UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL
INSTITUTO DE BIOCIÊNCIAS - COMISSÃO DE GRADUAÇÃO
BACHARELADO EM CIÊNCIAS BIOLÓGICAS
TRABALHO DE CONCLUSÃO DE CURSO

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Genus *Anoxybacillus* as source of high value biotechnological products

Uma revisão de literatura acerca do gênero *Anoxybacillus* e seu potencial biotecnológico

Porto Alegre
Outubro/2022

PERIÓDICO ESCOLHIDO PARA PUBLICAÇÃO

Revista**: Extremophiles** Editora: Springer

ISSN eletrônico: 1433-4909 ISSN impresso: 1431-0651

Fator de impacto (2021): 3.035

CIP - Catalogação na Publicação

da Rosa, Deisiane Fernanda
Genus Anoxybacillus as source of high value biotechnological products / Deisiane Fernanda da Rosa. -- 2022.
33 f.
Orientador: Alexandre José Macedo.

Trabalho de conclusão de curso (Graduação) -- Universidade Federal do Rio Grande do Sul, Instituto de Biociências, Bacharelado em Ciências Biológicas, Porto Alegre, BR-RS, 2022.

1. Extremófilos. 2. Biotecnologia. 3. Anoxybacillus. 4. Enzimas. 5. Biorremediação. I. Macedo, Alexandre José, orient. II. Título.

Elaborada pelo Sistema de Geração Automática de Ficha Catalográfica da UFRGS com os dados fornecidos pelo(a) autor(a).

Sumário

RESUMO

Bactérias alcalófilas e termofílicas são um tópico interessante de estudos de procariontes, não apenas pela peculiaridade de ambas as condições de sobrevivência, mas como aliadas para o desenvolvimento tecnológico. O gênero *Anoxybacillus* (“An” preposição negação,” oxy” oxigênio, “bacillus” em referência a morfologia e à família) compõe o universo de termófilos da família Bacillaceae e atualmente é um dos gêneros está em ascensão nos estudos de aplicação biotecnológica. Desde a sua descrição em 2000, diferentes trabalhos associando bactérias do gênero a soluções industriais e ambientais. O artigo desenvolvido propõe uma revisão de literatura acerca do potencial biotecnológico do gênero, destacando os trabalhos desenvolvidos ao longo dos 22 anos. Para tal, desenvolveu-se uma pesquisa nos repositórios de artigos, selecionando estudos tendo a *Anoxybacillus* como foco. Da análise da literatura, percebeu-se a diversidade de enzimas descritas com possibilidade de aplicação em diferentes setores industriais, variando de produção de alimentos a produção de combustíveis, sendo a termoestabilidade e alcalinidade duas características muito ressaltada e explorada de modo geral. No âmbito ambiental, grupo se destaca por serem viáveis o uso de enzimas lacases para degradação de diferentes corantes e sequestro de metais pesados, quando aplicadas em tratamento de águas residuais (oriundas de setores industriais como setor de vestimenta). Além disso, relatos do uso de metabólitos secondarios de *Anoxybacillus* demonstram um novo seguimento que precisa ser mais explorado, visto que os poucos trabalhos publicados demonstram valores efetivos e significativos para fins de tratamento de células cancerosas e biofilms patogênicos. Apesar de toda literatura reunida indicar o quão promissor o grupo aparenta ser, muito ainda há ser estudado e aprimorado, ainda mais se aliada com os avanços das técnicas de biologia molecular e bioinformática.

INTRODUÇÃO GERAL

Através de uma concepção antropocêntrica, os ambientes hostis ao ser humano foram designados como extremos, e por muito tempo acreditou-se na inexistência de vida nesses locais. Contudo, com o avanço científico, verificou-se que seletos grupos de organismos conseguiam habitar esses ambientes e assim surge o grupo dos extremófilos, formado por diferentes táxons, mas predominantemente composto por microrganismos. Geralmente, são os principais focos de estudos e de aplicações biotecnológicas, devido a capacidade de resposta ao estresse através da produção de compostos muito estáveis, frente a diferentes condições muito semelhantes às encontradas em processos industriais e por serem economicamente viáveis quando comparados a outros organismos como animais e plantas (PEEPLES, 2014).

Existem diferentes classes extremófilos, sendo a sua classificação correspondente à maneira como seu nicho ambiental difere das condições mesofílicas (TIQUIA-ARASHIRO; RODRIGUES, 2016). Dentre as classificações, os organismos termófilos capazes de habitar locais de altas temperaturas (entre 45-80 °C), as bactérias e as arqueias são os grupos de termófilos mais conhecidos e explorados. As bactérias termófilas apresentam estratégias adaptativas relacionadas a formação de estruturas de resistência como os esporos, modificação na composição da membrana celular e produção de enzimas termoestáveis (termo-enzimas) (MERINO et al., 2019). As termo-enzimas são um dos alvos da biotecnologia, visto que alguns processos industriais acontecem em temperaturas acima de 40° (RADDADI et al., 2015). Enzimas como amilases, proteases, lipases, lacases e xilanases, tem ampla aplicação em diferentes setores industriais, variando de produtos alimentícios a produtos de limpeza, assim como aplicações em processos de biorremediação e tratamento de água.

A família Bacillaceae (filo Firmicutes) é reconhecida como uma família que abrange muitos gêneros de termófilos como *Geobacillus*, *Parageobacillus*, *Thermolonguibacillus* e *Anoxybacillus*. O gênero *Anoxybacillus* ("*An*" preposição negação," *oxy*" oxigênio, "*bacillus*" em referência à morfologia e à família) compõe o universo de termófilos da família Bacillaceae e conta com 24 espécies e 2 subespécies descritas oficialmente, sendo descrita pela primeira vez por Pikuta e colaboradores (2000). A maioria das espécies foram isoladas de fontes termais, sendo predominante em regiões da Ásia e Oriente Médio. Apesar disso, há registros de *Anoxybacillus* em diferentes locais como formações rochosas, solo e encanamentos de instalações industriais de laticínios, sendo inclusive considerada um problema para esterilização

de alimentos oriundos. Inicialmente, fora atribuída a característica anaeróbica, mas conforme estudos publicados posteriormente, demonstraram se tratar de organismos anaeróbios facultativos.

Diferentes trabalhos têm demonstrado variadas aplicações durante esses 22 anos de existência do gênero. O grupo vem recebendo atenção de pesquisas envolvendo inovação tecnológica, principalmente pelas enzimas termoestáveis, fácil manipulação molecular e a capacidade de atuar em processos de bioadsorção de metais pesados. Mesmo com a visibilidade crescente, há pouco material atualizado reunindo os estudos desenvolvidos e recentes descobertas, sendo o único trabalho exclusivo acerca do gênero fora publicado em 2013 (GOH et al., 2013).

Tendo em vista, o objetivo do trabalho consistiu na elaboração de um artigo de revisão de literatura acerca do potencial biotecnológico do gênero, destacando os trabalhos desenvolvidos ao longo dos 22 anos de existência desse grupo taxonômico que envolvesse a aplicação biotecnológica, assim como descrever descobertas importantes acerca do gênero, principalmente com o progresso da genômica, em que muito se descobriu acerca do comportamento do gênero na última década.

A pesquisa se desenvolveu a partir de análise de trabalhos publicados nos últimos 22 anos, período que compreende desde a data da descrição do gênero (2000), até agosto de 2022. A busca foi realizada nas bases de dados *Pubmed Central®*, *Web of Science* e *Science Direct*, utilizada como termo de busca: "*Anoxybacillus*", "*Anoxybacillus* biotechnology" e "*Anoxybacillus* genome", e aplicado os operadores lógicos "AND" e "OR", quando necessário. Foram selecionados apenas artigos científicos em que a Anoxybacillus fosse o foco do estudo. Para visualização dos membros do gênero, fora elaborada uma árvore filogenética agregando sequências do gene 16s RNA ribossomal de cepas tipos (o acesso ao gene obtida do artigo de descrição da espécie e reconhecida oficialmente pela *Microbiology Society*).

Os resultados da busca e refinamento da seleção resultou em cerca de 120 artigos e separados conforme o tópico possível a ser relacionado. No total, foi possível relacionar aplicações industriais (majoritariamente envolvendo enzimas), aplicações ambientais e biorremediação. Dois tópicos adicionais foram adicionados devido à importância em contextualizar as causas das características do grupo, os quais são um tópico acerca do estilo de vida (habitat) e as descobertas genômicas.

Ao analisar a literatura, é perceptível a abundância de enzimas já caracterizadas oriundas do grupo. Notável abundância por comportar poucas espécies quando comparadas a gêneros mais bem estudados como *Bacillus* e *Geobacillus*. Quando observado em relação às aplicações

ambientais, o grupo corresponde a característica desejada para tratar efluentes contaminados, principalmente com metais pesados. Além disso, apesar de apresentarem condições para produção de metabólitos secundários, ainda é uma área muito pouco explorada com as bactérias do gênero, uma vez que apenas nos últimos dois anos, trabalhos relacionados foram publicados. De modo geral, espera-se que a leitura do artigo consiga orientar pesquisadores que estejam conhecendo o grupo e desperte o interesse em desenvolver possíveis trabalhos correlacionados. Muito há ainda a ser estudado e descrito em relação ao gênero, porém com a análise realizada é possível afirmar que *Anoxybacillus* certamente é uma forte aliada para o Progresso da biotecnologia

Genus *Anoxybacillus* as source of high value biotechnological products

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Abstract:

Thermophilic and alkaliphilic microorganisms are singular organisms. As a result of survival strategies, these organisms can grow on a wide variety of substrates due to the composition of the cell wall and secreting of stable enzymes. *Anoxybacillus* is a genus of thermophilic and Alkaliphilic bacteria. The genus comprises 24 species and 2 subspecies and, over the years, has drawn attention with the advance of genomics techniques, it was identified important genes and molecular features which can be biotechnologically explored. As a relatively new genus, numerous studies identify a diversity of thermostable enzymes that can be used in industrial and environmental applications. Their biomass has been demonstrated as allied in bioremediation techniques, and recently, metabolites have been used in medicinal experiments. This review describes the principal genomics features and experimental discoveries related to potential biotechnological applications using bacteria from the genus.

Keywords: Bacillaceae**,** *Anoxybacillus*, thermophile, biotechnology, enzymes, bioremediation

Introduction:

Alkaliphilic and thermophilic bacteria are an exciting topic of prokaryotes studies. These organisms are known as a source of molecules useful for developing commercial biotechnology. In short, they can produce enzymes and proteins stable in extremes of pH and temperature. The use of these molecules is excelling in those providing benefits, such as solvent tolerance, substrate selectivity, and stability that represent a cheap and ecological alternative(Singh et al. 2016).

Anoxybacillus is a genus of thermophilic and alkaliphilic bacteria or tolerant in both conditions. All species are Gram-positive, spore-forming rods and facultative anaerobes. They belong to the Bacillaceae family, which comprises other groups of thermophiles such as *Geobacillus*, *Parageobacillus*, *Amphibacillus*, *Saccarococos*, and *Thermolonguibacillus*. The genus was proposed by Pikuta and collaborators (2000), until the paper data has 24 species and 2 subspecies. Over the years, the genus showed as a source of enzymes and an allied for environmental recovery.

The purpose of this review is to present the findings of enzymes, industrial applications, and environmental solutions through metabolites and molecules from members of the genus *Anoxybacillus*. The

group contains the ideal characteristics for obtaining potential molecules and has received attention in recent years, which intensifies the importance of analyzing the studies until described.

Materials and methods

Review literature

The Reviewing research was performed by searching the Databases "Web of science", "PubMed" and "Science Direct". The search criteria have performed using the combined fields of title, abstract, and keywords: "*Anoxybacillus*", "Biotechnology", and "*Anoxybacillus* genome", applying the Logical Operators "AND" and "OR". The selected articles have published between 2000-2022 (August). The genomes data size, was retrieve from NCBI and used only valid species.

Phylogeny

The molecular phylogenetic analysis was performed through the evaluation of highly similar sequences of type species, retrieved from the NCBI (GenBank) and subjected to multiple sequence alignment (MSA) using MAFFT and TRimm-AI used to eliminate poorly aligned positions and divergent regions of aligned sequences. The Neighbor-Joining phylogenetic tree was constructed using the Kimura two parameters, as a nucleotide model substitution.

1. Occurrence

Several *Anoxybacillus* spp. have been isolated from many countries since the first description of the genus (Pikuta et al. 2000). Most strains were isolated from hot springs, interestingly, it was found to be predominant with a large number of species in two hot springs in India (Najar et al. 2018). Some studies indicated occurrence in other extreme environments such as arid soils, and geothermal sediments (Figure 1).

Despite the occurrence typically in natural environments, these bacteria are known as a problem in dairy industries, they are commonly found as contaminant in gelatin and milk samples, to their sporulation ability which can modify the product quality (Goh et al. 2013). Furthermore, *Anoxybacillus* and *Geobacillus* frequently form biofilm consortia in milk processing plant. Interestingly, a report demonstrated that the supply of glucose and galactose following lactose degradation by *Anoxybacillus flavithermus* provided growth of *Geobacillus thermoglucosidans*, the dynamic within microbiological communities followed by these two genera, which are common to occur together (Zhao et al. 2018).

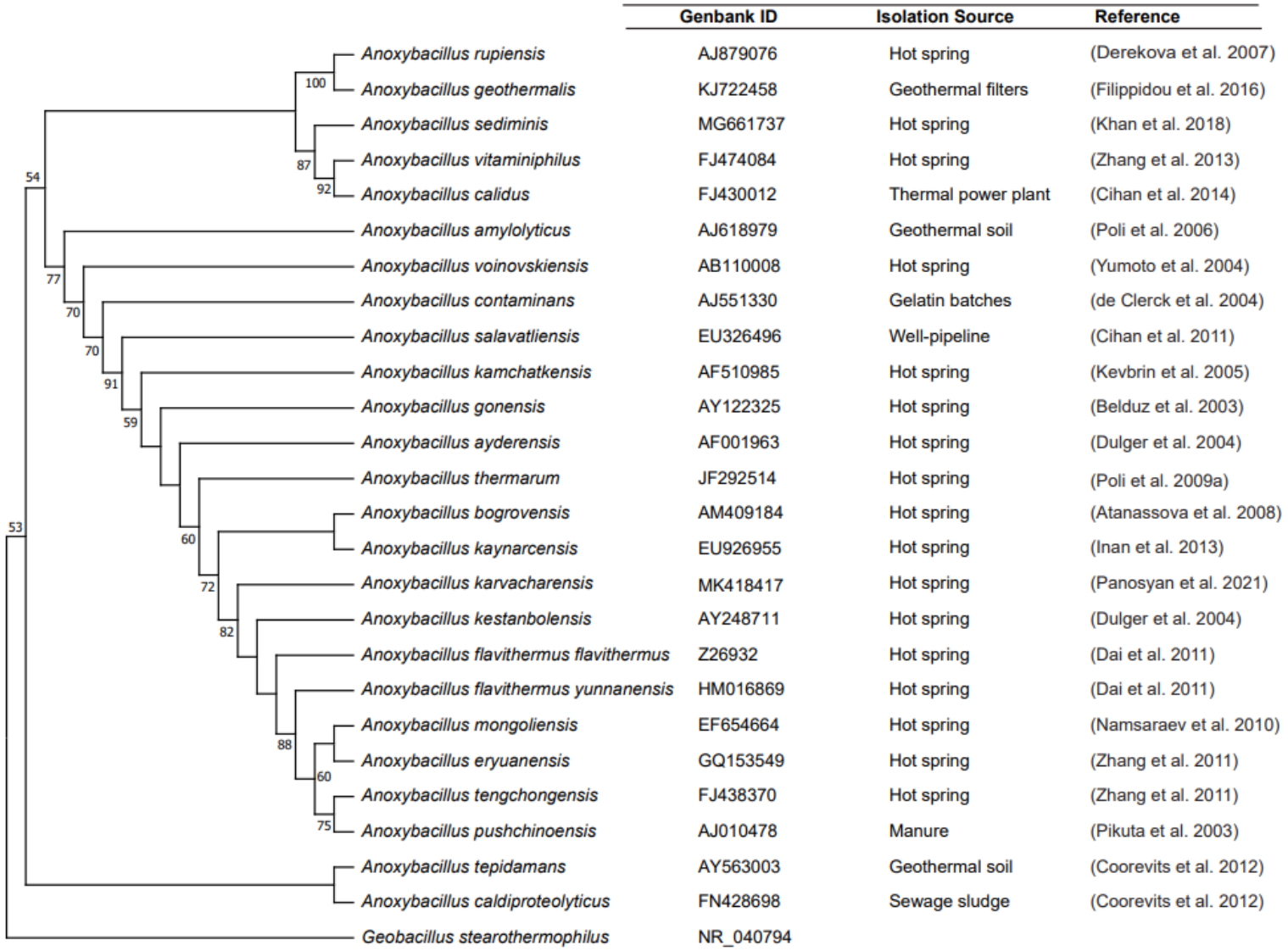

Fig. 1 – Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences. *Geobacillus stearothermophilus* was used as an outgroup. Multiple alignment was made using MAFFT. Bootstrap values based on 1000 replications were calculated and expressed as percentages. Significant bootstrapping values (>50%) are shown on the nodes. The Genbank access and the origin of isolation were obtained from literature.

2. Genome

To the date, 18 of 24 species *Anoxybacillus* have been their genome sequenced available in public databases. In general, the genomes are shorter, about 3.2 Mb (table S1), when compared with other bacteria of Bacillaceae family. *Anoxybacillus sediminis* has the the largest of all members of genus, about 3,931 Mb. The genomic DNA G+C content in media is estimated to 42%. Some genomes from *Anoxybacillus* encode numerous proteins involved chemiotaxis and motility (Saw et al. 2008; Goh et al. 2014; Khalil et al. 2019) . As expected, as a free-living environmental microorganism, the genes that encode the flagellar assembly are complete and include various ring, motor protein, hook, and filament sequences was found in *Anoxybacillus* spp. SK3-4 and DT3-1. Despite experimental motility were non-motile in certain growth media (Saw et al. 2008).

Analyzing the genome annotation, is observed a variety of genes associated to Carbohydrate metabolism, mostly glycosyl hydrolase enzymes(Goh et al. 2014; Lim et al. 2015a; Khalil et al. 2019; Yadav et al. 2021). In *A. flavithermus* AK1 strain was identify genes associated with 21 secondary metabolic pathways, as Terpenoid, caroteinod, Novabiocin and Prenyltransferases (Khalil et al. 2019), the authors reforces that the genome sequence of AK1 strain is not fully assembled and out of the total 21 secondary metabolic pathways, 5 pathways have 4 or more genes, and the remaining 16 pathways have only 3 or fewer genes. Recently, a study using several strains of *A. flavithermus,* predicted that the classes of genes most frequently involved in secondary metabolite production were polyketide synthase (PKS) and non-ribosomal peptide synthetase (NRPS) (Yadav et al. 2021). Moreover, Susuki and collaborators (2018) identified six genes involved in encoding enzymes responsible for acetic acid formation.

Anoxybacillus sp. PDR2 demonstrated as a great source to degraded important industrial azo dyes, it was found 27 genes as responsible for xenobiotic biodegradation and metabolism related to the degradation of azo dyes (Chen et al. 2020). Likewise, genes involved in metabolism of aromatic compounds were identify in *A. kamchatkensis* (Yadav et al. 2021). *Anoxybacillus ayderensis* AB04^T contains at least six heavy metal resistance genes, four genes are related to mercuric ion reduction, two of these are mercury resistance (mer) operons and the two other genes encode mercuric reductases, which catalyze the reduction of Hg^{2+} to Hg^0, addition, AB04^T carries genes for an arsenate reductase and an arsenic efflux pump protein (Belduz et al. 2015). Similarly, arsenate reductase genes were found in *Anoxybacillus gonensis* G2T (Lim et al. 2015b). Some genes related to aluminum resistance was found in *A. gonensis* G2(Beris et al. 2011) and *Anoxybacillus* sp. SK 3-4(Lim et al. 2015a).

3. Anoxybacillus applications

3.1. Industrial applications

Thermo-enzymes are one of the biotechnology aims since industrial processes occur above temperatures of 40 °C (Raddadi et al. 2015). As indicated by genomic analysis, several studies also reported and characterized enzymes from representatives of the genus. In general, Anoxybacillus enzymes

demonstrated activity in a temperature range of 10-8 and a pH of 8-10, revealing a thermostability and alkali tolerance, compiling industrial demands (table 1).

3.1.1. Detergents

Enzymes are usually used in the detergent as bio-additive, increasing the washing process efficiency, providing organic molecules degradation such as fats, proteins, and starch. Some enzymatic properties are required for the formulation, as thermostability, alkaline-tolerance and stability in the presence of surfactant (Gürkök 2019) several studies observed such proprieties from *Anoxybacillus* enzymes (Agüloğlu Fincan et al. 2014; Özdemir et al. 2016a), moreover, improved the thermostability (Chiş et al. 2013) and increased the activity (Acer et al. 2016; Bakir and Metin 2016).

In the case of laundry detergent, the challenge is to remove the stain and preserve the fabric. A protease from *Anoxybacillus kamchatkensis* MK1 removed blood and chocolate stain after one hour of incubation with commercial detergents at 40°C and showed a superior cleaning process when compared with only commercial laundry detergent action (Mechri et al. 2019). Other reported protease from *Anoxybacillus* showed this compatibility with laundry commercial detergents (Bekler et al. 2015). In addition, commercial detergents enhanced the Lipase activity of *Anoxybacillus* ARS-1 (Sahoo et al. 2020) and α- Amylase from *A. rupiensis* TS-4 (Kikani et al. 2020).

Baltas and collaborators (Baltas et al. 2016) simulated washing conditions with α- amylase with Solid detergents in diluting tap water was used at final concentrations of 0.5, 1, and 2% (w/v), and liquid detergents at 40°C. The results indicated that the enzyme was highly stable in the presence of commercial liquid washing machine detergents, laundry detergents, and oxidizing agents.

3.1.2. Food processing

Bacterial enzymes have been applied in many sectors of the food industry. Carbohydrate-degrading enzymes are required since sugar, starch, and syrups are the main ingredients of various products. *Anoxybacillus* species demonstrate notable starch-degrading enzymes that can improve the liquefaction step (Colak et al. 2008; Özdemir et al. 2012; Acer et al. 2015) and saccharification step (Pang et al. 2020) thereby reducing production time. Pullulanases are well-known starch-debranching enzyme, several reports have been improved their thermostability and the catalytic efficiency, to turning more suitable for industrial application (Li et al. 2015; Kahar et al. 2017; Wang et al. 2017; Pang et al. 2020).

Syrups are a versatile sweetener and ever-present in industrial candies and drinks. High-fructose corn syrup (HFCS) is a cheaper and popular sweetener in industrial food processing. Viksø-Nielsen and collaborators (2006) tested an α-amylase from *Anoxybacillus contaminans* and a glucoamylase from *Aspergillus niger*, this combination simplified the HFCS liquefaction and saccharification process, reducing

the input needed and reducing manufacturing equipment. Also, a glucose isomerase from *A. gonensis* showed as an ally for the Isomerization process (Karaoglu et al. 2013).

Even most high maltose-forming α-amylases require acidic to neutral pH for production, Chai and collaborators (Chai et al. 2012) described an α-amylase that produced under experimental conditions a syrup 69% maltose in alkaline conditions, suggesting a suitable enzyme in the bakery and maltose syrup production. Another efficient alpha-amylase was able to hydrolyze raw starch at temperatures below the starch gelatinization temperature, indicating the use in Glucose syrup production (Tawil et al. 2012)

Beyond conventional sweeteners, *A. flavithermus* produced L arabinose isomerase that was able to convert a D-galactose to D-tagatose, considered a rare sugar and low-calorie sugar-substituting sweetener, a promissory compound in the dairy food industry (Li et al. 2011).

A variety of calcium-independent enzymes have been identified from *Anoxybacillus* (Kikani and Singh 2012; Wang et al. 2018; Aliakbari et al. 2019; Kikani et al. 2020; Timilsina et al. 2020)Some products, like beer, require calcium-independent enzymes to avoid calcium oxalate formation that can be acting as a blocker of pipes and heat exchangers (Haki and Rakshit 2003), indicating potential enzymes that may be tested. In apple and pear juice production applies enzymes in the clarification process to treat Polymeric Carbohydrates, like starch. This process ensures a shining and good texture liquid. A clarification process was tested with an alpha-amylase from *A. flavithermus* that demonstrated degradation in 82% of starch present in pasteurized apple juice and keeping its conformation during the process at 70°C, as expected by authors. (Özdemir et al. 2016b).

3.1.3. Pulp and paper

Kraft process results in pulp with black color and needs a bleaching process. The bio-bleaching process occurs by enzymes as an alternative to the traditional bleaching, which uses chlorine and chlorine-based compounds that are not eco-friendly(Sharma et al. 2020). Besides the thermostability, *Anoxybacillus* members are an interesting source of enzymes for this commercial sector since the pulp is an alkaline substance. Xylanases isolated from *Anoxybacillus* species show stability in a range of 6.0 -12.0 pH (Kacagan et al. 2008) and free of cellulose activity (Wang et al. 2010; Hauli et al. 2013) particularly important to avoid hydrolysis of the cellulose fibers.

3.1.4. Biofuel

Enzymatic saccharification is an essential step for second-generation ethanol production that commonly uses agricultural waste. These materials are compound by cellulose and xylan residues. Cellulolytic and xylanolytic activities suitable to this sector were detected in some *Anoxybacillus* strains (Ellis and Magnuson 2012; Yadav et al. 2018). The β -glucosidases have been significantly used in biofuel conversion since they hydrolyze cello-oligosaccharides into glucose units important to the final steps of cellulose degradation. It was observed a common characteristic about the secreted β-glucosidases by

Anoxybacillus, as high tolerance glucose and a significant range of thermal stability (Chan et al. 2016; Almeida et al. 2020).

In practice, these assignments can improve commercial cocktails present in the enzyme market. Also, these enzymes demonstrated excellent synergism and cooperation, which reinforce this purpose. Celluclast® 1.5 L showed a lower hydrolytic efficiency in the presence of high concentrations of Glucose in the conversion of sugarcane bagasse, the supplementation of β-glucosidase from *Anoxybacillus flavithermus yunnanensis* E13 significantly enhanced the conversion under all the concentrations of Glucose that was 3.4-fold higher than that of Celluclast® 1.5 L alone (Liu et al. 2017). A Similar cooperation was observed by xylanase from *A. flavithermus* BC and β- xylosidase /α-arabinosidase from *Sulfolobus solfataricus* (Kambourova et al. 2007).

Anoxybacillus species can produce hydrogen from monosaccharides, as observed by Pikuta and collaborators (2000). In some related studies, the thermophilic community bacterial produced hydrogen from raw starch using products as cassava and sago, one of the dominant groups was *Anoxybacillus* (Hasyim et al. 2011; Sompong et al. 2011). The production of Hydrogen by microorganisms it is a promising field in removable energy production.

Table 1 All isolated enzymes from *Anoxybacillus* species. Their general characteristics related to temperature and pH.

Enzyme	Specie	strain	Optimum conditions	Thermostability (Half-life)	Reference
Amylase	*A. ayderensis*	FMB1	pH: 8.0 T: 55 °C	ND	(Bekler et al. 2020)
	A. flavithermus	-	pH: 5.0 – 8.0 T: 60 – 80 °C	ND	(Özdemir et al. 2012)
	A. flavithermus	-	pH: 7.0 T: 55 °C	ND	(Agüloğlu Fincan et al. 2014)
	A. flavithermus	SO-19	pH: 6.0 T: 70 °C	ND	(Özdemir et al. 2016)
	A. gonensis *	A4	pH: 7.5 T: 50 °C	ND	(Colak et al. 2008)
	A. rupiensis	TS4	pH: 8.0 T: 80 °C	22h at 50°C 13h at 70°C 5h at 90°C	(Kikani et al. 2020)
	A. thermarum	A4	pH: 5.5 – 10.5 T: 70 °C	ND	(Baltas et al. 2016)
	Anoxybacillus sp.	AH1	pH: 7.0 T: 60 °C	2h at 60°C	(Acer et al. 2016)
	Anoxybacillus sp.	DT3–1	pH: 8.0 T: 60 °C	3h at 65°C	(Chai et al. 2012)
	Anoxybacillus sp.	KP1	pH: 8.0 T: 60 °C	ND	(Bekler and Güven 2014)
	Anoxybacillus sp.	SK3–4	pH: 8.0 T: 60 °C	48h at 65°C	(Chai et al. 2012)
	Anoxybacillus sp.	YIM 342	pH: 9.0 T: 80 °C	ND	(Zhang et al. 2016)
	Anoxybacillus sp.	TSSC-1	pH: 7.0 T: 55 °C	24 h at 50°C	(Kikani and Singh 2012)
Carboxylesterase	*Anoxybacillus* sp.	PDF1	pH: 8.0 T: 60 °C	ND	(Ay et al. 2011)
Cellulase	*A. gonensis*	O9	pH: 3.0 – 10 T: 50 ◦C	ND	(Genc et al. 2015)

*Crude enzymes ND: No data

Table 1 Continuation

Enzyme	Specie	strain	Optimum conditions	Thermostability (Half-life)	Reference
Diphenolase	*A. kestanbolensis*	K1	pH: 9.5 T: 80◦C	ND	(Yildirim et al. 2005)
	A. kestanbolensis	$K4^T$	pH: 9.5 T: 70◦C	ND	(Yildirim et al. 2005)
Esterase	*A. Gonensis*	G2	pH: 7.5–9.5 T: 60 °C	ND	(Çolak et al. 2005)
	A. Gonensis	A4	pH: 5.5 T: 60 – 80°C	ND	(Faiz et al. 2007)
Galactosidase	*Anoxybacillus* sp.	KP1	pH: 9.0 T:60 °C	ND	(Bekler et al. 2017)
Glucosidase	*A. flavithermus yunnanensis*	$E13^T$	pH: 7.0 T: 60◦C	10h at 60°C	(Liu et al. 2017)
	Anoxybacillus sp.	. DT3-1	pH: 8.5 T: 70◦C	24h at 60°C	(Chan et al. 2016)
	A. thermarum	-	pH: 7.0 T: 65◦C	ND	(Almeida et al. 2020)
keratinase	*Anoxybacillus* sp.	PC2	pH: 5.0–10.0 T: 50–60°C	ND	(Reis et al. 2020)
Laccase	*A. ayderensis*	SK3-4	pH: 7.5 T: 75°C	155 min at 65°C	(Wang et al. 2020)
	A. gonensis	P39	pH: 5.0 T: 60°C	60 min at 30-90 °C	(Yanmis et al. 2016)
	Anoxybacillus sp.	UARK-01	pH: 9.0 T: 90°C	ND	(Al-balawi et al. 2017)

*Crude enzymes ND: No data

Table 1 Continuation

Enzyme	Specie	strain	Optimum conditions	Thermostability (Half-life)	Reference
Lipase	*A. flavithermus*	HBB 134	pH: 9.0 T: 50°C	ND	(Bakir and Metin 2016)
	A. flavithermus	-	pH: 6.0 – 8.5 T: 60 – 65 °C	5h at 60 °C	(Chiş et al. 2013)
	Anoxybacillus sp.	HBB16	pH:9.5 T: 55 °C	ND	(Burcu Bakir and Metin 2017)
Protease	*A. kamchatkensis*	-	pH: 11.0 T: 70 °C	8h at 80 °C 18h at 70°C 24h at 60°C	(Mechri et al. 2019)
	A. mongoliensis	-	pH: 10.5 – 10.8 T: 65 °C	ND	(Namsaraev et al. 2010)
	Anoxybacillus sp.	KP1	pH: 9.0 T: 50 – 60 °C	ND	(Bekler et al. 2015)
	Anoxybacillus sp.	MU3	pH: 6.0 T: 60 °C	ND	(Nakamichi et al. 2010)
	Anoxybacillus sp.	P1	pH: 10.5 T: 60 °C	ND	(Lavrenteva et al. 2009)
Pullulanase	*Anoxybacillus* sp	LM18-11	pH: 6.0 T: 60 °C	48 h at 60°C	(Li et al. 2015)
	Anoxybacillus sp.	SK3-4	pH: 7.5 T: 60 °C	4h at 65°C	(Kahar et al. 2013)
	Anoxybacillus sp.	WB42	pH: 5.8 T: 55 - 65 °C	ND	(Wang et al. 2018)
Xylanase	*A. flavithermus**	TWXYL3	pH: 6.0 T: 65 °C	ND	(Ellis and Magnuson 2012)
	A. kamchatkensis	NASTPD13	pH: 9.0 T: 65 °C	ND	(Yadav et al. 2018)
	*A. kaynarcensis**	D1021	pH: 7.0 – 9.0 T: 65 °C	ND	(Inan et al. 2013)

*Crude enzymes ND: No data

Table 1 Continuation

Enzyme	Specie	strain	Optimum conditions	Thermostability (Half-life)	Ref.
Xylanase	*A. pushchinoensis*	A8	pH: 6.5 – 11.0 T: 50 – 60◦C	ND	(Kacagan et al. 2008)
	Anoxybacillus sp	E2	pH: 6.6 – 8.6 T: 60 °C	ND	(Wang et al. 2010)
Xylosidase	*Anoxybacillus sp.*	3M	pH: 5.5 T: 65 °C	10 h at 60 °C	(Marcolongo et al. 2019)
Glucose isomerase	*A. gonensis*	$G2^T$	pH: 6.5 T: 85°C	3h at 85°C	(Karaoglu et al. 2013)
Xylose Isomerase	*A. gonensis*	$G2^T$	pH: 6.5 T: 85 °C	ND	(YANMIŞ et al. 2014)
	A. Kamchatkensis	G10	pH: 7.5 T: 80°C	30 min at 70°C	(Park et al. 2018)

*Crude enzymes ND: No data

3.2. Environmental solutions

The main pollutants are from industrial effluents, that comprises compounds such as heavy metals and dyes. The members of the genus possess biocatalytic systems and cellular structures resistant to xenobiotic compounds. Due to adaptation to harsh conditions in aquatic systems, several *Anoxybacillus* sp. have been applied experimentally in wastewater treatment.

3.2.1. Decolorification

The decolorization process occurs by adsorption, enzymatic degradation, or a combination of both mechanisms (Solís et al. 2012). Jardine and collaborators (2018) isolated an *Anoxybacillus* sp. 19S that demonstrated to be a source of enzymes with wastewater bioremediation applicability, such as oxidoreductases (catalase-peroxidase, superoxide dismutase, azoreductase) and hydrolytic enzymes (amylase, protease, phosphatase, and ribonuclease), as well as showing the ability to reduce phenol in the laboratory, a common structure found in many pollutants.

An Isolated and cloned *Anoxybacillus ayderensis* SK3-4 Laccase showed high decolorization capability toward five dyes (direct blue 6, acid black 1, direct green 6, direct black 19, and acid blue 93) (Wang et al. 2020), the same was observed against Reactive Black5, Fuchsine, Allura Red and Acid Red by *Anoxybacillus gonensis* P39 (Yanmis et al. 2016) . Microbial Communities might promote a synergic degradation secreting different enzymes, this phenomenon was observed in a community composed by *Clostridium* sp., *Bacillus* sp, *Tepidiphilus* sp. and *Anoxybacillus* sp. The authors showed a dye Direct Black G decolorization process and conclude that derive by azoreductase and laccase action (Chen et al. 2019).

Alvarez and collaborators (2013) proposed a new system treatment using an *A. flavithermus* selected from a colony from geothermal sites in Galicia (Spain)(Deive et al. 2010) based on biosorption mechanism, the decolorization ability of A. *flavithermus* in an aqueous effluent containing two textile finishing dyes (Reactive Black 5 and Acid Black 48), the decolorization efficiency for a mixture of both dyes reached almost 60% in less than 12 h.

3.2.2. Bioremediation of Heavy metals

A portion of heavy metals are toxicity to living organisms, considering cumulative capability, it is difficult to remove from ecosystems. A study using *Anoxybacillus mongoliensis* was detected that Ni (II) had a more toxic effect than Co (II) to SOD and CAT enzymes that are the important elements of the antioxidant defense system of *A. mongoliensi*s were induced linearly in the presence of different amounts (0, 2.5, 5 and 10 mg/L) of Ni (II) and Co (II) (Akkoyun et al. 2020). A similar analysis was performed using *Anoxybacillus amylolyticus*, the bacteria grown in the presence of heavy metals and had a decrease in a-amylase activity, correlated with a decrease in α-amylase production, suggesting an effect on the biosynthesis of the enzyme, as a result Hg^{2+} showed the most toxic

and Fe^{3+} the last one, thus, α-amylase could represent a potential sensitive bioassay for detecting trace heavy metals (Poli et al. 2009b).

Biosorption is a rapid mechanism of passive metal sequestration by the non-growing biomass/adsorbents and demonstrated a great solution for decontamination systems (Abbas et al. 2014). To the application of this strategy, is necessary a significant affinity by the cells. Different techniques employing *Anoxybacillus* strains has successful result in remove metal ions (table 2), in general, the dead cells are used with a resin and applied in contaminant sample. A new biosorbent, composed of Amberlite XAD-4 loaded with *Anoxybacillus kestanboliensis* was efficient to Co (II) and Hg (II), the developed a solid phase extraction column composed of bacteria loaded resin was used for over 35 cycles of biosorption/desorption without any loss in its biosorption behavior, according to authors, the system might be a method for routine analysis since the low cost and ecofriendly alternative (Ozdemir et al. 2020a).

Table 2 Metals ions removed using *Anoxybacillus* species by adsorption technique.

Specie (strain)	Heavy metal	Reference
Anoxybacillus kestanboliensis	Co (II) and Hg (II)	(Ozdemir et al. 2020)
Anoxybacillus flavithermus	Cu^{2+}	(Swedlund et al. 2015)
	Th (IV) and Ce (III)	(Yener et al. 2017)
Anoxybacillus amylolyticus	Cd^{2+}, Cu^{2+}, Co^{2+} and Mn^{2+}	(Özdemir et al. 2013a)
Anoxybacillus mongoliensis	Ni (II) and Co (II)	(Akkoyun et al. 2020)
Anoxybacillus gonensis	Zn^{2+}, Fe^{3+}, Cu^{2+}, Cd^{2+}, Ni^{2+}, $Co^{2+,}$ and Pb^{2+}	(Duran et al. 2009)

3.3. Metabolites production for medicinal applications

Over the years, several studies have shown metabolites produced by *Anoxybacillus* that could be used in medicinal treatment. *Anoxybacillus* sp. JT-12 was found as a producer of 4-O-methyl-a-D-glucuronosyl-xylotriose from xylan, this acidic xylotriose can be used in cosmetic and pharmaceutical field as antimicrobial compound, collagen production promoters, treatment agents for atopic dermatitis, topical anti-inflammatory, and others (Thitikorn-amorn et al. 2012).

Recently, Exopolysaccharides (EPS/EPSs) from *Anoxybacillus* have been rouse the attention. *Anoxybacillus gonensis* YK25 EPS was tested with three EPS concentrations (1, 1.25 and 2.5 mg/mL) on lung cancer cells, two EPS concentrations (1.25 and 2.5 mg/mL) on colon cancer cells, and one EPS concentration (2.5 mg/mL) on prostate cancer cells and neuroblastoma cancer cells and showed a significant anticancer activity (Karadayi et al. 2021). Similar tested was performed using EPS from *Anoxybacillus pushchinoensis* G11 in lung, colon, and neuroblastoma cancer cell lines that resulted in a significant anticancer activity, analysis by Fourier transform infrared spectroscope revealed the presence of sulfate ester that might be related with this activity. In

addition, the same study tested antibiofilm experiments, as a result, the EPS showed a significant activity against *Staphylococcus aureus* (Genc et al. 2021).

Conclusion and prospects

In contrast to advances in the Genomics analysis of *Anoxybacillus*, much headway has been made to identify the genes with a biotechnological application, including genes related to secondary metabolite pathways. Some reports have improved the thermostability of pullulanase and aim to get high catalytic efficiency more suitable for industrial application, opening the possibility to other enzymes from members of the genus. Additionally, these bacteria could be used in enzymatic consortia to improve the catalytic process in different fields, as observed in biofuel production, or as a microbial cell factory.

In addition, the members of the genus have interesting features useful to environmental applications. As previously noted, the genus has various enzymes isolated however, laccases have special attention to their efficiency to degrade several azo dyes. In addition, the biomass of Anoxybacillus has demonstrated significant efficiency in the cleanup process demanded by bioremediation methods, their cellular structure allows the recovery of metal ions. Thus, the genus is a great ally in improving and developing new bioremediation processes.

To conclude, *Anoxybacillus* is promising in biotechnology applications. The species are widespread in different environments their adaptation resulting in diversity in their genes and genomes. Last 20 years, the members of the genus have proved to be a great source of enzymes and demonstrated efficiency when applied to real industrial problems.

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Anexo:

Supplementary data:

Table S1 Genomes size related to *Anoxybacillus* species and their ID in the Genbank

Specie	**Strain**	**Genome size**	**Genbank acession**
A. amylolyticus	DSM 15939	3.158	ASM163428v1
A. ayderensis	MT-Cab	2.577	ASM211756v1
A. ayderensis	SK3-4	2.672	SK3-4dn
A. ayderensis	AB04	2.832	ASM83360v1
A. ayderensis	HOT.CON.111	2.126	ASM2351249v1
A. caldiproteolyticus	U458	3.789	ASM1532672v1
A. caldiproteolyticus	DSM 15730	3.633	ASM1376124v1
A. caldiproteolyticus	1A02591	3.894	ASM1646471v1
A. calidus	DSM 25220	3.406	ASM1376084v1
A. flavithermus	25	2.839	ASM75377v1
A. flavithermus	AF14	2.563	ASM165152v1
A. flavithermus	AF16	2.649	ASM165154v1
A. flavithermus	52-1A	2.830	ASM219748v1
A. flavithermus	KU2-6_11	2.646	ASM274268v1
A. flavithermus	FHS-PPAM212	2.645	ASM402530v1
A. flavithermus	WS5497	2.678	ASM1489011v1
A. flavithermus	WS5496	2.662	ASM1489013v1
A. flavithermus	WS5495	2.631	ASM1489014v1
A. flavithermus	WS5494	2.656	ASM1489015v1
A. flavithermus	WS5493	2.811	ASM1489018v1
A. flavithermus	WS5492	2.713	ASM1489021v1
A. flavithermus	WS5491	2.751	ASM1489023v1
A. flavithermus	WS5490	2.764	ASM1489025v1
A. flavithermus	WS5449	2.649	ASM1489026v1
A. flavithermus	WS5448	2.648	ASM1489027v1

Table 1 Continuation

Specie	**Strain**	**Genome size**	**Genbank acession**
A. flavithermus	WS5446	2.697	ASM1489031v1
A. flavithermus	WS5284	2.724	ASM1489033v1
A flavithermus	WS5364	2.620	ASM1489034v1
A. flavithermus	WS5292	2.660	ASM1489037v1
A. flavithermus	WS5290	2.667	ASM1489038v1
A. flavithermus	WS5291	2.674	ASM1489039v1
A. flavithermus	WS5286	2.660	ASM1489043v1
A. flavithermus	WS5285	2.671	ASM1489045v1
A. flavithermus	WS5287	2.912	ASM1489046v1
A. flavithermus	WS5279	2.665	ASM1489047v1
A. flavithermus	WS5281	2.792	ASM1489051v1
A. flavithermus	FB4_88	2.902	ASM2213475v1
A. flavithermus	AK1	2.631	GCA_000353425.1
A. flavithermus	NBRC 109594	2.773	ASM36750v1
A. flavithermus	TNO-09.006	2.658	GCA_000327465.1
A. flavithermus	WK1	2.847	ASM1904v1
A. gonensis	G2	2.804	ASM118759v1
A. gonensis	DT3-1	2.597	DT3-1dn
A. gonensis	G2	2.758	ASM77037v1
A. kamchatkensis	DSM 14988	2.561	ASM1376101v1
A. kamchatkensis	PD13	2.867	ASM479913v1
A. kamchatkensis	G10	2.963	ASM28341v2
A. karvacharensis	K1	2.722	ASM199628v1
A. kestanbolensis	NCIMB 13971	2.710	ASM2365301v1
A. mongoliensis	DSM 19169	2.634	ASM1420151v1
A. mongoliensis	MB4	2.808	ASM191443v1

Table 1 Continuation

Specie	**Strain**	**Genome size**	**Genbank acession**
A. pushchinoensis	K1	2.625	GCA_900111795.1
A. rupiensis	DSM 17127	3.706	ASM1419619v1
A. rupiensis	NL1.2	3.619	ASM1827466v1
A. rupiensis	TPH1	3.710	ASM2245703v1
A. salavatliensis	DSM 22626	2.758	ASM2446467v1
A. sediminis	PCH 117	3.931	ASM2092349v1
Anoxybacillus sp.	HOT.CON.20	3.282	ASM2351231v1
Anoxybacillus sp.	b2m1	3.819	ASM163426v1
Anoxybacillus sp.	b7m1	3.869	ASM163430v1
Anoxybacillus sp.	BCO1	2.809	Anoxy_BCO1
Anoxybacillus sp.	CHMUD	2.730	ASM1309696v1
Anoxybacillus sp.	EFIL	2.832	ASM1309695v1
Anoxybacillus sp.	KU2-6(11)	2.884	ASM75387v1
Anoxybacillus sp.	LAT_27	3.152	ASM2203007v1
Anoxybacillus sp.	LAT_11	3.285	ASM2226735v1
Anoxybacillus sp.	LAT_26	3.568	ASM2226727v1
Anoxybacillus sp.	LAT_31	2.712	ASM2226725v1
Anoxybacillus sp.	LAT_33	2.730	ASM2226721v1
Anoxybacillus sp.	LAT_35	2.720	ASM2226722v1
Anoxybacillus sp.	LAT_38	3.307	ASM2226729v1
Anoxybacillus sp.	MB8	2.899	ASM1100675v1
Anoxybacillus sp.	P3H1B	3.541	ASM156085v1
Anoxybacillus sp.	PDR2	3.791	ASM983404v1
Anoxybacillus sp.	ST4	2.835	ASM1944854v1
Anoxybacillus sp.	ST70	2.856	ASM1955362v1
Anoxybacillus sp.	UARK-01	3.669	ASM207536v1

Table 1 Continuation

Specie	**Strain**	**Genome size**	**Genbank acession**
A. tengchongensis	DSM 23211	2.727	ASM1420158v1
A. tepidamans	DSM 16325	3.386	ASM1420146v1
A. tepidamans	PS2	3.364	ASM62016v1
A. thermarum	AF/04	2.737	ASM83672v1
A. vitaminiphilus	CGMCC 1.8979	3.556	ASM325993v1
A. voinovskiensis	JCM 12111	3.244	ASM1464661v1
A. voinovskiensis	DSM 17075	3.231	ASM1419620v1