 2009, **107**(3):858-866.
14. Rodrigues Neto J, Malavolta Júnior VA, Victor O: **Meio simples para isolamento e cultivo de *Xanthomonas campestris* pv. citri tipo B**. *Summa Phytopathol* 1986, **12** 16.
15. Pavan M.A., M.F.D. Bloch, H.C.D. Zempulski, M. Miyazawa, and D.C. Zocoler. 1992. **Manual de análise química de solo e controle de qualidade**, p. 51. *In* Instituto Agronomico do Paraná, Londrina.
16. Gram, H.C. (1884). "Über die isolierte Färbung der Schizomyceten in Schnitt- und Trockenpräparaten". **Fortschritte der Medizin** (in German) **2**: 185–189.
17. Sambrook J, Fritsch EF, Maniatis T: **Molecular Cloning. A Laboratory Manual**, 2 edn: Cold Spring Harbor: Cold Spring Harbor Laboratory Press; 1989.
18. Weisburg WG, Barns SM, Pelletier DA, Lane DJ: **16S ribosomal DNA amplification for phylogenetic study**. *J Bacteriol* 1991, **173**:697-703.
19. Menna P, Hungria M, Barcellosa FG, Bangele EV, Hessf PN, Martinez-Romero E: **Molecular phylogeny based on the 16S rRNA gene of elite rhizobial strains used in Brazilian commercial inoculants**. *Syst Appl Microbiol* 2006, **29**:315-332.
20. Young JPW, Downer HL, Eardly BD: **Phylogeny of the phototrophic *Rhizobium* strain BTAi1 by polymerase chain reaction-based sequencing of a 16S rRNA gene segment**. *J Bacteriol* 1991, **73**:2271-2277.
21. Huang X: **A contig assembly program based on sensitive detection of fragment overlaps**. In: *Genomics* 1991.
21. Hall TA: **BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT**. *Nucleic Acids Symposium Series* 1999, **41**:95-98.
22. Larkin MA, Blackshields G, Brown NP, Chenna R, Mcgettigan PA, Mcwilliam H, Valentin F, Wallace IM, Wilm A, Lopez R *et al*: **Clustal W and Clustal X version 2.0**. *Bioinformatics Applications Note* 2007, **23** (21):2947–2948.
23. Kimura M: **A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences**. *J Mol Evol* 1980, **16** (2):111–120.
24. Saitou N, Nei M: **The neighbor-joining method: a new method for reconstructing phylogenetic trees**. *Mol Biol Evol* 1987, **4**:406–425.
25. Tamura K, Stecher G, Peterson D, Filipski A, Kumar S: **MEGA6: Molecular Evolutionary Genetics Analysis Version 6.0**. *Mol Biol Evol* 2013, **30**(12):2725-2729.
26. Andrade DS, Murphy PJ, Giller KE: **The diversity of Phaseolus-nodulating rhizobial populations is altered by liming of acid soils planted with**

***Phaseolus vulgaris* L. in Brazil**. *Appl Environ Microbiol* 2002, **68**(8):4025-4034.
27. Sneath P, Sokal R: **Numerical taxonomy: the principles and practice of numerical classification**. San Francisco: Freeman; 1973.
28. Hammer Ø, Harper DAT, Ryan PD: **PAST: Paleontological statistics software package for education and data analysis**. *Palaeontologia Electronica* 2001, **4**(1):9.
29. Bhandari V, Ahmod NZ, Shah HN, Gupta RS: **Molecular signatures for Bacillus species: demarcation of the *Bacillus subtilis* and Bacillus cereus clades in molecular terms and proposal to limit the placement of new species into the genus *Bacillus***. *Int J Syst Evol Microbiol* 2013, **63**:2712-2726.
30. Logan NA: **Bacillus and relatives in foodborne illness**. *Journal of Applied Microbiology* 2012, **112**(3):417-429.
31. Sanchis V, Bourguet D: ***Bacillus thuringiensis*: applications in agriculture and insect resistance management: A review**. *Agron Sustain Dev* 2008, **28** 28 : 11-20.
32. Eppinger M, Bunk B, Johns MA, Edirisinghe JN, Kutumbaka KK, Koenig SS, Creasy HH, Rosovitz MJ, Riley DR, Daugherty S *et al*: **Genome sequences of the biotechnologically important Bacillus megaterium strains QM B1551 and DSM319**. *J Bacteriol* 2011, **193**(16):4199-4213.
33. Ramesh A, Sharma SK, Sharma MP, Yadav N, Joshi OP: **Inoculation of zinc solubilizing *Bacillus aryabhattai* strains for improved growth, mobilization and biofortification of zinc in soybean and wheat cultivated in Vertisols of central India**. *Applied Soil Ecology* 2014, **73**:87-96.
34. Farbiszewska-Kiczma J, Farbiszewska T: **Bioleaching of metals from Polish Black Shale in neutral medium**. *Physicochem Probl Miner Process* 2004, **38**(1).
35. Ash C, Priest FG, Collins MD: **Molecular identification of rRNA group 3 bacilli (Ash, Farrow, Wallbanks and Collins) using a PCR probe test**. *Antonie van Leeuwenhoek* 1993, **64**(3):253-260.
36. Andreeva IS, Morozov IV, Pechurkina NI, Morozova OV, Ryabchikova EI, Saranina IV, Emel'yanova EK, Puchkova LI, Torok TT, Vlasov VV *et al*: **Isolation of bacteria of the genus Paenibacillus from soil and springs of the Valley of Geysers (Kamchatka)**. *Microbiology* 2010, **79**(5):696-704.
37. Daane LL, Harjono I, Barns SM, Launen LA, Palleron NJ, Häggblom MM: **PAH-degradation by Paenibacillus spp. and description of Paenibacillus naphthalenovorans sp. nov., a naphthalene-degrading bacterium from the rhizosphere of salt marsh plants**. *International Journal of Systematic and Evolutionary Microbiology* 2002, **52**(1):131-139.
38. Jemli S, Ben Messaoud E, Ben Mabrouk S, Bejar S: **The Cyclodextrin glycosyltransferase of *Paenibacillus pabuli* US132 strain: Molecular characterization and overproduction of the recombinant enzyme**. *Journal of Biomedicine and Biotechnology* 2008, **2008**:9.
39. Chen H-B, Kao P-M, Huang H-C, Shieh C-J, Chen C-I, Liu Y-C: **Effects of using various bioreactors on chitinolytic enzymes production by *Paenibacillus taichungensis***. *Biochemical Engineering Journal* 2010, **49**(3):337-342.
40. Lorentz RH, Ártico S, Da Silveira AB, Einsfeld A, Corção G: **Evaluation of antimicrobial activity in *Paenibacillus* spp. strains isolated from natural**

environment. *Letters in Applied Microbiology* 2006, **43**(5):541-547.
41. Coenye T, Goris J, De Vos P, Vandamme P, LiPuma JJ: **Classification of Ralstonia pickettii-like isolates from the environment and clinical samples as Ralstonia insidiosa sp. nov**. *International Journal of Systematic and Evolutionary Microbiology* 2003, **53**(4):1075-1080.
42. Berlendis S, Beyssac O, Derenne S, Benzerara K, Anquetil C, Guillaumet M, Estève I, Capelle B: **Comparative mineralogy, organic geochemistry and microbial diversity of the Autun black shale and Graissessac coal (France)**. *International Journal of Coal Geology* 2014, **132**:147-157.
43. Swanner ED, Nell RM, Templeton AS: ***Ralstonia* species mediate Fe-oxidation in circumneutral, metal-rich subsurface fluids of Henderson mine, CO**. *Chemical Geology* 2011, **284**(3–4):339-350.
44. Ryan MP, Pembroke JT, Adley CC: ***Ralstonia pickettii* in environmental biotechnology: potential and applications**. *Journal of Applied Microbiology* 2007, **103**(4):754-764.
45. Nicolini J, Pereira BF, Pillon CN, Machado VG, Lopes WA, de Andrade JB, Mangrich AS: **Characterization of Brazilian oil shale byproducts planned for use as soil conditioners for food and agro-energy production**. *Journal of Analytical and Applied Pyrolysis* 2011, **90**(2):112-117.
46. Li J, Sun W, Wang S, Sun Z, Lin S, Peng X: **Bacteria diversity, distribution and insight into their role in S and Fe biogeochemical cycling during black shale weathering**. *Environ Microbiol* 2014, **16**(11):3533-3547.
47. Yabuuchi E, Yano I, Oyaizu H, Hashimoto Y, Ezaki T, Yamamoto H: **Proposals of Sphingomonas paucimobilis gen. nov. and comb. nov., Sphingomonas parapaucimobilis sp. nov., Sphingomonas yanoikuyae sp. nov., Sphingomonas adhaesiva sp. nov., Sphingomonas capsulata comb, nov., and Two Genospecies of the Genus Sphingomonas**. *Microbiology and Immunology* 1990, **34**(2):99-119.
48. Takeuchi M, Hamana K, Hiraishi A: **Proposal of the genus *Sphingomonas* sensu stricto and three new genera, *Sphingobium*, *Novosphingobium* and *Sphingopyxis*, on the basis of phylogenetic and chemotaxonomic analyses**. *International Journal of Systematic and Evolutionary Microbiology* 2001, **51**:1405–1417.
49. Collins MD, Dorsch M, Stackebrandt E: **Transfer of *Pimelobacter tumescens* to *Terrabacte*r gen. nov. as *Terrabacter tumescens* comb. nov. and of *Pimelobacter jensenii* to *Nocardioides* as *Nocardioides jensenii* comb. nov**. *International Journal of Systematic and Evolutionary Microbiology* 1989, **39**(1):1-6.
50. Won K, Kook M, Yi T-H: ***Terrabacter koreensis* sp. nov., isolated from soil of a flowerbed**. *Int J Syst Evol Microbiol* 2014, **64**:3335-3340.
51. Li M, Cheng X, Guo H, Yang Z: **Biomineralization of Carbonate by Terrabacter Tumescens for Heavy Metal Removal and Biogrouting Applications**. *Journal of Environmental Engineering* 2015, **0**(0):C4015005.
52. Wiegel J: **The Genus *Xanthobacter***. In: *The Prokaryotes.* Edited by Dworkin M, Falkow S, Rosenberg E, Schleifer K-H, Stackebrandt E: Springer New York; 2006: 290-314.
53. Torz M, Beschkov V: **Biodegradation of monochloroacetic acid used as a sole carbon and energy source by *Xanthobacter autotrophicus* GJ10 strain in batch and continuous culture**. *Biodegradation* 2005, **16**(5):423-433.

54. Conn HJ, Dimmick I: **Soil Bacteria Similar in Morphology to Mycobacterium and Corynebacterium**. *Journal of Bacteriology* 1947, **54**(3):291-303.
55. Matlakowska R, Skłodowska A, Nejbert K: **Bioweathering of Kupferschiefer black shale (Fore-Sudetic Monocline, SW Poland) by indigenous bacteria: implication for dissolution and precipitation of minerals in deep underground mine**. *FEMS Microbiol Ecol* 2012, **81**(1):99-110.
56. Kovacik WP, Takai K, Mormile MR, McKinley JP, Brockman FJ, Fredrickson JK, Holben WE: **Molecular analysis of deep subsurface Cretaceous rock indicates abundant Fe(III)- and S°-reducing bacteria in a sulfate-rich environment**. *Environ Microbiol* 2006, **8**(1):141-155.
57. Matlakowska R, Narkiewicz W, Sklodowska A: **Biotransformation of organic-rich copper-bearing black shale by indigenous microorganisms isolated from Lubin copper mine (Poland)**. *Environ Sci & Technol* 2010, **44**(7):2433-2440.

5.2.6 Tables

Table 1 Strain designation (IPR code) from fine shale (FS) and from retorted shale (RS), temperature of bacteria isolation, Gram staining, proposed taxonomic position (identification) and access number in NCBI database.

IPR strain	Temp. (°C)	Gram	Identification	Access number
Fine Shale				
4894	28	+	*Bacillus cereus*	KT933203
4895	28	+	*Bacillus cereus*	KT933204
4897	28	+	*Bacillus megaterium*	KT933205
4893	28	+	*Paenibacillus taichungensis*	KT933216
4896	28	+	*Paenibacillus pabuli*	KT933217
4884	37	+	*Bacillus megaterium*	KT933200
4883	37	+	*Bacillus aryabhattai*	KX355760
4886	37	-	*Paenibacillus chibensis*	KX355757
4881	37	+	*Paenibacillus pabuli*	KT933214
4880	37	-	*Paenibacillus taichungensis*	KT933213
4882	37	+	*Bacillus thuringiensis*	KT933199
Retorted Shale				
4900	28	+	*Bacillus aryabhattai*	KX355761
4909	28	+	*Bacillus cereus*	KT933207
4898	28	+	*Bacillus megaterium*	KX355754
4899	28	+	*Bacillus aryabhattai*	KX355762
4911	28	+	*Bacillus megaterium*	KX355753
4908	28	+	*Bacillus muralis*	KX355763
4919	28	+	*Paenibacillus pabuli*	KX355755
4916	28	+	*Bacillus thuringiensis*	KT933210
4910	28	+	*Bacillus megaterium*	KT933208
4920	28	-	*Xanthobacter autotrophicus*	KT933230
4915	28	+	*Bacillus cereus*	KT933209
4912	28	+	*Arthrobacter oryzae*	KT933198

4921	28	+	*Bacillus aryabhattai*	KX355759
4905	28	-	*Paenibacillus pabuli*	KX355756
4901	28	-	*Paenibacillus taichungensis*	KT933218
4906	28	-	*Paenibacillus taichungensis*	KT933220
4917	28	+	*Bacillus cereus*	KT933211
4902	28	-	*Ralstonia pickettii*	KT933222
4903	28	-	*Ralstonia mannitolilytica*	KT933223
4904	28	-	*Ralstonia pickettii*	KT933224
4907	28	-	*Ralstonia pickettii*	KT933225
4918	28	-	*Paenibacillus chibensis*	KX355758
4885	37	+	*Bacillus megaterium*	KT933201
4890	37	+	*Bacillus megaterium*	KT933202
4887	37	+	*Paenibacillus chibensis*	KT933215
4888	37	-	*Serratia nematodiphila*	KT933226
4889	37	-	*Serratia marcescens*	KT933227
4891	37	-	*Sphingomonas* sp.	KT933228
4892	37	+	*Terrabacter tumescens*	KT933229

Table 2 Molecular techniques, byproducts samples, numbers of isolates and groups formed, and diversity indexes of isolates IPR from FS and RS samples.

Molecular techniques	Origin of isolates	Number of isolates	Number of groups	Shannon (H')	Margalef
ARDRA	*Bacillus*	19	11	2.306	3.396
	Paenibacillus	11	8	2.02	2.919
16S rRNA sequence	FS	11	2	0.689	0.417
	RS[a]	29	8	1.608	2.079

[a]Isolates identified as *Serratia*, *Arthrobacter*, *Xanthobacter*, *Sphingomonas*, *Terrabacter and Ralstonia* from RS are included in indexes.

5.2.7 Figure legends

Figure 1. Neighbor-Joining tree reconstructed from 16S rRNA gene sequences of *Bacillus* clado *cereus* IPR isolates from shale byproducts. GenBank accession numbers of type strains are given in parentheses and of IPR strains are in Table 1. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is show next to the branches. The evolutionary distances were computed using the Tamura 3-parameter method. *Escherichia coli* NBRC 102203 16S rRNA gene sequence was used as an out-group sequence.

Figure 2. Neighbor-Joining tree reconstructed from 16S rRNA gene sequences of *Paenibacillus* IPR isolates from shale byproducts. GenBank accession numbers of type strains are given in parentheses and of IPR strains are in Table 1. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is show next to the branches. The evolutionary distances were computed using the Tamura 3-parameter method. *Bacillus subtilis* DSM10 16S rRNA gene sequence was used as an out-group sequence.

Figure 3 UPGMA dendrogram based on Jaccard's coefficient from ARDRA profiles of *Paenibacillus* isolated from two different byproducts, retorted shale (RS) and fine shale (FS).

Figure 4 UPGMA dendrogram based on Jaccard's coefficient from ARDRA profiles of *Bacillus* isolated from two different byproducts, retorted shale (RS) and fine shale (FS).

5.2.8 Figures

Figure 1.

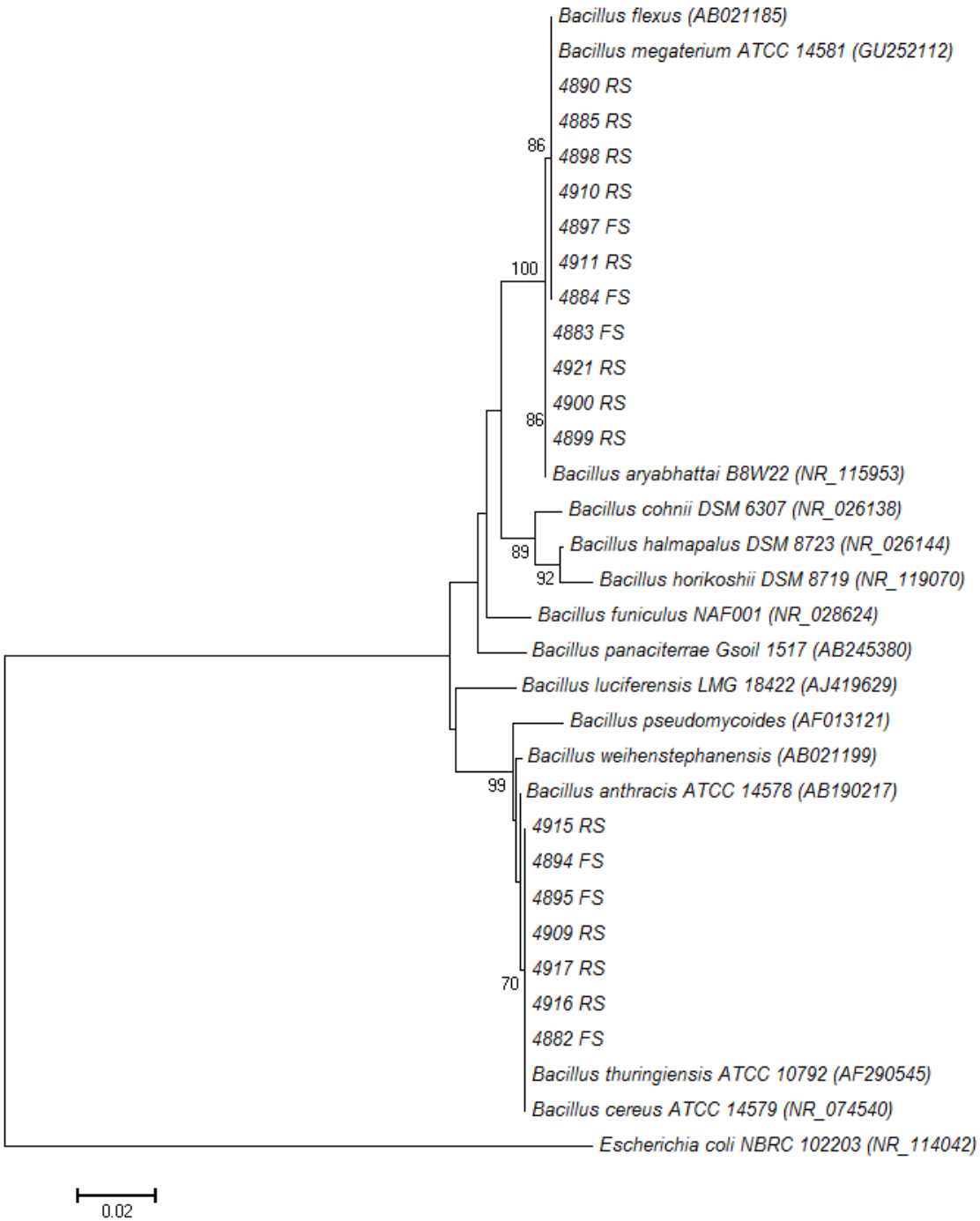


Figure 2.

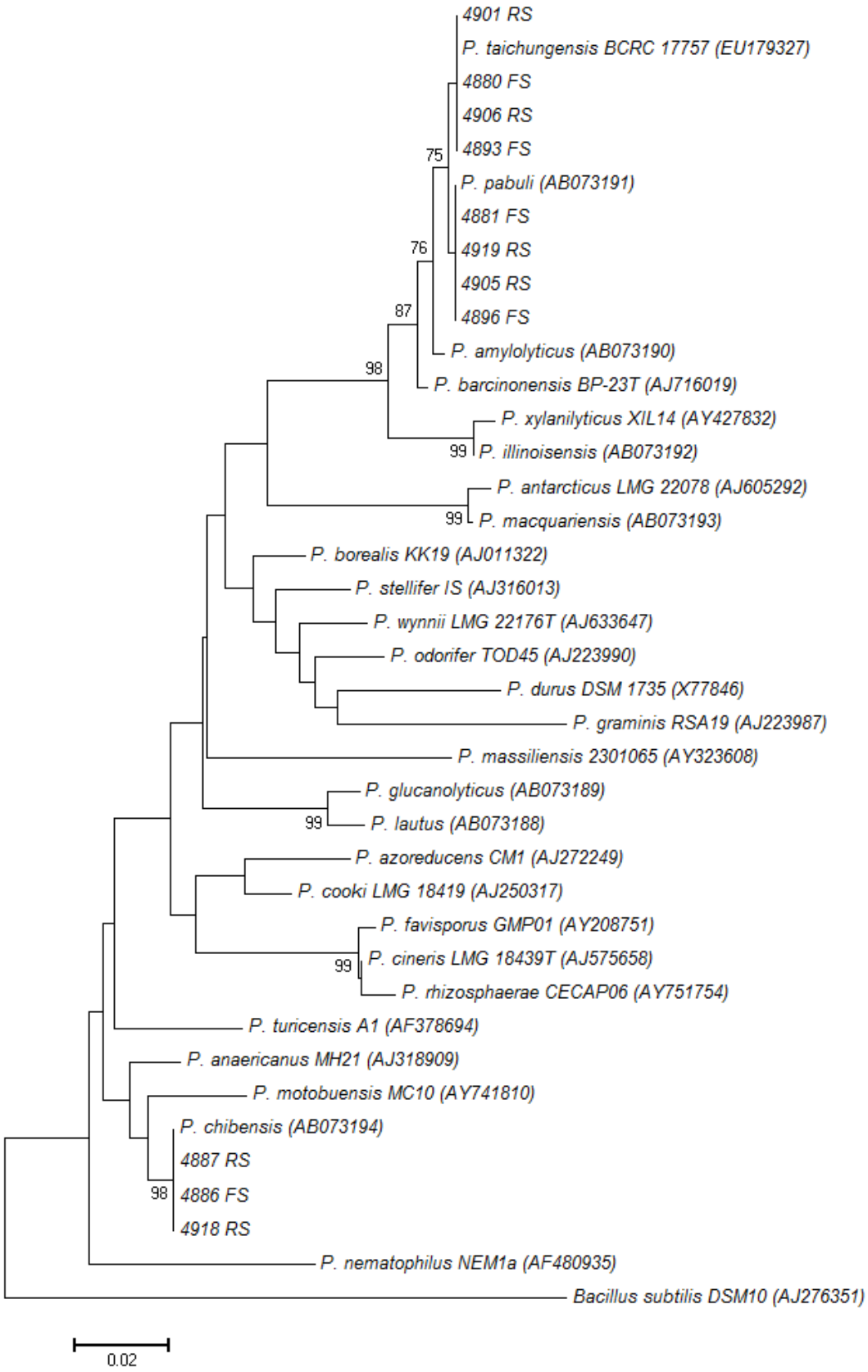

4901 RS
P. taichungensis BCRC 17757 (EU179327)
4880 FS
4906 RS
4893 FS
P. pabuli (AB073191)
4881 FS
4919 RS
4905 RS
4896 FS
P. amylolyticus (AB073190)
P. barcinonensis BP-23T (AJ716019)
P. xylanilyticus XIL14 (AY427832)
P. illinoisensis (AB073192)
P. antarcticus LMG 22078 (AJ605292)
P. macquariensis (AB073193)
P. borealis KK19 (AJ011322)
P. stellifer IS (AJ316013)
P. wynnii LMG 22176T (AJ633647)
P. odorifer TOD45 (AJ223990)
P. durus DSM 1735 (X77846)
P. graminis RSA19 (AJ223987)
P. massiliensis 2301065 (AY323608)
P. glucanolyticus (AB073189)
P. lautus (AB073188)
P. azoreducens CM1 (AJ272249)
P. cooki LMG 18419 (AJ250317)
P. favisporus GMP01 (AY208751)
P. cineris LMG 18439T (AJ575658)
P. rhizosphaerae CECAP06 (AY751754)
P. turicensis A1 (AF378694)
P. anaericanus MH21 (AJ318909)
P. motobuensis MC10 (AY741810)
P. chibensis (AB073194)
4887 RS
4886 FS
4918 RS
P. nematophilus NEM1a (AF480935)
Bacillus subtilis DSM10 (AJ276351)
75
76
87
98
99
99
99
98
0.02

Figure 3.

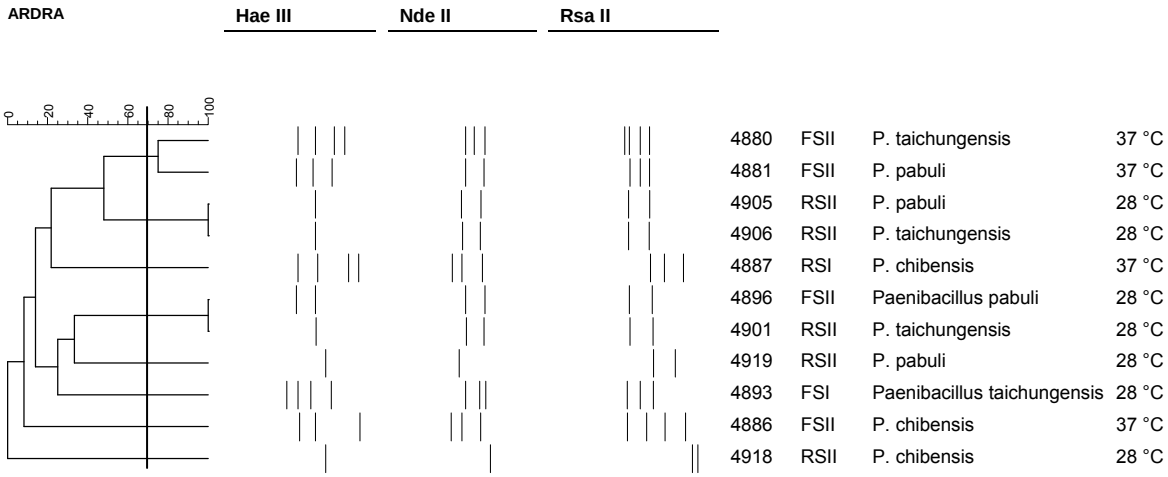
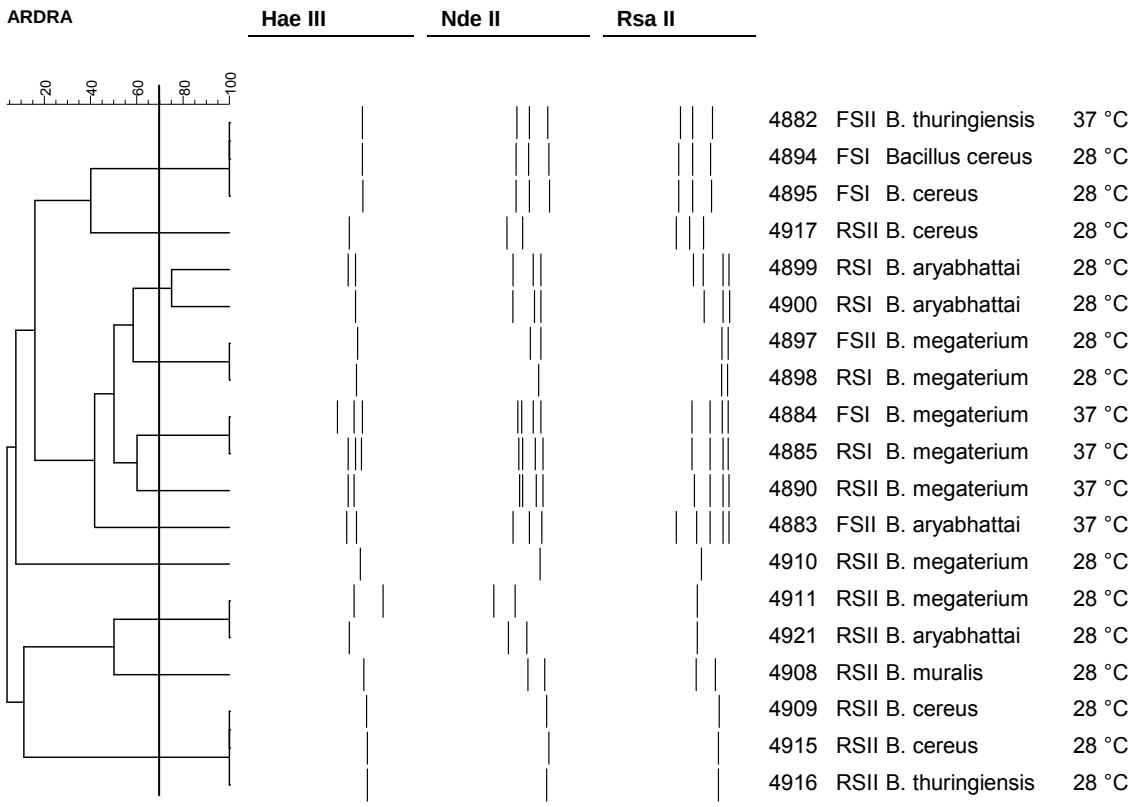

ARDRA
Hae III
Nde II
Rsa II
0
20
40
60
80
100
4880 FSII P. taichungensis 37 °C
4881 FSII P. pabuli 37 °C
4905 RSII P. pabuli 28 °C
4906 RSII P. taichungensis 28 °C
4887 RSI P. chibensis 37 °C
4896 FSII Paenibacillus pabuli 28 °C
4901 RSII P. taichungensis 28 °C
4919 RSII P. pabuli 28 °C
4893 FSI Paenibacillus taichungensis 28 °C
4886 FSII P. chibensis 37 °C
4918 RSII P. chibensis 28 °C

Figure 4.


ARDRA
Hae III
Nde II
Rsa II
20
40
60
80
100
4882 FSII B. thuringiensis 37 °C
4894 FSI Bacillus cereus 28 °C
4895 FSI B. cereus 28 °C
4917 RSII B. cereus 28 °C
4899 RSI B. aryabhattai 28 °C
4900 RSI B. aryabhattai 28 °C
4897 FSII B. megaterium 28 °C
4898 RSI B. megaterium 28 °C
4884 FSI B. megaterium 37 °C
4885 RSI B. megaterium 37 °C
4890 RSII B. megaterium 37 °C
4883 FSII B. aryabhattai 37 °C
4910 RSII B. megaterium 28 °C
4911 RSII B. megaterium 28 °C
4921 RSII B. aryabhattai 28 °C
4908 RSII B. muralis 28 °C
4909 RSII B. cereus 28 °C
4915 RSII B. cereus 28 °C
4916 RSII B. thuringiensis 28 °C

5.3 Artigo III: Bacteria isolated from shale byproducts can enhance *Brachiaria ruziziensis* growth

5.3.1 Abstract

Some soil/sediment bacterial isolates produce a wide gamma of beneficial metabolites for plant growth. We aimed to analyse the biochemical traits and the potential plant growth-promoting of selected bacteria isolated from shale byproducts as microbial inoculant to *Brachiaria ruziziensis*. In total, 40 bacteria were isolated from fine shale and retorted shale byproducts by serial dilution. Among these 40 bacterial strains, 39 were able to produce indole-3-acetic acid (IAA) in tryptophan-supplemented medium, with values ranging from 0.02 to 86.12 mM mL^{-1} IAA, while 50% showed potential of phosphate solubilization and 35% was able to produce siderophore in specific agar medium. The ability to survive in mineral medium containing the byproducts were demonstrated by strains of *Paenibacillus pabuli* (4881), *B. aryabhattai* (4883) and *B. megaterium* (4884 and 4897) after 40 days of incubation. To verify the *e*ffect of some selected isolates based on the plant growth traits a pot experiment was conducted with *Brachiaria ruziziensis.* The isolate of *Bacillus aryabhattai* (IPR4883), a mix of five strains (grown separately – GS and grown together – GT) and *Azospirillum brasiliense* strains (AbV5 and AbV6) were inoculated on *Brachiaria ruziziensis*. Plant inoculation study indicated that *B. aryabhattai* strain IPR4883 isolated from RS provides increases in tiller numbers, shoot and root mass of *B. ruziziensis* similar to treatment of nitrogen fertilizer (urea). This study indicates the potential of this strain from RS for inoculant production using the shale byproducts as microbial carrier for enhancing plant growth and nutrients uptake.

Keywords: fine shale, retorted shale, 16S rRNA, IAA, PGPB, siderophore.

5.3.2 Introduction

Shale is a sedimentary rock that contains kerogen as organic matter and decomposes under heating, producing oil and gas (BRENDOW, 2003; PEREIRA & VITTI et al., 2003). Brazil holds the Irati Formation, a large shale reserve with a significant part is located in São Mateus do Sul – Paraná. Petrobras explore this reserve applying the Petrosix® process to mining and extract oil from shale through the pyrolysis process. This exploration generates tons of solid byproducts, such as the fine shale particles (FS) that constitutes 20% of mined rock not usable in the oil extraction in addition to the retorted shale (RS) originated after pyrolysis. The RS returns to the mine after oil extraction and cooling, where is exposed to air until receiving its final destination (COSTA et al., 1996; PIMENTEL, 2005; PIMENTEL et al., 2006). These byproducts have shown potential to be used in agriculture as fertilizers (CHAVES & VASCONCELOS, 2006; ANDRADE, 2013).

World agricultural practice is moving toward a more sustainable and environmentally friendly approach without decreasing productivity. This includes the increasing use of transgenic plants, plant growth-promoting bacteria (PGPB) and new fertilizers as a part of mainstream agricultural practice, mainly considering that the indiscriminate use of fertilizers has led to substantial pollution of soil, air and water; besides the deleterious effects on soil microorganism and losses by leaching and groundwater pollution.

PGPB have various effect mechanisms in plant growth, and the prospects in the not too distant future will begin to replace the use of chemicals in different agriculture practices and environmental cleanup strategies (GLICK, 2012). These bacteria may exist in the rhizosphere, rhizoplane or in the spaces between the cells of root cortex and inside specialized structures of root cells. They mediate plant growth promotion directly by their ability for nutrient supply (nitrogen, phosphorus, potassium and essential minerals) or by modulating plant hormone levels, or indirectly by decreasing the inhibitory effects of various pathogens on plant growth and development in the forms of biocontrol agents, root colonizers, and environmental protectors (GUPTA et al., 2015). It is very important to search for specific microbial strains which can be

used as a growth promoting/enhancing inoculum to achieve desired crop production (DEEPA et al., 2010).

Microorganisms in different sources and types of shale have been reported in literature (JOHNSON and HALLBERG, 2007; MATLAKOWSKA and SKLODOWSKA, 2009; DRAGUTINOVIC and VRVIC, 2012), however there aren't studies about the microbial community from Irati Formation. Due the possibility of RS and FS use in Brazilian agriculture, it is necessary identify its microbial community; which impacts and/or benefits microorganisms can bring to the soil and crops; suggest how bacteria can act providing shale complexed nutrients or investigate its plant growth promoting potential adding value to these byproducts. Matlakowska et al. (2010; 2012) demonstrated that indigenous bacteria play a prominent role in the weathering of black shale and in the biogeochemical cycles of elements occurring in this rock.
In this study we suggest that indigenous or settler's microorganisms can be used as PGPB or in a shale biodisponibilization process, mainly considering that microorganisms isolated from byproducts have advantages of surviving in its material. In this context, the aims of this study were to characterize by biochemical techniques the cultivable bacteria isolated from FS and RS byproducts and to evaluate the potential of selected strains as plant growth promoting bacteria when inoculated on *Brachiaria ruziziensis*.

5.3.3 Material and methods

5.3.3.1 Sampling and chemical characterization

Samples of shale byproducts, FS and RS were collected from twelve different points in storage piles at Schist Industrialization Business Unit (Six) – Petrobras, in São Mateus, Paraná - Brazil (25° 52' 26" S, 50° 22' 58" W). Two samples of FS and RS were sent to the Soil Microbiology Laboratory of Agronomic Institute of Paraná - IAPAR and stored at 25°C until be processed. Chemical analyses were conducted at Soils Laboratory (IAPAR) according to the routine procedures (PAVAN et al., 1992). The following characteristics were determined: phosphorus (P), potassium (K), calcium (Ca), magnesium (Mg), sulphur (S), copper (Cu), zinc (Zn), boron (B),

manganese (Mn), iron (Fe), chromo (Cr), cobalt (Co), nickel (Ni), lead (Pb), cadmium (Cd), barium (Ba) and total carbon (C) (Table 1). The byproducts moisture determination was made by gravimetric method, based on the sample weight loss after oven-dried at 105°C overnight (ANDRADE & HAMAKAWA, 1994) and the electrical conductivity (μS cm^{-1}) was determined using conductivity, adjusted to ambient temperature.

5.3.3.2 Bacterial isolation

Viable bacteria were isolated by serial dilution plating method of each byproduct. Approximately 10 g of each byproduct were grounded in 1:10 (w/v) saline solution (0.85% NaCl) with glass beads and serially diluted up to 10^{-7}. One hundred microliters of the 10^{-2} to 10^{-4} dilutions were seeded in modified DYGS agar medium (g L^{-1}): 2.0 glucose; 1.5 peptone; 2 yeast extract; 0.5 KH_2PO_4 7.H_2O; 0.5g $MgSO_4$ 7.H_2O; pH 6.0 (RODRIGUES NETO & MALAVOLTA, 1986). Plates were incubated for 7 days at 28 °C and 37 °C to expand the growth of some mesophilic bacteria groups. Macroscopically unique colonies were subcultured on DYGS agar to establish pure cultures for further experimentation. Isolates from single colonies were deposited in IPR Collection of Microorganisms of Agricultural Interest - Soil Microbiology Lab - IAPAR. The maintenance of bacterial isolates being in liquid glycerol at -20°C and lyophilized ampoules. These strains are also deposited lyophilized at the Diazotrophic and Plant Growth Promoting Bacteria Culture Collection of Embrapa Soja (WFCC Collection # 1213, WDCM Collection # 1054) with the same IPR number designation.

All strains were identified by analysis of 16S rRNA gene sequence. The partial 16S rRNA region was amplified using the universal bacterial fD1 and rD1 primers as described in GOES et al., 2015. The gene sequences were compared with sequences in NCBI (National Center for Biotechnology Information) [http://www.ncbi.nlm.nih.gov/] with BLASTn (Basic Local Alignment Search Tool) and against the RDP (Ribosomal Database Project) [http://rdp.cme.msu.edu/index.jsp] database with the aid of Classifier tool for taxonomic identification of strains.

5.3.3.3 Biochemical characterization

Indole-3-acetic acid (IAA) production

To detect and quantify IAA production, each strain was inoculated in DYGS liquid medium supplemented with D-L-tryptophan (100 µg mL^{-1}). The cultures were incubated at 28ºC for 5 days in the dark. Then, the cell suspensions were centrifuged at 8000 rpm for 5 min to remove cells, and the concentration of IAA in 1 mL of supernatant was determined by Salkowsky colorimetric method (GORDON & WEBER, 1951) [1 mL of $FeCl_3.6H_2O$ 0.5 M in 50 mL of $HClO_4$ 35%] by measuring the absorbance at 540 nm with a spectrophotometer (Genesys 10 uv - Thermo Electron Corporation). A standard curve of 0 to 200 µM mL^{-1} of indol-3-acetic acid was used for IAA quantification. All assays were performed in triplicate.

Protein quantification

After determination of IAA analysis, the cells sediments were used for protein quantification by Bradford method (BRADFORD, 1976). Cells suspension (1 mL) was mixed with NaOH 1 M (1 mL), boiled (90°C, 15 min) to disrupt the membranes and release the total cellular contents. After disrupt 300 µl of Comassie Brilliant Blue G-250 reagent (1 M) was added. After five min of incubation the protein content of each sample was measured by the absorbance at 595 nm using spectrophotometer. A standard curve of 0 to 500 µg mL^{-1} of serum albumin was used for protein quantification. Experiments were performed in triplicate.

Siderophore production

Siderophore production was assayed using the modified SCHWYN & NEILANDS, (1987) method. One drop of bacterial cultures was inoculated onto plates containing DYGS solid medium supplemented with Chrome Azurol S (CAS), Cetyl Trimethyl Ammonium Bromide (CTAB) and Fe^{+3} solutions (1 mM $FeCl_3.6H_2O$, 10 mM HCl). The plates were incubated at 28°C for 3 days and detection of orange halo zone around

the colonies was assumed as the ability to produce siderophores. Evaluations were performed in triplicate.

Phosphate solubilization potential

Each bacterial culture was tested to phosphate solubilization using a solid medium containing insoluble calcium phosphate $Ca_3(PO_4)_2$ according to Nautiyal (2000). One drop of bacterial cultures was inoculated onto plates containing medium and were incubated at 28°C for 3 days. Formation of a clear halo around the bacterial colony indicated the phosphate solubilization potential. Evaluations were performed in triplicate.

5.3.3.4 Bacterial survival in shale byproducts

To test the ability of *P. pabuli* (IPR4881), *Bacillus aryabhattai* (IPR4883) and *B. megaterium* (IPR4897 and 4884) strains to survive in shale byproducts, a liquid medium containing FS and RS byproducts were used. Isolates were growth in 10 mL of Luria Bertani (LB) medium, for 24 h at 28 °C under constant shaking (100 rpm). The two byproducts (proportions of 10 g L^{-1} and 15 g L^{-1}) were sterilized by autoclaving for 20 min at 121 °C. Aseptically was added to byproducts 45 mL of J.E. mineral medium (g L^{-1}): 0.5 K_2HPO_4; 0.5 $(NH_4)_2SO_4$; 0.5 $MgSO_4.7H_2O$; 0.01 $FeCl_2.1H_2O$; 0.01 $CaCl_2$; 0.0001 $MnCl_2$; 0.0001 $ZnSO_4$ (JONES & EDINGTON, 1968) and inoculated 5 mL of each strain. Samples were incubated at room temperature. After 5, 10 and 40 days of incubation, survival were analyzed by serial dilution plating technique on LB agar and number of viable cells was estimated as colony forming units (CFU). All experiments were performed in triplicate.

5.3.3.5 Effects of PGPB strains inoculation in *Brachiaria ruziziensis*

Effect of bacterial inoculation on plant growth was examined in *B. ruziziensis*, in a greenhouse experiment using a sandy texture soil (Table 2), during September to December 2014.

Table 2. Selected chemical characteristics of soil used in the plant growth promoting bioassay.

P	C	pH	Al	H+Al	Ca	Mg	K	Base sum	CEC	Base sat	Al sat
$mg.dm^3$	$g.dm^3$			……$cmol_c.dm^3$……						…..%.....	
5.6	5.37	4.6	0.15	3.68	1	0.65	0.1	1.75	5.43	32.22	7.89

Total Carbon by Walkley and Black; P and K in Mehlich-1; H+Al in SMP; Ca, Mg and Al by KCl 1 mol L^{-1}; CEC – Cation exchange capacity; Base Sat –saturation.

Treatments evaluated were: i. control without inoculation; ii. nitrogen fertilized control; iii. *B. aryabhattai* strain IPR4883; iv. a mix of five bacteria strains (grown separately – GS); v. a mix of five bacteria strains (grown together – GT); and vi. *Azospirillum brasilense* strains AbV5 and AbV6 in a complete randomized design with five replications. The bacteria mixture contained the following strains: IPR4881 (*Paenibacillus pabuli*), 4883 (*B.aryabhattai*), 4884 (*Bacillus megaterium*) and 4897 (*B. megaterium*), grown separated or grown in the same medium to prepare the inoculum. Twenty seeds were sown in four liter pots. The phosphate fertilization was performed in all treatments, using 24 ppm phosphorus, which resulted in 480 mg per pot. Seeds were inoculated according to the treatments at planting adding 0.5 g of inoculant per pot. In the nitrogen fertilizer treatment it was applied 120 mg dm^3 of urea per pot.

Inoculum and plant analyses

Bacterial cultures were grown in 250 mL Erlenmeyer flasks filled with 100 mL of LB medium that were kept on shaker at 200 rpm for 72 h. The inoculant was prepared by adding 40 mL of bacterial cultures in 160 g of RS or FS byproducts sterilized by gamma radiation at 50 kGy. The number of cells in the inoculum used was approximately $2x10^{10}$ CFU g^{-1}. The growth parameters evaluated were height, number of leaves, number of tillers and root length, and biomass parameters was dry weight of shoot and root. The nutrition parameters evaluated were nitrogen (N), phosphorus (P), potassium (K), magnesium (Mg), calcium (Ca), and sulfur (S) in the aerial part, analysed according to Pavan et al., (1992) method, after ninety days of

growth in a greenhouse. All data were submitted to analysis of variance and t test at 5 % significance level to compare means using SASM-Agri version 8.2 (CANTERI et al., 2001).

5.3.4 Results

5.3.4.1 Chemical characteristics

Samples of FS (I and II) and RS (I and II) showed different physicochemical characteristics (Table 1). The total carbon and phosphorus contents had a slight difference among FS and RS samples. The major values of electrical conductivity (I=2.675 and II=3.451 μS cm^{-1}) and moisture content (I=5.96 and II=7.67) were found to FS byproducts samples. The byproducts differed to calcium, sulphur, zinc and iron content, the major values were found in RSII sample (Ca=26.97 and S=29.52 g Kg^{-1}; Zn=188.52 and Fe=20329.41 mg Kg^{-1}). The two RS samples had major values of Cu, Mn, Cr, Co and Ni content comparing to the FS samples. Barium was present in both sample types, but the FSI and RSII samples exhibited a higher level (I= 96.68 and II=92.14) than the FSII (87.63) and the RSI (I=50.66) samples.

5.3.4.2 Culturable bacteria isolated from shale byproducts

A total of 40 bacterial strains were obtained from FS and RS byproducts as well in the two temperatures using cultivation based analysis. Isolates were obtained in all dilutions mostly from the 10^{-3} dilution and from the plates that were incubated at 28 °C. A difference in the number of isolates among the byproducts was observed, five isolates were obtained from FS at 28°C and six from 37°C (Table 3). A higher number of different colony morphotypes was isolated from the RS that harbored the largest number of bacteria, at 28°C of incubation were obtained twenty-two isolates and six from 37°C (Table 3).

Among the byproducts, bacteria belonging to the genus *Bacillus* and *Paenibacillus*, which occur abundantly in most rhizospheric soils, prevailed in the RS and FS byproducts. With respect to the bacterial strains defined by the phylogenetic analysis (data not show), six *Bacillus* (1 *B. aryabhattai*, 2 *B. cereus*, 2 *B. megaterium* and 1 *B. thuringiensis*) and five *Paenibacillus* (1 *P. chibensis*, 2 *P. pabuli* and 2 *P.*

thuringiensis) were obtained from FS samples. On the other hand, an *Arthrobacter oryzae*, thirteen *Bacillus* (3 *B. aryabhattai*, 3 *B. cereus*, 5 *B. megaterium,* 1 *B. muralis* and 1 *B. thuringiensis*), six *Paenibacillus* (2 *P. chibensis,* 2 *P. pabuli* and 2 *P. taichungensis*), four *Ralstonia* (3 *R. pickettii* and 1 *R. mannitolilytica*), one *Sphingomonas* sp., one *Serratia marcescens*, one *S. nematodiphila*, one *Terrabacter tumescens* and one *Xanthobacter autotrophicus* were isolated from the RS byproducts (Table 3).

5.3.4.3 Biochemical characterization

We evaluated the abilities of the isolates to produce IAA, protein, siderophores, and solubilize P. The ability to produce IAA is associated to bacterial plant-promoting activity and the ability to solubilize calcium phosphate, the potential to provide nutrition to plants. These tests were used to evaluate the potential use of isolates as biofertilizers. Of the 40 isolates, 38 were able to produce IAA and the amount of IAA produced by individual isolates ranged from 0.02 to 86.12 mM mL^{-1} IAA (Table 3). Among isolates from FS byproduct 90% were able to produce IAA and all isolates from RS isolates were able to produce IAA. The highest value was recorded for the IPR-4887 isolated from RS and phylogenetically related to *Paenibacillus chibensis*. Strains isolated from FS present the lower levels of IAA production. The amount of protein produced by individual isolates ranged from 9.23 to 360 µg mL^{-1} protein.

The ability to produce siderophores was identified in a total of fourteen strains (five from FS and nine from RS), based on the observation of an orange halo around the colonies in the plate assay (Table 3). Most isolates from RS did not produce siderophores. The potential P-solubilization was observed in 20 isolates, being 12 from RS and eight from FS that formed a clear halo around colonies in the plate assay.

5.3.4.4 Bacterial survival test in shale byproducts

Bacterial survival in mineral medium containing FS and RS byproducts was conducted for 40 days with *Bacillus* (IPR-4883 FS and 4884 FS) and *Paenibacillus* (4881 FS and 4896 FS) strains. After five days, strains from FS showed the major CFU (82.21 to 114.15 CFU x 10^7 mL^{-1}) in the RS and FS supplemented medium, it

was observed an increase in the CFU number at the higher byproducts doses. After 10 and 40 days, strains maintained the cells viability but decrease the CFU number. Strains IPR4881 (*P. pabulli*) and IPR4883 (*B. aryabhattai*) remained viable after 40 days showing the higher numbers of CFU (37.49 and 63.32 CFU x 10^7 mL^{-1}) in RS and FS medium, respectively.

Considering the results of plant growth-promotion characterization, potential candidates were selected to plant inoculation experiments. From the total 43 strains, four of them presented at least three plant growth-promotion traits in potential: IPR4883 (*Bacillus aryabhattai*), 4884 (*B. megaterium*), 4881 (*P. pabuli*) and 4896 (*P. pabuli*).

5.3.4.5 Effects of bacteria inoculation on *Brachiaria ruziziensis* growth

To prove the effects of bacterial inoculation on plant growth and biomass of *ruzi grass* plants, four selected isolates were inoculated using as vehicle the gamma-sterilized byproducts. We included one positive control in the experiment, the strains AbV5 and AbV6 (*A. brasilense*) which are used as a biofertilizer, one fertilizer control and an absolute control. The chemical characteristics of the soil used in bioassay for plant growth promoting evaluations were present in Table 2.

To *B. ruziziensis* growth and biomass the inoculation of Bacterial mixture (GT), in FS vehicle, provided the major plant height comparing others treatments. To number of leaves, results not differ statistically. Results of tillers and shoot dry weight shows that the inoculation of isolate IPR-4883 (*B. aryabhattai*), in FS and RS vehicle, bacteria mixture GS in RS, AbV5 and AbV6 RS not differ to fertilizer treatment. To dry weight of roots only AbV5 and AbV6 FS inoculation and absolute control provided smaller statistical values compared to the others treatments that differ statistically (Table 4).

Only K nutrition parameter was influenced by inoculation of bacteria growing in FS and RS inoculant in *B. ruziziensis*, all treatments showed significant major value than absolute control. In relation of others nutritionals parameters, N, P, Mg, Ca and S, the fertilizer control treatment provided the major values (Table 5).

5.3.5 Discussion

The role of byproduct in the associated bacterial population is unclear, but a low number of bacteria recovery from shale samples has been previously reported (MATLAKOWSKA & SKLODOWSKA, 2009; MATLAKOWSKA et al., 2012). The FS generated a total of 11 bacterial strains, corresponding to 27.5% of the total isolated. This differed from the bacterial associated to the RS byproduct, where a total of 29 different colony morphotypes were isolated (corresponding to 72.5%). This higher number of isolates recovery from RS maybe due to recolonization process, mainly considering the pyrolysis process suffered by this byproduct.

A number of different bacterial groups being considered as PGPB include *Acinetobacter*, *Agrobacterium*, *Arthrobacter*, *Azotobacter*, *Azospirillum*, *Burkholderia*, *Bradyrhizobium*, *Rhizobium*, *Frankia*, *Serratia*, *Thiobacillus*, *Pseudomonads* and *Bacillus* (GLICK, 1995; VESSEY, 2003).

In the rhizosphere, species of these two genera (*Bacillus* and *Paenibacillus*) are involved in atmospheric nitrogen fixation, solubilization of soil phosphorus and uptake of micronutrients, production of phytohormones and antimicrobial metabolites. Multiple species of the genus affect the crop growth and its health by different ecological mechanisms. In recent years, *Bacillus* and *Paenibacillus* spp. attracted considerable attention because of their advantages over other PGPB strains in inoculant formulations, stable maintenance in rhizosphere soil, and greater potentials in sustainable agriculture (GOVINDASAMY et al., 2010). The other genus isolated in this study, as *Arthrobacter* and *Serratia* belongs to PGPB list and have been reported in literature being analyzed for some plant-growth promoting traits (ALMAGHRABI et al., 2013; DONG et al., 2014; AGBODJATO et al., 2015).

Results of this study suggest that an inoculation of bacteria mixture composed by *Bacillus* and *Paenibacillus* strains isolated from FS and RS, using the FS as vehicle, provided the major plant height of *B. ruziziensis* in a greenhouse condition. Araújo et al. (2012) inoculated 45 isolates of *Bacillus* sp. in *Brachiaria brizantha* seed during 180 days in a greenhouse experiment. As final evaluation seven isolates were able to root colonization and three were able to promote the growth of *B. brizantha*. Results

in literature prove the plant-growth promoting potential by *Bacillus* sp. in different cultures (ARAUJO & GUERREIRO, 2010; FIGUEIREDO et al., 2010).

Considering that some PGPB strains can improve plant growth with others mechanisms, the association of beneficial bacteria with *B. ruziziensis* can be a good alternative to supply and improve the plant nutrition. Kelemu et al. (2011) studied three bacterial strains previous isolated from *Brachiaria* hybrid CIAT 36062 and inoculated into *Brachiaria* hybrid cv. Mulato. Under conditions of nutrient deficiency, inoculated Mulato plants had significantly higher biomass production, chlorophyll and total nitrogen contents in leaves than to control plants and were darker green, confirming effects of benefit bacteria in plant-growth. Hungria et al. (2016) observed increases of 5.4% and 22.1% of two genotypes of *Brachiaria* spp. (=*Urochloa* spp.) in response to N-fertilizer alone and to N-fertilizer in combination with *Azospirillum brasilense*, respectively, The inoculation with this PGPB may represent a key component of programs to reclaim degraded pastures according to these authors.
In conclusion, the strains tested in this study exhibited some characteristics of PGPB. Selected *Bacillus* and *Paenibacillus* strains provided increase in *Brachiaria ruziziensis* growth and nutrition in greenhouse experiment. This study indicates the potential of some strains for inoculums using the shale byproducts as microbial vehicle inoculant for enhancing plant growth. However, there are needs for field experiments where these selected strains should be assessed.

5.3.6 References

AGBODJATO, N. A., NOUMAVO, P. A., BABA-MOUSSA, F., SALAMI, H. A., SINA, H., SÈZAN, A., BANKOLÉ, H., ADJANOHOUN, A., BABA-MOUSSA, L. Characterization of Potential Plant Growth Promoting Rhizobacteria Isolated from Maize (*Zea mays* L.) in Central and Northern Benin (West Africa). **Applied and Environmental Soil Science**, v. 2015, , p. 9, 2015.

ALMAGHRABI, O. A., MASSOUD, S. I., ABDELMONEIM, T. Influence of inoculation with plant growth promoting rhizobacteria (PGPR) on tomato plant growth and nematode reproduction under greenhouse conditions. **Saudi Journal of Biological Sciences**, Jan; v. 20, n. 1, p. 57–61, 2013.

ANDRADE, D. S.; HAMAKAWA, P. J. Estimativa do número de células rizóbio no solo e inoculantes por infecção em plantas. In: HUNGRIA, M.; ARAÚJO, R. S. **Manual de métodos empregados em estudos de microbiologia agrícola**. Brasília: EMBRAPA, cap.3, p. 63-94, 1994.

ANDRADE, D. S. **Processo de obtenção de veículo sólidos para inoculantes microbianos de fixadores biológicos de nitrogênio.** Brazil, 2013. Disponível em: <https://gru.inpi.gov.br/pePI/servlet/PatenteServletController?Action=detail&CodPedido=851793&SearchParameter=PI 1100001-5 A2>

ARAUJO, F.F.; GUERREIRO, R.T. Biopropspecção de isolados de *Bacillus* promotores de crescimento de milho cultivado em solo autoclavado e natural. **Ciência e Agrotecnologia**, v. 34, p. 837-844, 2010.

BRADFORD, M. M. Rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, **Analytical Biochemistry**, v. 72, p. 248–25, 1976.

BRENDOW, K. Global oil shale issues and perspectives. Synthesis of the Symposium on oil shale held in Tallinn (Estonia) on 18 and 19 November 2002. **Oil Shale**, v. 20, n. 1, p. 81-92, 2003.

CANTERI, M. G. et al. "SASM-Agri: Sistema Pará Análise e Separação de Médias em Experimentos Agrícolas Pelos Métodos Scoft-Knott, Tukey e Duncan" **Revista Brasileira de Agrocomputação**, v. 1, n. 2, p. 18-24, 2001.

CHAVES, L. H. G.; VASCONCELOS, A. C. F. Changes in soil chemical attributes and corn growth due to schist application. n. 83, p. 84–88, 2006.

COSTA, A. C. A; MESQUITA, L. M. S.; TORNOVSKY, J. Bath and continuous heavy metals biosorsion by brown seaweed from a zinc-production plant. **Minerals engineering**, Rio de Janeiro, v. 9, p. 811-824, 1996.

DEEPA, C. K.; DASTAGER, S.G.; PANDEY, A. Isolation and characterization of plant growth promoting bacteria from non-rhizospheric soil and their effect on cowpea (*Vigna unguiculata* (L.) Walp.) seedling growth*.* **World Journal of Microbiology and Biotechnology***,* v. 26, p. 1233–1240, 2010.

DONG, R., GU, L., GUO, C., XUN, F., LIU, J. Effect of PGPR *Serratia marcescens* BC-3 and AMF *Glomus intraradices* on phytoremediation of petroleum contaminated soil. **Ecotoxicology**, v. 23, n. 4, p. 674-680, 2014.

DRAGUTINOVIĆ, V.; VRVIĆ, M. M.; SWIECICKA, I.; CVETKOVIĆ, O.; BERIĆ, T.; STANKOVIĆ, S. Characterization of New *Bacillus circulans* Strain from Oil Shale, **Food Technology and Biotechnology**, v. 50, n. 1, p. 123–127, 2012.

FIGUEIREDO, M.V.B.; SELDIN, L.; ARAUJO, F.F. et al. Plant growth promoting rhizobacteria: fundamentals and applications In: MAHESHWARI, D.K. (Ed.) **Plant growth and health promoting bacteria**.1.ed. Berlin: Springer-Verlag, v. 18, p. 45-68, 2010.

GLICK, B.R. The enhancement of plant growth by free living bacteria. **Canadian Journal of Microbiology**, v. 4, p. 1109–1114, 1995.

GLICK, B. R. Plant growth-promoting bacteria: mechanisms and application. **Scientifica**, v. 2012, p. 15, 2012.

GOES, K. C. G. P. Caracterização de microrganismos isolados do xisto pirobetuminosos da área de mineração em São Mateus do Sul, Paraná. Universidade Estadual de Londrina, 2016.

GORDON, S. A.; WEBER, R. P. Colorimetric estimation of indoleacetic acid. **Plant Physiology**, Baltimore, v. 26, n. 1, p. 192-195, 1951.

GOVINDASAMY, V., SENTHILKUMAR, M., MAGHESHWARAN, V., KUMAR, U., BOSE, P., SHARMA, V., ANNAPURNA, K. *Bacillus* and *Paenibacillus* spp.: Potential PGPR for Sustainable Agriculture**. Plant Growth and Health Promoting Bacteria**, v. 18 of the series Microbiology Monographs, p. 333-364, 2010.

GUPTA, G., PARIHAR, S. S., AHIRWAR, N. K., SNEHI, S. K., SINGH, V. Plant Growth Promoting Rhizobacteria (PGPR): Current and Future Prospects for Development of Sustainable Agriculture. **Journal of Microbial & Biochemical Technology**, v. 7, p. 96-102, 2015.

HUNGRIA, M., NOGUEIRA, M. A., ARAUJO, R. S. Inoculation of *Brachiaria* spp. with the plant growth-promoting bacterium *Azospirillum brasilense*: An environment-friendly component in the reclamation of degraded pastures in the tropics. **Agriculture, Ecosystems & Environment**, v. 221, n. 1, p. 125-131, 2016.

JONES, J. G.; EDINGTON, M. A. An ecologic survey of hydrocarbon-oxidizing microorganisms. **Journal of Genetic Microbiology**, v. 52, p. 381-390, 1968.

JOHNSON, D. B., K. B. HALLBERG. "Bioprospecting for micro-organisms associated with black shale ores. Publishable Activity Report of Bioshale Project." **Bioshale project: A Specific Targeted Research Project co-funded by the Europen Commission (FP6 programme)**, 54, 2007.

KELEMU, S. et al. Detecting bacterial endophytes in tropical grasses of the Brachiaria genus and determining their role in improving plant growth. **African Journal of Biotechnology**, v. 10, n. 6, p. 965-976, 2011.

MATLAKOWSKA, R.; SKLODOWSKA, A. The culturable bacteria isolated from organic-rich Black shale potentially useful in biometallurgical procedures. **Journal of Applied Microbiology**, v. 107, p. 858-866, 2009.

MATLAKOWSKA, R., NARKIEWICZ, W., SKLODOWSKA, A. Biotransformation of organic-rich copper-bearing black shale by indigenous microorganisms isolated from Lubin copper mine (Poland). **Environmental Science & Technology**, v. 44, p. 2433-2440, 2010.

MATLAKOWSKA, R., SKŁODOWSKA, A., NEJBERT, K. Bioweathering of Kupferschiefer black shale (Fore-Sudetic Monocline, SW Poland) by indigenous bacteria: implication for dissolution and precipitation of minerals in deep underground mine. **FEMS Microbiology Ecology**, v. 81, p. 99-110, 2012.

NAUTIYAL, C. S., S. BHADAURIA, et al. "Stress induced phosphate solubilization in bacteria isolated from alkaline soils." **FEMS Microbiology Letters**, 182(2): 7, 2000.

PAVAN, M.A.; BLOCH, M.F.M.; ZEMPULSKI, H.C.; MIYAZAWA, M. & ZOCOLER, D.C. **Manual de análise química de solo e controle de qualidade**. Londrina, Instituto Agronômico do Paraná, 1992, p.40.

PEREIRA, H. S.; VITTI, G. C.; KORNDORFER, G. H. Comportamento de diferentes fontes de silício no solo e na cultura do tomateiro. **Revista Brasileira de Ciência do Solo**, vol. 27, num. 1, p. 102-108, 2003.

PEREIRA, H. S.; VITTI, G. C. Efeito do uso do xisto em características químicas do solo e nutrição do tomateiro. **Horticultura Brasileira**, v. 22, p. 317-322, 2004.

PIMENTEL, P. M. Remoção de metais pesados em águas produzidas usando xisto retortado como adsorvente. Tese (Doutorado em Ciência e Engenharia de Materiais)-Centro de Ciências Exatas e da Terra, Universidade Federal do Rio Grande do Norte, Natal, 2005.

PIMENTEL, P. M., SILVA JR, C. N., MELO, D. M. A., MELO, M. A. F., MALDONADO, G., HENRIQUE, D. M. Caracterização e uso de xisto para adsorção de chumbo (II) em solução. **Cerâmica,** v. 52, p. 194-199, 2006.

RODRIGUES NETO, J., V. A. MALAVOLTA JR., et al. "Meio simples para isolamento e cultivo de *Xanthomonas campestris* pv. citri tipo B." **Summa Phytopathologica**, v. 12, p.1-2, 1986.

SCHWYN, B., NEILANDS, J. B. Universal chemical assay for the detection and determination of siderophores. **Analytical Biochemistry**, v. 160, n. 1, p. 47-56, 1987.

VESSEY, J. K. Plant growth promoting rhizobacteria as biofertilizers. **Plant and Soil**, v. 255, p. 571-586, 2003.

5.3.7 Tables

Table 1. Physicochemical characteristics of shale byproducts samples of fine shale (FS) particles and retorted shale (RS).

Characteristics	FSI	FSII	RSI	RSII
Total C (g dm^{-3})	29.22	27.23	31.79	31.63
P (mg dm^{-3})	145.30	134.00	150.20	154.10
Moisture content (%)	5.96	7.67	3.68	3.68
Electrical Conductivity (µS.cm^{-1})	2.675	3.451	2.074	1.396
K (g Kg^{-1})	12.24	10.50	11.90	12.51
Ca (g Kg^{-1})	14.37	13.92	16.53	26.97
Mg (g Kg^{-1})	9.95	8.42	9.43	7.99
S (g Kg^{-1})	17.92	10.85	19.47	29.52
Cu (mg Kg^{-1})	36.43	31.37	59.27	54.50
Zn (mg Kg^{-1})	49.19	90.41	85.32	188.52
Mn (mg Kg^{-1})	323.76	144.38	454.61	571.62
Fe (mg Kg^{-1})	13505.00	9866.51	15566.89	20329.41
Cr (mg Kg^{-1})	34.20	33.98	45.53	43.02
Co (mg Kg^{-1})	10.77	6.71	16.79	17.08
Ni (mg Kg^{-1})	13.91	10.71	20.84	21.55
Pb (mg Kg^{-1})	12.64	11.67	13.74	12.96
Cd (mg Kg^{-1})	0.90	0.65	1.16	1.26
Ba (mg Kg^{-1})	96.68	87.63	50.66	92.14

Total Carbon by Walkley and Black; P and K in Mehlich-1; H+Al in SMP; Ca, Mg and Al by KCl 1 mol L^{-1}. K, Ca, Mg, S, Cu, Zn, Mn, Fe, Cr, Co, Ni, Pb, Cd and Ba in nitro-perchloric digestion.

Table 3. Strain designation (IPR code) from fine shale (FSI=sampling in 2008 and FSII=sampling in 2011) and retorted shale (RSI=sampling in 2008 and RSII=sampling in 2011), temperature of bacteria isolation (°C), siderophore production (Sidero), phosphate production (Phosph), protein production, proposed of taxonomic position (identification).

IPR	Source	(°C)	Sidero	Phosph	IAA mM mL^{-1}	Protein mg mL^{-1}	Identification
4894	FSI	28	-	-	0.33	73.68	*B. cereus*
4895	FSI	28	-	-	2.34	73.62	*B. cereus*
4897	FSII	28	+	+	14.55	53.73	*B. megaterium*
4893	FSI	28	-	+	0.02	56.71	*P. taichungensis*
4896	FSII	28	+	+	0	44.42	*P. pabuli*
4884	FSI	37	NG	+	11.23	58.14	*B. megaterium*
4883	FSII	37	+	+	7.41	46.75	*B. aryabhattai*
4881	FSII	37	+	+	1.33	56.73	*P. pabuli*
4880	FSII	37	+	+	7.53	58.25	*P. taichungensis*
4882	FSI	37	-	-	0.65	77.91	*B. thuringiensis*
4886	FSII	37	-	+	0.96	77.72	*P. chibensis*
4899	RSI	28	-	-	37.96	114.8	*B. aryabhattai*
4900	RSI	28	-	+	6.72	35.23	*B. aryabhattai*
4909	RSII	28	-	-	32.39	227.69	*B. cereus*
4911	RSII	28	-	-	15.3	94.49	*B. megaterium*
4916	RSII	28	-	-	7.45	69.39	*B. thuringiensis*
4910	RSII	28	-	-	5.13	9.83	*B. megaterium*
4908	RSII	28	-	-	8.99	88.88	*B. muralis*
4920	RSII	28	+	-	22.34	107.39	*Xanthobacter autotrophicus*
4915	RSII	28	-	-	2.96	88.09	*B. cereus*
4912	RSII	28	-	-	8.07	138.1	*Arthrobacter oryzae*
4921	RSII	28	-	+	4.7	64.66	*B. aryabhattai*
4898	RSI	28	-	-	7.41	ND	*B. megaterium*
4905	RSII	28	-	+	5.07	60.43	*P. pabuli*
4901	RSII	28	+	+	6.05	22.82	*P. taichungensis*
4906	RSII	28	-	+	7.09	47.24	*P. taichungensis*
4917	RSII	28	-	+	30.61	195.6	*Bacillus cereus*
4902	RSII	28	-	-	16.74	115.07	*Ralstonia pickettii*
4903	RSII	28	-	-	21.81	84.06	*R. pickettii*
4904	RSII	28	NG	-	28.26	122.45	*R. pickettii*
4907	RSII	28	-	+	8.43	103.16	*R. mannitolilytica*
4918	RSII	28	+	+	7.39	161.44	*P. chibensis*
4919	RSI	28	+	-	9.11	360	*P. pabuli*
4885	RSI	37	+	-	6.59	86.47	*B. megaterium*
4890	RSII	37	+	+	1.46	67	*B. megaterium*
4887	RSI	37	+	-	86.12	90.76	*P. chibensis*
4888	RSII	37	+	+	8.85	218.05	*Serratia nematodiphila*
4889	RSII	37	+	+	3.21	222.68	*S. marcescens*
4891	RSII	37	NG	-	47.23	113.65	*Sphingomonas sp.*
4892	RSII	37	-	+	38.4	226.67	*Terrabacter tumescens*

NG= not growth; ND= not determined. Phosphate solubilization potential was determined in vitro by the ability to solubilize $Ca_3(PO_4)_2$ in solid medium; Total indoles in the supernatant of isolates cultured in Dygs liquid medium supplemented with 100 µg/ml DL-tryptophan; Siderophore production determined in Dygs solid medium supplemented with chrome azurol S; ND = not determined; IAA = Indole-3-Acetic Acid; (-) = negative for evaluated characteristic; (+) = positive for evaluated characteristic.

Table 4. Influence of inoculation of selected bacteria isolated from FS and RS in *Brachiaria ruziziensis* growth and biomass. GS: strains growth separately and GT: growth together.

Treatments	Height	Leaves	Tillers	Shoot	Root
	cm	.....number.....		.....g.....	
1- IPR-4883 FS (*B. aryabhattai*)	82,4 bc	40,2	6,8 ab	10,5 ab	4,0 ab
2- Mix of Bacteria GS FS	65,9 c	25,3	4,8 b	8,5 bc	3,0 abc
3- Mix of Bacteria GT FS	111,3 a	33,0	4,75 b	9,2 bc	3,2 abc
4- AbV5 and AbV6 FS	76,3 bc	26,8	4,5 b	8,3 bc	1,9 c
5- IPR-4883 RS (*B. aryabhattai*)	84,2 bc	34,8	5,8 ab	11,3 ab	4,4 a
6- Mix of Bacteria GS RS	91 ab	32,7	5,5 ab	8,2 bc	3,0 abc
7- Mix of Bacteria GT RS	83,6 bc	31,4	5,2 b	9,1 bc	3,4 abc
8- AbV5 and AbV6 RS	91,4 ab	35,8	6,3 ab	9,9 bc	3,5 ab
9- Nitrogen fertilizer	71,6 bc	40,8	8,2 a	13,6 a	3,9 ab
10- Control	62,2 c	27,8	5,2 b	6,9 c	2,4 bc
C.V. (%)	22,2	36,8	38,1	26,7	37,9

Means followed by different letters in each column are different ($p<0.05$) by t test.

Table 5. Influence of inoculation of selected bacteria isolated from FS and RS in *Brachiaria ruziziensis* nutrition. GS: strains growth separately and GT: growth together.

Treatments	N	P	K	Mg	Ca	S
			mg.planta^{-1}			
1- IPR-4883 FS (*B. aryabhattai*)	65,9 bc	49,6 bc	88,7 a	59,9 b	57,8 bc	14,6 bc
2- Mix of Bacteria GS FS	55,7 bc	45,6 bc	82,6 ab	51,9 bc	44,1 bc	11,3 bc
3- Mix of Bacteria GT FS	64,8 bc	45,6 bc	87,7 a	52,9 bc	55,7 bc	13,8 bc
4- AbV5 and AbV6 FS	51,7 bc	37,0 c	81,0 ab	45,9 bc	42,4 bc	10,8 bc
5- IPR-4883 RS (*B. aryabhattai*)	74,7 b	52,2 bc	85,8 a	65,1 b	61,7 b	16,7 ab
6- Mix of Bacteria GS RS	56,8 bc	49,0 bc	83,0 ab	52,3 bc	49,5 bc	12,5 bc
7- Mix of Bacteria GT RS	56,9 bc	53,4 b	79,7 ab	53,0 bc	55,6 bc	13,7 bc
8- AbV5 and AbV6 RS	64,5 bc	44,9 bc	81,5 ab	58,0 bc	60,3 b	12,2 bc
9- Nitrogen fertilizer	133,1 a	79,8 a	72,9 ab	133,3 a	114,2 a	22,7 a
10- Control	45,8 c	38,9 bc	65,2 b	33,2 c	33,4 c	7,8 c
C.V. (%)	27,5	24,2	17,4	33,9	33,2	39,0

Means followed by different letters in each column are different ($p<0.05$) by t test.

6 CONSIDERAÇÕES FINAIS

Este estudo fornece os primeiros dados sobre a ocorrência e caracterização de microrganismos cultiváveis presentes nos coprodutos do xisto da Formação Irati, o que contribui para uma melhor compreensão da microbiota presente nos finos do xisto e xisto retortado.

A presença de microrganismos cultiváveis nos coprodutos é baixa, porém foram recuperadas pelo plaqueamento direto de colônias morfologicamente distintas. O xisto retortado apresentou o maior número de microrganismos cultiváveis quando comparado às amostras de finos de xisto.

Combinando os dados morfológicos e as sequências de DNA da região ITS foi possível descrever pela primeira vez, um panorama da diversidade de fungos que habitam os coprodutos do xisto da Formação Irati no Brasil. *T. sayulitensis* foi à espécie mais frequentemente encontrada no xisto retortado e *A. bohemica* nas amostras dos finos de xisto.

Entre as bactérias cultiváveis, *Bacillus* e *Paenibacillus* foram os gêneros prevalentes encontrados nos dois coprodutos.

Dentre as bactérias isoladas dos coprodutos foram encontradas estirpes com potencial para serem aplicadas como promotoras do crescimento de *Brachiaria ruziziensis*.