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24. Цекова К., Денчева В., Петров К. (2010) "Интегриран биотехнологичен процес за получаване на биологично активни вещества от нативно нишесте и очистване на води, замърсени с тежки метали"

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IV. МИКРООРГАНИЗМИ И ОКОЛНА СРЕДА

ИНТЕГРИРАН БИОТЕХНОЛОГИЧЕН ПРОЦЕС ЗА ПОЛУЧАВАНЕ НА БИОЛОГИЧНО АКТИВНИ ВЕЩЕСТВА ОТ НАТИВНО НИШЕСТЕ И ОЧИСТВАНЕ НА ВОДИ, ЗАМЪРСЕНИ С ТЕЖКИ МЕТАЛИ

К. Цекова, В. Денчева, К. Петров

INTEGRATED BIOTECHNOLOGICAL PROCESS FOR PRODUCTION OF BIOLOGICALLY ACTIVE COMPOUNDS FROM RAW STARCH AND HEAVY METALS REMOVING FROM WASTEWATER

K. Tsekova, V. Dencheva, K. Petrov

Abstract: Strains of *Rhizopus* fungi were investigated for lactic acid production from raw starch. *R. arrhizus* and *R. oryzae* showed higher biochemical activity for starch conversion to lactic acid and ethanol while *R. delemar* produced more fungal biomass as a by-product. Growth conditions: 35 g/L starch, pH 6 and 30°C were favorable for both starch saccharification and valuable products formation, resulting in lactic acid yield of 0.71 g/g starch associated with 0.36 g/g ethanol and 0.73 g/g fungal biomass with *R. arrhizus* as a producer. The ability of waste mycelia to remove Cu, Co, Mn, Ni and Zn ions from single as well as mixed solutions was assessed. The results obtained showed that the waste fungal biomasses may be successfully used as biosorbents for treatment of metal-polluted industrial wastewaters.

Keywords: *Rhizopus sp.*, raw starch, lactic acid, ethanol, heavy metals, biosorbtion

ВЪВЕДЕНИЕ

Нишестето е основна форма за резервни полизахариди в растенията и евтин източник за получаване на хидролизати, съдържащи глюкоза, фруктоза или малтоза, широко използвани в хранителните индустрии [1, 2]. Захарите, получени от нишесте могат да бъдат ферментирани до етанол, аминокиселини, органични киселини и други ценни за практиката продукти [3–10]. Конверсията на нишесте до ферментируеми захари се осъществява чрез предварителна хидролиза (втечняване и озахаряване) на субстрата по киселинен, киселинно-ензимен или ензим-ензимен метод, което изисква разходи за енергия, химикали и амилолитични ензими. От гледна точка на понижаване на енергийните разходи и намаляване на цената на биопродуктите, изследванията са насочени към селекция на микроорганизми с висок потенциал да разграждат нишестето и да трансформират разградните продукти до ценни за практиката ферментационни продукти, при което двата процеса – озахаряване и ферментация, се осъществяват в един биореактор [11, 12, 13, 14, 15].

Използването на селекционирани плесенни щамове като продуценти на биоетанол, млечна киселина, хитин, хитозан и други продукти е интересна и ефективна алтернатива пред използването на бактериални и дрождеви щамове, поради способността им да продуцират амилолитични ензими, по-евтино струващи компоненти на хранителни среди, улеснени процеси за отделяне на биомасата и възможност за използване на отпадната биомаса като добавка към храна за животни, суровина за извличане на хитин, хитозан и др., и като евтин сорбент за извличане на тежки метали от отпадни води [15, 16, 17, 18, 19]. Тези значителни предимства на плесенните гъби ги характеризират като перспективни източници за получаване на биопродукти от нишесте, като най-често използвани за тази цел се очертават видовете на род *Rhizopus* [4, 9, 11–15, 20].

Целта на настоящата работа е да се изследва възможността за получаване на биологично активни вещества от нативно нишесте, чрез култивиране на селекционирани плесенни гъби от род *Rhizopus* и отпадната биомаса да се използва като евтин биосорбент за третиране на води, замърсени с тежки метали.

МАТЕРИАЛИ И МЕТОДИ

Микроорганизми, хранителни среди и условия на култивиране

Изследвани са видове от род *Rhizopus*: *R. arrhizus, R. oryzae, R. delemar, R. niveus и R. chinensis*, от

25. Vasileva E., Stoyanov A., Petrov K., Beschkov V. (2011) "Influence of aeration on biodegradation of monochloroacetic acid", *Journal of International Scientific Publications: Ecology & Safety* vol. 5 (1), 190-195. (ISSN 1313-2563)

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INFLUENCE OF AERATION ON BIODEGRADATION OF MONOCHLOROACETIC ACID

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Abstract

The biodegradation of high amounts of the toxic monochloroacetic acid (MCA) was studied in a series of fed-batch processes by the use of Xanthobacter autotrophicus GJ 10 cells. The influence of the oxygen supply on biodegradation capabilities of the strain was investigated by airflow and the stirring rate variations. The highest conversion of MCA – 300 mM was achieved at airflow 1.0 L/min (2.2 vvm) and agitation rate of 500 rpm.

Key words: *biodegradation, monochloroacetic acid, Xanthobacter autotrophicus GJ 10, fed-batch processes*

1. INTRODUCTION

Protecting the purity of the water resources and their prudently and environmentally use is one of the main problems of contemporary humanity. When the water used up for drinking industrial and other purposes is contaminated by various substances and alters its natural physical properties and chemical composition, it becomes wastewater. In recent years, scientists are searching for more novel approaches for wastewater treatment. The advantages of biotechnological methods are: ability to separate the majority of organic pollutants, simple apparatus design and relatively low operation costs. The drawbacks here are the strict need of control of the technological regime of treatment, toxicity of some of the treated organic compounds and requirement to pre-dilution of highly concentrated toxic waste.

The biological treatment is the conversion of the contaminants contained in the organic wastewater to simpler compounds through the vital activity of particular cultivated microorganisms under certain conditions. Some of them degrade halogenated aliphatic hydrocarbons through specific enzyme systems that break or abate the carbon-halogen bands. These microorganisms use contaminants rather as carbon source and energy, and have the capacity to transform the intermediate products in digestible substrates.

Significant contaminant of wastewater is ethylene chloride [1]. Intermediate product of its degradation is monochloroacetic acid (MCA), which is also very toxic halogenated compound. It has a relatively long life in nature and it can be found in rainwater, surface and drinking water as well in the snow and underground water. In literature there is evidence that MCA is the most toxic halogenated fatty acids especially for some algae [2].

There is a variety of bacterial strains that are able to break the carbon-halogen band of the fatty acid molecules [3, 4]. One of them is *Xanthobacter autotrophicus GJ10*. Cells of the strain used halogenated aliphatic hydrocarbons (HAH) as carbon and energy sources and are able to decompose ethylene chloride to 2-dichloroetanol, MCA and glycolic acid (GA).

26. Petrova P., Petrov K. (2011) "Antimicrobial activity of starch-degrading *Lactobacillus* strains isolated from boza". *Biotechnology & Biotechnological Equipment* vol. 25 (4), 114-116. (IF 0.760)

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ANTIMICROBIAL ACTIVITY OF STARCH-DEGRADING *LACTOBACILLUS* STRAINS ISOLATED FROM BOZA

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ABSTRACT

The proved probiotic effects of boza consummation prompted the increasing interest in the microflora taking part in its fermentation. Boza is a rich source of probiotic lactic acid bacteria that are able to produce bacteriocins active against a number of pathogens. In the present study, three strains – Lactobacillus paracasei B41, Lactobacillus plantarum Bom 816, and Lactobacillus pentosus N3, isolated from boza and possessing significant amylolytic activity, were studied for their ability to repress the growth of common foodborne pathogens. All strains demonstrated antimicrobial activity against Escherichia coli, Klebsiella pneumoniae, Vibrio cholerae and Bacillus subtilis. The results indicated possible bacteriocin production and made the strains desirable starters with application in cereals processing. None of the tested lactobacilli inhibited the growth of Saccharomyces cerevisiae or Pichia strains, suggesting the existence of stable microbial community of yeasts and lactobacilli in boza starters.

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Keywords: *Lactobacillus*, antimicrobial activity, foodborne pathogens, boza

Introduction

Lactic acid fermentation is a centuries-old method of food preservation and processing. It is the main approach to increase the nutritional value and the taste and to save energy for the preparation of foods and beverages of plant origin, cereals, and bread. Million tonnes of fermented foods, based on manioc, taro, sorghum, rice, millet and maize in the form of over 90 different products such as bakery bread, pastas, traditional drinks or snacks are obtained with the participation of amylolytic lactic acid bacteria (ALAB) (1, 8). ALAB were initially found in tropical starch-containing fermented foods, prepared mainly from manioc and cereals (maize and sorghum). Consumer's expectations that besides the improved aroma and taste the LAB fermented cereal foods possess probiotic properties are not always justified. The majority of the LAB strains, evaluated as probiotic do not possess amylolytic activity, i.e. their survival and growth in starchy environment are questionable. In addition, the amylase-positive strains usually are not probiotic. Driven by the fact, that some of the starch-based beverages, as boza, yosa or ogi, have been claimed for their reference to the functional foods because of LAB content (1), we focused our investigation on the finding of potentially probiotic, and at the same time, amylolytic LAB strain.

Boza is an ancient cereal beverage which originated in Mesopotamia about 9000 years ago. Starting from the 13th century, with the Ottoman invasion of Anatolia, the Turks introduced the drink under the name "boza" to the Balkans. The name derives from the Persian word "buze", meaning "millet". Indeed, it is a malt drink, made from maize, bulgur, millet, barley or chick peas in Albania, fermented wheat in Turkey and wheat or millet in Bulgaria and Romania. It has a creamy light to dark yellow colour and is rather thick in consistency.

Boza has been found to have several health benefits: the drink helps to balance blood pressure, to increase milk production in lactating women and to facilitate digestion. It is a valuable nutrient to physically active people, as it contains vitamins A, C, E and four types of vitamin B. Boza is especially suitable for vegetarians, as it is entirely plant-based and a good source of vitamins and thus constitutes a good substitute for dairy-based drinks.

Recently, boza is being increasingly introduced to the EU countries. This requires an implementation of scientific assessment of the potential probiotic microbial strains taking part in boza manufacturing. The species diversity of *Lactobacillus* strains isolated from boza included *L. sanfrancisco*, *L. coryniformis*, *L. fermentum*, *L. confusus*, *L. plantarum* (6), *L. paracasei*, *L. pentosus*, *L. brevis*, and *L. rhamnosus* (2). Some of them are potential or proved probiotics (5, 10). The isolation of a *Lactobacillus* strain, holding both features – amylolytic and antibacterial activities would contribute to the development of new kind of valuable starters for cereal foods manufacturing.

Materials and Methods

Bacterial strains

Three *Lactobacillus* strains were included in this study: *L. paracasei* B41, *L. plantarum* Bom 816 and *L. pentosus* N3. The first strain was deposited in the German Collection of Microorganisms and Cell Cultures (DSMZ) under registration

27. Petrova P., Petrov K. (2012) „Direct starch conversion into L (+) lactic acid by a novel amylolytic strain of *Lactobacillus paracasei* B41" *Starch-Starke* vol. 64 (1), 10-17. (IF 1.220)

RESEARCH ARTICLE

Direct starch conversion into L-(+)-lactic acid by a novel amylolytic strain of *Lactobacillus paracasei* B41

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A new amylolytic strain of *Lactobacillus paracasei* able to convert starch directly into L-(+)-lactic acid (LA) was isolated. The identification of the by 16S rDNA sequencing proved that this strain, B41, is the first amylolytic representative of *Lactobacillus casei* group. The amylase activity assay revealed that *L. paracasei* B41 produced extracellular amylolytic enzyme, reaching 62 U/mL in the cell-free supernatants. The optimal conditions for its action were pH 5.0 and temperature 45°C. The gene *amy1* (1779 bp) encoding the putative B41 amylopullulanase was cloned, sequenced, and analyzed. The deduced protein contained a leader peptide of 28 amino acids and a mature peptide of 564 amino acids. Compared to the amylases of closely related species, B41 enzyme had several amino acid substitutions. An inducible control at *amy1* expression was demonstrated. The starch fermentation abilities of *L. paracasei* B41 were studied in batch processes performed with and without pH control. The highest amount of LA from starch was obtained during 48 h fermentation from 40 g/L substrate at pH maintained at 5.0–37.3 g/L. In addition, 93.3% starch conversion into LA and the highest reported productivity for 24 h were achieved – 1.30 g/L/h.

Keywords:
Amylase / L-(+)-Lactic acid / *Lactobacillus paracasei* / Starch

Supporting information available online

1 Introduction

Lactic acid (LA) is widely used by the modern industry. It is a common reagent in chemical syntheses of lactate esters, propylene glycol, 2,3-pentanedione, propanoic acid, acrylic acid, acetaldehyde degradable polymers, based on the poly – lactates [1]. However, the major consumers of LA remain to be the food and pharmaceutical industries. The former uses it as acidulant, preservative (which ensures up to 50% pathogens growth inhibition), taste-enhancer in margarines, butters and yoghurts, as a pickling agent for vegetables, olives, and sauerkraut. The latter applies LA for medical uses: as a surgical sutures and controlled drug release [2]. Whereas, the chemical syntheses use both LA stereoisomers [3], the food industry needs only the human – harmless L-(+)-isomer that is provided in optical pure form only by microbial synthesis [4]. The development of new biotechnologies for L-(+)-LA production determined the choice of starch as a cheap and abundant carbohydrate source [5, 6] and the search for strains, able to convert it.

The amylolytic lactic acid bacteria (ALAB) were initially found in tropical various starch-containing fermented foods, prepared mainly from manioc and cereals (maize and sorghum). Strains of *Lactobacillus plantarum* and *Lactobacillus manihotivorans* have been isolated from African cassava-based fermented products [7–9], *Lactobacillus amylovorus* [10], *Lactobacillus amylophilus* [11], and *Streptococcus bovis* [12] were isolated from cattle waste-corn fermentations, starch-containing wastes, or bovine rumen. Amylolytic *Lactobacillus fermentum* strains

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Abbreviations: ALAB, amylolytic lactic acid bacteria; **LA,** lactic acid

Colour online: See the article online to view Fig. 4 in colour.

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28. Petrov K., Stoyanov A. (2012) "Accelerated production of 1,3-propanediol from glycerol by *Klebsiella pneumoniae* using the method of forced pH fluctuations", *Bioprocess Biosyst Eng*, vol. 35 (3), 317-321. (IF 1.869)

Bioprocess Biosyst Eng (2012) 35:317–321
DOI 10.1007/s00449-011-0569-0

ORIGINAL PAPER

Accelerated production of 1,3-propanediol from glycerol by *Klebsiella pneumoniae* using the method of forced pH fluctuations

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Abstract 1,3-Propanediol (1,3-PD) is a bivalent alcohol, used in a number of chemical syntheses. It could be produced from glycerol in course of microbial fermentation by *Klebsiella pneumoniae* along with more than five minor liquid products. With the purpose to enhance 1,3-PD production and to eliminate by-products formation, principally new pH control on the process was applied. The method, named "forced pH fluctuations" was realized by consecutive raisings of pH with definite ΔpH amplitude (ranging from 1.0 to 2.0) at time intervals between 2 and 4 h, during a series of fed batch processes. The fermentation performed by forced pH fluctuations with ΔpH = 1.0, risen at every 3 h was evaluated as the most successful. Increase by 10% of the maximal amount of 1,3-PD (g/l), 22% higher productivity [g/(l h)], and 29% increase in 1,3-PD molar yield were achieved, compared to the referent fed batch (with constant pH = 7.0). In addition, significant decrease in by-products formation was obtained. The most important reduction was observed in the lactic and acetic acids yields, where 50 and 70% decrease were reached. The results suggested the potential of pH to manage the share and quantity of product spectrum in mixed diols–acids fermentations. The application of "forced pH fluctuations method" achieves the desirable increase in 1,3-PD formation and decrease in by-products accumulation at the same time by a comparatively simple approach by adjustment of one bioprocess parameter only.

Keywords 1,3-Propanediol · Glycerol · *Klebsiella pneumoniae* · pH fluctuations

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Introduction

1,3-Propanediol (1,3-PD) is a valuable organic chemical with wide application in polymer synthesis; in manufacturing of adhesives, laminates, coatings, solvents, and antifreezes [1, 2], and in cosmetics and medicines as a bifunctional compound [3]. Two main ways for 1,3-PD production are available: chemical—from acrolein and biotechnological—from the waste glycerol, abundantly delivered by the biodiesel industry. While the chemical synthesis requires high temperature, high pressure, and catalysts, the microbial conversion of glycerol provides minimum energy losses along with the usage of waste carbohydrate source.

1,3-PD is a typical product of glycerol fermentation, but only few microorganisms are able to form it. They belong to either of these genera: *Klebsiella* (the species *K. pneumoniae* and *K. oxytoca*), *Enterobacter*, *Citrobacter*, *Lactobacillus* and *Clostridium*. The fermentation of glycerol by *K. pneumoniae* occurs with the accumulation of more than five liquid metabolites—diols, organic acids, and ethanol which ratio depends on the process conditions. The most significant factors, affecting the fermentation are the medium content, the aeration regime, and pH. The first two influencing factors were a subject of detailed research by a number of scientific experiments [4–7] and were optimized to a great extend. In contrast, pH requirements for predominant 1,3-PD production and low by-products receiving at the same time, still to be obscure until now [8–10]. The majority of authors supported the pH maintenance at 7.0 for high 1,3-PD synthesis. Other, focused at by-products accumulation decrease, suggested pH maintenance at two different levels—6.3 and 7.0, changed periodically [11].

Here, we report a principally new and highly effective pH control of the process, based on externally provoked

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REGULATION OF AMYLASE GENES EXPRESSION IN LACTIC ACID BACTERIA

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Abstract

The enzymes α-amylases (EC 3.2.1.1) are endo-glycosyl hydrolases that randomly cleave the α-1, 4-glycosidic bonds in starch. Their family in lactic acid bacteria (LAB) consists of several enzymes with unclear structures, because of the long-term opinion that this type of bacteria lack starch-degrading ability. On the other hand, the sugar utilization has been extensively studied in LAB, because of its significant role in the industrial fermentations. Most genes involved in sugar catabolism are organized into operons that are strongly expressed and controlled at the level of transcription initiation and are subjected to CcpA-dependent catabolite repression.

In the present study the molecular structures of different amylase genes, presented in the genomes of *Lactococcus lactis* ssp. *lactis* B84 and *Lactobacillus paracasei* B41, are described. Four genes for enzymes, involved in starch degradation were detected in B84 genome: *amyL*, *amyY*, *glgP* and *apu*, coding cytoplasmic and extracellular alpha-amylases, glycogen phosphorylase and amylopullulanase. Reverse transcription PCR experiments showed that both genes, encoding alpha-amylases (*amyL* and *amyY*), were expressed into mRNAs. The genetic basis of *Lactobacillus paracasei* B41 amylolytic properties were investigated and discussed. *CRE*-site for CcpA-recognition was found and the putative mechanism of gene expression regulation was proved by Northern hybridization experiments.

Keywords: amylase, *Lactococcus lactis*, *Lactobacillus paracasei*, expression, starch

30. Tsvetanova F.V., Petrov K.K. (2012) "Fermentative biofuels production" *Journal of International Scientific Publications: Materials, Methods & Technology* vol. 6 (2), 425-439. (ISSN 1313-2563)

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FERMENTATIVE BIOFUELS PRODUCTION

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Abstract

The limited reserves and increasing prices of fossil carbohydrates, as well as the global warming due to their utilization, impose the finding of renewable energy sources. Because of this, since decades an increasing interest in production of alcohols, which can be used as a fuel additives or fuels for direct replacement in gasoline engines, is observed.

Alcohols can be obtained chemically or as products of microbial metabolism of different species in fermentation of sugars or starchy materials. In the present review are summarized different fermentative pathways for production of all alcohols, which are or could be used as biofuels. The focus of the paper is on production limitations, strains development and economical perspectives.

Key words: *fermentation, biofuel, alcohols*

1. INTRODUCTION

The increasing energy demand and running low stocks of fossil fuels in last decades shift the attention to alcohols production in respect of their fuel properties. Alcohols can be produced from renewable sources and reply both to energy crisis and environmental problems. A number of alcohols are candidates to replace the existed fuels, but to date only ethanol and methanol fuels are on race. Having in mind that methanol fuel is produced mainly from coal or natural gas, it remains that only ethanol fuel is received by fermentation. Nevertheless, many other alcohols have fuel properties, very similar to those of gasoline and could be used as a fuel, much better than ethanol (Table 1). The higher alcohols as butanol and branched-chain alcohols (2-methyl-1-butanol (2MB) and 3-methyl-1-butanol (3MB)) have higher energy density and lower hygroscopicity than the ethanol (Atsumi et al. 2008a). Moreover, one of the main problems of ethanol fuel - the insufficient vaporization, is partially solved, using butanol. The heat of vaporization of butanol is less than half of those of ethanol, which makes butanol preferable fuel in cold weather. Other advantage of higher alcohols is that they can be transported in already existing pipelines (Ladisch 1991, Dürre 2007).

Table 1. Properties of alcohol fuels

	Gasoline	Methanol	Ethanol	Isopropanol	Butanol
Research octane number	95	109	109	118	96
Motor octane number	85	89	90	98	78
Anti knock index	90	99	99.5	108	87
Energy density (MJ/L)	32.6	15.8	19.6	23.9	29.2
Heat of combustion (kJ/mol)	4817	725	1378	2030	2683
Heat of vaporization (MJ/kg)	0.36	1.20	0.92		0.43
Air-fuel ratio	14.6	6.4	9.0		11.1
Boiling point (°C)		65	78	82.5	118

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МЕТАБОЛИТНИ ПЪТИЩА ЗА ПОЛУЧАВАНЕ НА АЛКОХОЛИ С РАЗКЛОНЕНА ВЕРИГА С ЦЕЛ ИЗПОЛЗВАНЕТО ИМ КАТО БИОГОРИВА

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METABOLIC ENGINEERED PATHWAYS FOR BRANCHED-CHAIN ALCOHOLS AS BIOFUELS

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Abstract *Since the loads of natural resources are going down and fossil fuel prices are increasing, many attempts have been made for producing branched chain alcohols. They have some priorities over traditional biofuel-ethanol, that include higher energy density, lower hygroscopicity and higher octane number. Such alcohols are 2-methyl-1-butanol and 3-methyl-1-butanol. These alcohols however can't be produced economically so metabolic inputs are needed in order to be transformed the native organism to synthesize them. There have been used host's native amino acid biosynthetic pathway and transforms the 2-keto acid intermediates for alcohol synthesis.*

Key words: branched chain alcohols, metabolic engineering, *E. coli*

Fuel properties of branched-chain alcohols

Because of its hygroscopicity which poses a risk for storage and distribution and its lower energy density than gasoline, ethanol is not the most proper candidate for biofuel of the future. By contrast, higher alcohols as C4 and C5 alcohols have energy densities much closer to gasoline, they are not hygroscopic, less volatile than ethanol and have lower vapor pressure. 2-methyl-1-butanol (2MB) and 3-methyl-1-butanol (3MB) however have not been detected to be produced from renewable sources in a yields high enough to be considered as a gasoline substitute. In addition, no microorganism has been observed to produce these higher alcohols in industrially economic quantities. Small amounts of these alcohols however have been detected to be produced as byproducts [16].

The approach of production higher alcohols from amino acid precursors in *E. coli* has proven to be promising as it exploits the host's native amino acid synthesis pathways [15,4]. At least four natural 2-ketoacids which can be converted to potentially useful higher alcohols can be produced through the biosynthetic pathways of the branched-chain amino acids [16].

32. Petrova P., Tonkova A., Petrov K. (2012) "Sequence analysis, cloning and extracellular expression of cyclodextrin glucanotrasferase gene from the alkaliphilic *Bacillus pseudalcaliphilus* 8SB in *E. Coli*", *Process Biochemistry* vol. 47 (12), 2139-2145, (IF 2.414)

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Sequence analysis, cloning and extracellular expression of cyclodextrin glucanotransferase gene from the alkaliphilic *Bacillus pseudalcaliphilus* 8SB in *Escherichia coli*

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ABSTRACT

The *cgt*-gene from the alkaliphilic halotolerant *Bacillus pseudalcaliphilus* 8SB was isolated and sequenced. An open reading frame (ORF) of 2112 bp encoding a polypeptide of 704 amino acids, composed of a 29-amino acid signal sequence and a 675-amino acid mature enzyme was found. The established low level of homology with nucleotide sequences of other *Bacillus* CGTases (less than 82%) suggested that the *cgt*-gene from *Bacillus pseudalcaliphilus* 8SB encodes a new enzyme. The *cgt*-gene was cloned as a PCR amplicon and thereby the construction of genome library was avoided. This is the first evidence for the use of pJET vector as an expression vector. The opportunity to apply its T7 promoter for efficient extracellular production of heterologous proteins in *Escherichia coli* BL21 (DE3) was demonstrated. The expression of extracellular recombinant CGTase improved 23-fold, concerning β-CGTase activity and 4.5-fold concerning γ-CGTase activity after IPTG induction and glycine supplementation was achieved.

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1. Introduction

Cyclodextrin glucanotransferase (CGTase, EC 2.4.1.19) is industrially important enzyme that converts starch and related α-1,4-glucans into cyclodextrins (CDs). The ability of CDs to encapsulate hydrophobic molecules within their hydrophobic cavity determines their wide applications in medicine, pharmaceutical, food, cosmetic industries, agriculture and for environmental protection [1–5].

The enzyme product is usually a mixture of CDs, including mainly α-, β- and γ-cyclodextrins and trace amount of large-ring cyclodextrins [6]. To date, CGTases of about fifty microorganisms have been characterized for CD specificity and classified as α-, β- and γ-CGTases according to the major type of CD produced by the cyclizing reaction [7]. Among the bacterial producers, the alkaliphilic bacilli are the main source of β- and γ-CGTases [6–8]. The nucleotide sequences of the relevant genes have been determined, compared and the genes have been cloned into *Escherichia coli* or *Bacillus subtilis* since the CGTases from the alkaliphilic bacilli are more active and stable in wide pH and temperature ranges than those of the other bacterial CGTases, and are potent for industrial use [3,9–14].

Recently, we have succeeded in isolation of a CGTase-producing alkaliphilic halotolerant *Bacillus pseudalcaliphilus* 8SB [15]. The enzyme from this strain converted starch into β- and γ-CDs [16]. With the aim for an improved enzyme production, the *cgt*-gene from *B. pseudalcaliphilus* 8SB was isolated and cloned in a pJET vector under control of T7 promoter and extracellular expression by heterologous host *E. coli* BL21 (DE3) was achieved. Other reports in respect to sequencing and cloning of the *cgt*-gene from the species *B. pseudalcaliphilus* are not available.

2. Materials and methods

2.1. Bacterial strains, plasmid and culture media

Alkaliphilic halotolerant *B. pseudalcaliphilus* 8SB that was used for the chromosomal DNA source has been described in our previous papers [15,16]. *E. coli* DH5α [F– endA1 glnV44 thi-1 recA1 relA1 gyrA96 deoR nupG Φ80d*lacZ*ΔM15 Δ (*lacZYA-argF*) U169, hsdR 17 (r_K^- m_K^+), λ–] was used for a cloning host. *E. coli* BL21 (DE3) [F– *ompT hsdS*B (rB–, mB–) *gal dcm* (DE3)] was used as an expression host purchased from Invitrogen™ (Life Technologies, Carlsbad, CA, USA). Plasmid pJET 2.1/blunt was used as a cloning and expression vector.

B. pseudalcaliphilus 8SB was cultivated in medium containing ($g l^{-1}$): soluble starch (Poland), 2; peptone (Oxoid, Basingstoke, UK), 5; yeast extract (Oxoid), 5; $MgSO_4$, 0.2; and K_2HPO_4, 1. Sterile sodium carbonate was added to adjust

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RESEARCH ARTICLE

Covalent attachment of cyclodextrin glucanotransferase from genetically modified Escherichia coli on surface functionalized silica coated carriers and magnetic particles

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ABSTRACT

Recombinant cyclodextrin glucanotransferase JCGT8-5 (CGTase, EC 2.4.1.19) was effectively immobilized by formation of chemical bounds on polyethylenimine (PEI)-activated silica particles, on silanized Spherosils 5, XOA200, XOC 005, Arylamine CPG and magnetite Fe_3O_4 ($Fe(II)O.Fe(III)_2O_3$) micro-particles and by adsorption on magnetite/activated carbon support. The immobilization capacities of the carriers were determined. Binding yields reached from 50% to 93%. The amount of bound enzyme was considerably higher when this protein was immobilized on polyethylenimine (PEI)-activated silica particles and silanized Spherosil 5, XOA and XOC micro-particles and reached 1.33-1.77 mg/g support. Arylamine CPG and the activated carbon/magnetite support also possessed high binding capacities – 0.73-0.78 mg enzyme/g carrier. Analysis of formed CDs revealed changes in the β-cyclodextrin: γ-cyclodextrin ratio due to immobilization.

Key words: Immobilization, recombinant CGTase, magnetic and silica carriers, products ratio

Introduction

Enzyme immobilization greatly simplifies the whole bioprocess because the immobilized enzyme can be recovered and reused again in the subsequent reaction cycle, as long as the enzyme still maintains its functionality and operational stability. The choice of the immobilization method depends on the enzyme stability (Kelly et al., 1977; Gemeiner, 1992) and the process of application. The most commonly used carrier binding methods are adsorption or covalent linkage to a carrier (Kierstan & Coughlan, 1991; Gemeiner, 1992; Katchalski-Katzir & Kraemer, 2000) because in the protein (enzyme) molecule a number of amino acid functional groups are suitable for covalent bond formation. Those that are most often involved are the amino group of lysine or arginine, the carboxyl group of aspartic and glutamic acids, the hydroxyl group of serine or threonine and the sulfhydryl groups of cycteine. Silica micro- and nanoparticles provide many surface reactive groups, substantially vi a silanol or Si–OH groups, to be directly employed in subsequent surface functionalization (Kierstan & Coughlan, 1991). Surface modification by silanization is very common method for particle functionalization. High density of free amino groups ($-NH_2$) lying outwards the particle surface provides an excellent media for further chemical surface modification such as enzyme cross-linking with glutaraldehyde.

In the recent years, an increased attention is particularly focused on production of functionalized magnetic micro- and nanoparticles (MMPs and MNPs) for uses in immobilization of various bioactive compounds such as proteins, DNA, various chemicals, viruses - key components in different areas like catalysis, environmental remediation, the biomedical field and sensing devices, cell labeling and immunomagnetic separations, cell isolation, drug targeting, waste water treatment and other applications (Šafařik & Šafařikova, 1999, 2002; Häfeli, 2001; Berensmeier, 2006; Luo et al., 2006; Wu et al., 2008; Stanciu et al., 2009; Šafařik et al., 2011). Iron oxides, mainly Fe_3O_4 have been the most widely used because of their simple preparation and

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FOOTROT IN CLAWED AND HOOFED ANIMALS: SYMPTOMS, CAUSES AND TREATMENTS

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ABSTRACT

Footrot is a chronic bacterial infection in cattle, horses, pigs, deer and mouflons. The disease leads to huge economical losses in the wool, meat and dairy industry. Although Dichelobacter nodosus is considered the essential transmitter of the disease, a complex synergism exists with Fusobacterium necrophorum. Recent reports suggest that F. necrophorum may manifest itself in more than one form, depending on the animal species. This review summarizes the characteristics of D. nodosus and F. necrophorum, discusses the genes involved in virulence, and addresses the methods used in prevention and treatment of the disease.

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Keywords: footrot, *Dichelobacter nodosus, Fusobacterium necrophorum*

Implications

Footrot is a necrotic disease in clawed and hoofed animals and is often difficult to treat. Although several bacteria and yeast species have been isolated from infected claws and hoofs, the Gram-negative bacteria *Dichelobacter nodosus* and *Fusobacterium necrophorum* are always associated with necrotic lesions. The animal industry suffers huge losses due to joint disorders, bone and tendon infections, lameness and weight loss. The exact interaction between the pathogens is not fully understood. This review summarizes the latest findings on *D. nodosus* and *F. necrophorum*, discusses methods used to detect footrot, and addresses the different treatments currently used.

Introduction

Footrot is a highly contagious disease of the feet of ruminants, specifically sheep and goats, but symptoms have also been reported in cattle, horses, pigs, deer and mouflon (9, 43, 108). Although several bacteria and yeast species have been isolated from claws and hoofs, the Gram-negative bacteria *Dichelobacter nodosus* and *Fusobacterium necrophorum* are always associated with necrotic lesions (63, 88). Associated signs of infection are lameness and severe weight loss (25, 96). If the disease is left untreated, the infection may spread to joints, bones and tendons.

By the severity of the disease footrot is described as severe (progressive), benign or intermediate (95). Progressive footrot begins with a mild inflammation of the skin, followed by eruption of the skin–horn junction and separation of the soft horn from the underlying epithelium (98). In horses, infection may also start at the white line around the ground surface of the hoof. In some cases, especially with overgrown hoof walls, this section of the hoof is more porous than the sole, frog, or wall tissue and serves as direct contact to the laminar and solar corium. In severe infections, separation of tissue at the axial margin of the sole or around the back of the heel develops within a few days. If left untreated, the horn may be separated across the entire sole to the toe and abaxial wall within five to ten days. Eventually the distal lamellar portion of the abaxial wall becomes infected, leaving the stratum corneum attached along the proximal section. At this stage the non-horny part of the infected sole is covered by soft, necrotic epithelial tissue and the shape of the hoof changes drastically. In the case of benign (mild) footrot, infection usually does not extend beyond the interdigital skin. Sheep with footrot may display separation of the soft horn at the heel and posterior sole, but with much less necrosis of the underlying soft tissue.

Pathogens

Beveridge (6) was the first to describe footrot in sheep and isolated *Spirohaeta penortha* from infected tissue. However, footrot could not be initiated by infecting sheep with a pure culture of *S. penortha* (7). Several years later Beveridge (8) isolated *Fusoformis nodosus* from infected sheep and was the first who described that a pure culture could cause footrot. This was later confirmed by Thomas (105). The species was reclassified as *Bacteroides nodosus* (74), and later as *Dichelobacter nodosus* based on 16S rRNA sequencing (22, 62).

In a later study, Egerton et al. (25) isolated coccobacilli and diphtheroids from infected tissue, but only from surface areas. The authors isolated strains of *D. nodosus* and *F. necrophorum* from the epidermis of freshly infected tissue, but could not isolate *F. necrophorum* from the same area a few days later. Strains of *F. necrophorum* were, however, isolated from decayed tissue (25). Based on these results, the authors

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Short communication

Fusobacterium necrophorum, and not *Dichelobacter nodosus*, is associated with equine hoof thrush

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ABSTRACT

The aim of this study was to determine which of the two species, *Fusobacterium necrophorum* or *Dichelobacter nodosus*, are associated with hoof thrush in horses. Fourteen hoof samples, collected from eight horses with thrush and 14 samples collected from eight horses with healthy hooves, were examined for the presence of *F. necrophorum*, *Fusobacterium equinum* and *D. nodosus*. Only isolates with phenotypic characteristics representing *Fusobacterium* could be cultured. Total DNA extracted from the 28 hoof samples was amplified by using DNA primers designed from gene *lktA*, present in *F. necrophorum* subsp. *necrophorum*, *F. necrophorum* subsp. *funduliforme* and *F. equinum*, and gene *fimA*, present in *D. nodosus*. The *lktA* gene was amplified from five of the 14 infected hoof samples and from one hoof sample without thrush. The DNA sequence of the amplified *ltkA* gene was identical to the *lktA* gene of the type strain of *F. necrophorum* (GenBank accession number AF312861). The isolates were phenotypically differentiated from *F. equinum*. No DNA was amplified using the *fimA* primer set, suggesting that *F. necrophorum*, and not *D. nodosus*, is associated with equine hoof thrush. Hoof thrush in horses is thus caused by *F. necrophorum* in the absence *D. nodosus*. This is different from footrot in sheep, goats, cattle and pigs, which is caused by the synergistic action of *F. necrophorum* and *D. nodosus*.

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1. Introduction

Thrush of the equine hoof is recognised as an infection of the frog adjacent to the sulci. In severe cases, thrush may spread to the white line, sole, and sensitive layers of the foot, which may result in permanent lameness. Despite being a general problem in horses, little is known about the microorganisms associated with hoof thrush. Most of our understanding of the infection is gained from research published on *Fusobacterium* spp. isolated from sheep (Egerton and Roberts, 1971), goats (Bennett et al., 2009b), cattle (Sun et al., 2011) and pigs (Zhou et al., 2010) diagnosed with footrot. In earlier literature, footrot is described as an infection caused by the synergistic action of *Fusobacterium necrophorum* and *Dichelobacter nodosus* (Roberts and Egerton, 1969). In cattle *F. necrophorum* subsp. *necrophorum* and *F. necrophorum* subsp. *funduliforme* have been associated with footrot (Shinjo et al., 1991).

Dorsch et al. (2001) have shown that *Fusobacterium* spp. isolated from oral-associated diseases in horses are genetically different from *F. necrophorum* and classified the strains as *Fusobacterium equinum*. *F. equinum* is a normal inhabitant of the gastrointestinal, respiratory, and genitourinary tracts of horses and is generally associated with abscesses and necrotic infections (Racklyeft and Love, 2000; Trevillian et al., 1998; Zicker et al., 1990). Although phenotypically similar than *F. necrophorum*, *F. equinum* does not produce alkaline phosphatase and tests negative for haemagglutination (Dorsch et al., 2001).

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REVIEW

Starch-modifying enzymes of lactic acid bacteria – structures, properties, and applications

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In spite that lactic acid bacteria (LAB) are used for production of fermented foods and drinks for millennia, their ability to grow using starch as a sole carbon source was noticed by the scientists in the last 30 years. A number of amylolytic LAB (ALAB) strains were isolated and several detailed investigations of biochemical and genetic basis of starch hydrolysis were performed. The purpose of this review is to summarize for the first time the available data about the starch-modifying enzymes in ALAB. The most important amylolytic representatives of the genera *Lactobacillus*, *Lactococcus*, *Streptococcus, Pediococcus*, *Carnobacterium*, and *Weissella* are described. Amino acid sequences, corresponding to ALAB amylase enzymes are compared and some features of the gene expression are analyzed. The possible application of ALAB strains for direct production of lactic acid from starch, as well as their participation in food manufacturing is discussed.

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1 Introduction

Starch is the second carbohydrate, after cellulose that is most abundant in terrestrial plants. As part of the food since the dawn of civilization, starch can be defined as the most valuable polysaccharide used by mankind. According to FAO statistics (http://faostat.fao.org), the world total agricultural production of the starch-rich plants maize, rice, wheat, potatoes and cassava reached 2.72 billion tons in 2010, with the largest share of maize (0.8 billion tons). Starch is harvested and used chemically or enzymatically processed into a variety of different products such as starch hydrolysates, glucose syrups, fructose, starch or maltodextrin derivatives, and cyclodextrins [1]. It is also used as a raw material with many industrial applications: for production of polyols used as sweeteners, in the paper industry, and as a cheap and abundant substrate for variety of microbial fermentations.

The ability of lactic acid bacteria (LAB) to live in starchy media without symbiotic partner's help was noticed in the last 30 years when two new species *Lactobacillus amylophilus* and *L. amylovorus* were described [2, 3]. LAB strains with starch-degrading activities are very rare: so far, representatives of only three LAB genera (*Lactobacillus*, *Lactococcus*, and *Streptococcus*) are reported to produce lactic acid directly from starch as a carbon source. On the other hand, the complete genome sequencing and annotations revealed the existence of amylase genes in almost all LAB. The purpose of this review is to summarize the available data about the starch-modifying enzymes in LAB. The most important amylolytic representatives of each genus are described and their potential industrial importance is evaluated. Amino acid sequences, corresponding to LAB amylase enzymes are compared and analyzed.

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Abbreviations: ALAB, amylolytic lactic acid bacteria; **LA,** lactic acid; **LAB,** lactic acid bacteria

Colour online: See the article online to view Fig. 1 in colour.

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INFLUENCE OF THE DIFFERENT MEDIA COMPOUNDS ON 2,3-BUTANEDIOL PRODUCTION IN GLUCOSE FERMENTATION

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Abstract

The Bulgarian isolate Klebsiella pneumoniae G31, an extra-producer of 2,3-butanediol (2,3-BD) from glycerol, was tested in glucose fermentation. In purpose to achieve maximum yield of the valuable chemical, the media content was optimized. The influence of the different compounds was tested in batch processes at appropriate conditions for 2,3-BD production – uncontrolled pH of the media, temperature 37°C and strong aeration rate. By the use of the optimized medium composition, 33.3 g/l 2,3-BD yield and 0.69 g/lh productivity were achieved. The conversion rate was close to the theoretical – 0.851 mol 2,3-BD per mol consumed glucose.

Key words: *2,3-butanediol, glucose fermentation, media content*

1. INTRODUCTION

2,3-butanediol is a valuable chemical, widely used in chemical, food and pharmaceutical industries (Xiu and Zeng 2008). It can be used as fuel additive, solvent or component in anti-freeze solutions. Also, it has application in the manufacture of printing ink, perfumes, moistening and softening agents and explosives (Garg and Jain 1995). Likewise, 2,3-BD can easily be converted to other worthy chemicals as 1,3-butadiene, diacetyl, and methyl ethyl ketone (Syu 2001).

2,3-BD is produced via carbohydrate fermentation by species of *Klebsiella pneumoniae* (Lee and Madox 1986), *Klebsiella oxytoca* (Afschar et al. 1993), *Enterobacter aerogenes* (Zeng et al. 1990) and *Enterobacter cloacae* (Saha and Bothast 1999). Among them, only the strains of *K. pneumoniae* and *K. oxytoca* are capable to produce 2,3-BD in amounts, sufficient for industrial production. The product can be obtained from different carbon source, but always in mix with many byproducts pursuing the fermentation to lactic, acetic and succinic acids, carbon dioxide and ethanol. Thus, the synthesis of 2,3-BD as a main product requires specific conditions. The most important of them are considered to be: the low pH value of the media, the high level of aeration rate and the specific media content.

2. MATERIALS AND METHODS

2.1. Bacterial strain

All presented experiments were carried out with the strain *Klebsiella pneumoniae* G31, extraproducer of 2,3-BD from glycerol (Petrov and Petrova 2009, 2010), isolated from an active slime (Petrova et al. 2009) and deposited in the Bulgarian National Collection for Microorganisms and Cell Cultures under registration № 8650.

2.2. Media and cultivation conditions

The strain was maintained at -20°C with of 20% (w/w) glycerol added. All presented experiments, including both inoculum and working cultures were performed in 500 ml flasks on rotary shaker with

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Production and Properties of Two Novel Exopolysaccharides Synthesized by a Thermophilic Bacterium *Aeribacillus pallidus* 418

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Abstract Synthesis of innovative exocellular polysaccharides (EPSs) was reported for few thermophilic microorganisms as one of the mechanisms for surviving at high temperature. Thermophilic aerobic spore-forming bacteria able to produce exopolysaccharides were isolated from hydrothermal springs in Bulgaria. They were referred to four species, such as *Aeribacillus pallidus*, *Geobacillus toebii*, *Brevibacillus thermoruber*, and *Anoxybacillus kestanbolensis*. The highest production was established for the strain 418, whose phylogenetic and phenotypic properties referred it to the species *A. pallidus*. Maltose and NH_4Cl were observed to be correspondingly the best carbon and nitrogen sources and production yield was increased more than twofold in the process of culture condition optimization. After purification of the polymer fraction, a presence of two different EPSs, electroneutral EPS 1 and negatively charged EPS 2, in a relative weight ratio 3:2.2 was established. They were heteropolysaccharides consisting of unusual high variety of sugars (six for EPS 1 and seven for EPS 2). Six of the sugars were common for both EPSs. The main sugar in EPS 1 was mannose (69.3 %); smaller quantities of glucose (11.2 %), galactosamine (6.3 %), glucosamine (5.4 %), galactose (4.7 %), and ribose (2.9 %) were also identified. The main sugar in EPS 2 was also mannose (33.9 %), followed by galactose (17.9 %), glucose (15.5 %),

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PURIFICATION AND PROPERTIES OF A NEW RECOMBINANT CYCLODEXTRIN GLUCANOTRANSFERASE FROM *E. COLI* BL21 (DE3) pJCGT8-5

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Abstract

A new recombinant cyclodextrin glucanotransferase JCGT8-5 (CGTase, EC 2.4.1.19) produced by *E. coli* BL21(DE3) cells was purified by ultrafiltration, starch adsorption and gel filtration with a yield of 34% and displayed a specific activity 35683 U mg^{-1}. The purified recombinant CGTase exhibited molecular weight of 75.5 kDa estimated by SDS-PAGE. It was active at 60–65 °C, stable at a broad pH range (5.0–11.0) and retained more than 50% of its original activities after a heat treatment at 70 °C for 1 h without additives. The enzyme produced high amounts of cyclodextrins (CDs) from raw starch (12.0–12.2 mg ml^{-1}) and the products formed were 22% γ-cyclodextrin (CD), 1% α-CD and 77% β-CD after 2 h incubation at 60 °C, without adding any selective agents.

Key words: cyclodextrin production, *E. coli* BL21(DE3), enzyme purification, pJCGT8-5, recombinant cyclodextrin glucanotransferase JCGT8-5

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40. Blagoeva G., Petrova P., Petrov K., Gotcheva V., Angelov A. (2013) "Amylolytic probiotics with application in food industry" ENGIHR "The Intestinal Microbiota and Gut Health: Valencia, Spain September 2013, p. 28-32.

Amylolytic lactic acid bacteria from Bulgarian cereal-based fermented products. By G. Blagoeva[1], P. Petrova[2], K. Petrov[3], V. Gotcheva[1] and A. Angelov[1], [1]*Department of Biotechnology, University of Food Technologies, Plovdiv 4002, Bulgaria,* [2]*Institute of Microbiology, Bulgarian Academy of Sciences, Sofia 1113, Bulgaria,* [3]*Institute of Chemical Engineering, Bulgarian Academy of Sciences, Sofia 1113, Bulgaria.*

Introduction

Fermented foods are associated with a number of health benefits related both to the food matrices and to the microorganisms and their metabolites[1,2,3]. Currently, most fermented foods at the market are dairy. However, the problems and limitations related to diary products, such as lactose intolerance, unfavourable cholesterol content, presence of allergens, and vegetarian preferences have triggered a trend for development of non-dairy fermented functional foods[1].

Fermentation of cereals by mixed microbial cultures of lactic acid bacteria (LAB) is the main process in the production of various traditional fermented foods. Consumption of cereal-based fermented products delivers prebiotics to gut microbiota and thus positively affects its composition[1,4,5], as well as the proportions and types of metabolites produced in the gastrointestinal tract. However, the efficient breakdown of starch in cereal matrices requires a range of catalytic activities, including amylases, glucoamylases, pullulanases and amylopullulanases[2,6]. Since the host human genome does not encode all carbohydrate-active enzymes for degradation of the structural polysaccharides found in starchy foods, current studies are focused on isolation and selection of microorganisms which are capable of assimilating digestible starch. In this view, LAB strains with amylolytic activities would make a valuable contribution to such fermented products[6].

Amylolytic LAB (aLAB) are able to convert starch into lactic acid (LA) in a single step process, and are, therefore, of high interest to science and food industry. Most aLAB are involved in the production of many cereal-based fermented foods. Few of them are used for the industrial production of lactic acid from starch[7]. Recent studies show that aLAB also have the potential to ferment different types of resistant starch (RS) in human large bowel, which results in numerous metabolites with health effects on the host[6,8]. The ability of aLAB to assimilate starchy matrices can successfully be applied in the development of novel cereal-based functional products and probiotics. This approach offers alternatives of the diary products, nutritional enhancement of foods, improved product safety and shelf life[5].

The aim of the present study was to select LAB strains from various cereal-based fermented sources and perform a screening for potential amylolytic activity and ability to convert starch into lactic acid in order to further develop starter cultures for specific food and biotechnology applications.

Materials and Methods

1. Isolation of LAB, phenotypic and biochemical characterization. LAB were isolated from samples of conventional white bread dough, sourdough, and a traditional Bulgarian fermented drink "Boza". Cultures were maintained in MRS and M17 broth at 4°C. All isolates were initially characterized by morphology, Gram staining and catalase activity (3% H_2O_2).

41. Popova L., Petrov K. (2013) "OPTIMIZED ELECTROPORATION PROCEDURE FOR *LACTOBACILLUS PARACASEI* TRANSFORMATION", Plovdiv, October 18-19, 2013" LX, 925-929. (ISSN 1314-7102)

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ОПТИМИЗАЦИЯ НА ЕЛЕКТРОПОРАЦИЯТА ПРИ ТРАНСФОРМИРАНЕ НА LACTOBACILLUS PARACASEI

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OPTIMIZED ELECTROPORATION PROCEDURE FOR LACTOBACILLUS PARACASEI TRANSFORMATION

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Abstract Lactobacillus paracasei B41 is known as a good L (+) lactic acid producer from starch, as well as possessing certain probiotic properties. The purpose of this work is the development of an electroporation procedure for an efficient gene transfer into L. paracasei cells. In the transformation experiments the shuttle vector pHT43 that is able to transform Gram-positive and Gram-negative hosts was used. The selective markers determination was done by the analysis of the *strain's resistance to several antibiotics. L. paracasei B41 was res*istant to kanamycin at different concentrations up to *100 μg/ml and was sensitive to vario*us amounts of chloramphenicol (5 - *25 μg/ml). The electroporation of the strain,* cultured in glycine-containing medium, was performed by the use of 0.5 M saccharose, 10% glycerol buffer. The influence of the following pulse parameters was investigated: the distance between the electrodes (0.2 - 0.4 cm cuvettes), pulse strength (0.9 - 3.0 kV), and pulse duration (4 – 6 ms). The number of positive transformants, obtained under different conditions was compared. The authors thank to the National Scientific Fund, Republic of Bulgaria, as this work was financially supported *by the Research grant ДМУ 03/45.*

Key words: Lactobacillus paracasei, electroporation, plasmids.

Introduction

Electroporation designates the use of short high-voltage pulses to overcome the barrier of the cell membrane. By applying an external electric field, which just surpasses the capacitance of the cell membrane, transient and reversible breakdown of the membrane can be induced. Initially developed for gene transfer, electroporation is now in use for delivery of a large variety of molecules: drugs, dyes, antibodies, oligonucleotides, RNA and DNA [5].

Whereas initial electroporation procedures causes cell damage, developments over the past decades have led to sophistication of equipment and optimization of protocols. Considerable effort has been directed toward the development of modern genetic and molecular techniques to analyze and manipulate gram-positive bacteria [4].

Lactobacillus paracasei B41, isolated from Bulgarian traditional beverage Boza is known as a starch degrading bacterium [7]. It was evaluated as probiotic, because it may confer a health benefit on the human host and for its proved antimicrobial activity against common food-borne pathogens such as Escherichia coli, Bacillus subtilis, Vibrio cholerae, and Klebsiella pneumoniae [8]. The probiotics are live microbial feed supplement which affects positively the host animal by improving its intestinal microbial balance. L. paracasei is widely used in the dairy industry as a probiotic starter for yogurt, fermented milk, and cheese production. Despite its importance as a food supplement, our understanding of the physiology and genetics of this bacterium is still limited because of the absence of a reliable transformation procedure.

The development of electroporation methods for several Lactobacillus species, such as Lactobacillus helveticus, Lactobacillus sakei [1, 2], Lactobacillus casei [3], and Lactobacillus acidophilus [6], showed that several parameters have to be tested in order to optimize electroporation efficiency for this group of organisms. Among these parameters are: 1) the growth stage at which cells are harvested, which depends on the species or even the strain used; 2) the composition of the electroporation buffer, which plays an important role in the transformation of lactobacilli; 3) the parameters of the electrical pulse; and 4) the source of the DNA used in transformations, since restriction - modification systems can severely inhibit transformation with foreign DNA [11].

42. Ivanova V., Petrov K., Safarikova M., Safarik I., Petrova P., Tonkova A. Delchev N. (2014) "Immobilization of recombinant CGTase JCGT8-5 on magnetically – modified silicates and natural supports" *International Review of Chemical Engineering* vol. 6 (1), (in press) (ISSN 2035-1755)

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Immobilization of recombinant CGTase JCGT8-5 on magnetically-modified silicates and natural supports

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Abstract – *Recombinant cyclodextrin glucanotransferase JCGT8-5 (CGTase, EC 2.4.1.19) was effectively immobilized by adsorption on magnetically-modified particles. Silicates (montmorillonite and halloysite), natural supports (oat straw, wheat bran, banana peel, sawdust, hazelnut and peanut shell, coffee beans, tea leaves, algae Chlorella vulgaris) and activated carbon were engaged as carriers, as the most of them were used for the first time for enzyme adsorption. The immobilization capacities of the carriers entrapping ultra-concentrate or purified enzyme were determined. Binding yields reached from 50% to 90% of the initial enzyme quantity. The analysis of the formed cyclodextrins (CDs) revealed that the highest amount of total CDs was obtained by the use of halloysite particles (10.1 mg/ml), followed by sawdust (8.6 mg/ml), algae (8.6 mg/ml) and peanut shell (8.2 mg/ml). A high degree of starch conversion into CDs, ranging from 21 to 25% was achieved for 20 min starch hydrolysis. Variations in γ-CD: α-CD: β-CD ratios due to the immobilization on the different carriers were observed. The six-fold reuse of the magnetic biocatalysts containing purified recombinant CGTase bound to halloysite, washed algae or sawdust provided 29-36 mg/ml CD yield without presence of α-CD for 120 min starch hydrolysis.* ***Copyright © 2014 Praise Worthy Prize S.r.l. - All rights reserved.***

Keywords: *Immobilization, Recombinant CGTase, Magnetic Carriers, CDs Production*

I. Introduction

In the recent years, an increased attention is focused on functionalized magnetic micro- and nanoparticles (MMPs and MNPs) production. They are extensively used in immobilization of bioactive compounds such as proteins, DNA, organic chemicals, and even entire viruses, engaged as key components in different areas. The immobilized molecules take part in catalysis, environmental remediation, biomedical and sensory devices, in cell labeling and immuno-magnetic separations, cells isolation, drug targeting, waste water treatment etc. [1-7].

Magnetic techniques are applied in numerous fields of bioscience and biotechnology [1], biomedicine [8], [9], protein isolation and purification and cell and enzyme immobilization [10-12]. The entrapment of enzymes in magnetically activated supports is a method that offers significant advantage over the conventional immobilization technique, namely the easy manipulation of the biocatalyst in an external magnetic field.

Cyclodextrin glucanotransferase (CGTase, EC 2.4.1.19) is an enzyme that catalyses conversion of starch into cyclodextrins (CDs). CGTase is a multifunctional enzyme and catalyzes four interrelated and reversible reactions: hydrolysis, cyclization, coupling, and disproportionation. The resulted CDs find numerous applications in medicine, agricultural, pharmaceutical and chemical industries because of their ability to encapsulate a wide range of organic and inorganic molecules and to change the stability, reactivity and solubility of the formed inclusion complexes with these molecules [13-15].

CGTases are primarily extracellular enzymes that vary in type and quantity of their products. They are capable of predominant synthesis of one or two of the three possible CDs: α-, β-and γ-CD, consisting of 6, 7 and 8 glucose units, respectively [16-18]. Bacterial strains from the genus Bacillus are one of the best CGTases producers, secreting mainly β-CGTase that converts starch into β-CDs [19, 20]. The weak or absent accumulation of α-CDs allow facilitated purification of the β-CDs from the reaction mixture.

In our previous studies the *cgt* gene of the alkaliphilic halotolerant *Bacillus pseudalcaliphilus* 8SB was identified and sequenced. It was cloned as PCR-amplicon under T7 promoter control and over expressed in *E. coli* BL21 (DE3) [21]. The recombinant strain *E. coli* BL21 (DE3) pJCGT8-5 was deposited in DSMZ Collection of Microorganisms, Germany, under accession number DSM 27107.

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43. Tsvetanova F., Petrova P., Petrov K. (2014) "2,3-butanediol production from starch by engineered *Klebsiella pneumoniae* G31-A" *Applied Microbiology and Biotechnology* (DOI: 10.1007/s00253-013-5418-4) (IF 3.689)

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BIOTECHNOLOGICAL PRODUCTS AND PROCESS ENGINEERING

2,3-Butanediol production from starch by engineered *Klebsiella pneumoniae* G31-A

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Abstract 2,3-Butanediol (2,3-BD) is an organic compound, which is widely used as a fuel and fuel additive and applied in chemical, food, and pharmaceutical industries. Contemporary strategies for its economic synthesis include the development of microbial technologies that use starch as cheap and renewable feedstock. The present work encompasses the metabolic engineering of the excellent 2,3-BD producer *Klebsiella pneumoniae* G31. In order to perform direct starch conversion into 2,3-BD, the *amyL* gene encoding quite active, liquefying α-amylase in *Bacillus licheniformis* was cloned under *lac* promoter control in the recombinant *K. pneumoniae* G31-A. The enhanced extracellular over-expression of *amyL* led to the highest extracellular amylase activity (68 U/ml) ever detected in *Klebsiella*. The recombinant strain was capable of simultaneous saccharification and fermentation (SSF) of potato starch to 2,3-BD. In SSF batch process by the use of 200 g/l starch, the amount of total diols produced was 60.9 g/l (53.8 g/l 2,3-BD and 7.1 g/l acetoin), corresponding to 0.31 g/g conversion rate. The presented results are the first to show successful starch conversion to 2,3-BD by *K. pneumoniae* in a one-step process.

Keywords 2,3-Butanediol · Starch · *Klebsiella pneumoniae* · Amylase · *Bacillus licheniformis*

Electronic supplementary material The online version of this article (doi:10.1007/s00253-013-5418-4) contains supplementary material, which is available to authorized users.

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Introduction

2,3-Butanediol (2,3-BD) is a bivalent alcohol with extensive industrial uses. The interest in its production increased remarkably in the last decade because of its application as a liquid fuel, along with its common usage in manufacture of antifreezes, printing inks, perfumes, moistening, and softening agents. 2,3-BD is also a chiral compound, reagent in a number of asymmetric chemical syntheses (Xiu and Zeng 2008; Zeng and Sabra 2011). It is platform chemical for valuable derivatives such as methyl ethyl ketone and 1,3-butadiene (Syu 2001).

The microbial production of 2,3-BD is an attractive alternative to the more costly chemical synthesis. Several bacterial species, belonging to *Klebsiella*, *Enterobacter*, *Serratia*, *Paenibacillus polymyxa*, *Bacillus licheniformis*, *Bacillus subtilis*, and *Bacillus amyloliquefaciens* are able to secrete 2,3-BD (Ji et al. 2011; Jurchescu et al. 2013). Among them, the species *Klebsiella pneumoniae* is known as the best for industrial use because of its more complete fermentation, broad substrate spectrum, and cultural adaptability (Garg and Jain 1995; Ma et al. 2009). It is capable of degrading a variety of hexoses, pentoses, and disaccharides and produces mainly meso-2,3-BD via mixed-acid pathway that yields several liquid by-products: lactic, succinic, and acetic acid and ethanol (Syu 2001). This is the reason that, so far, a number of engineered *Klebsiella* strains were manipulated with the purpose to redistribute the metabolic flux predominantly to 2,3-BD synthesis (Kumar et al. 2012; Kim et al. 2013; Ji et al. 2010, 2013), and at the same time, the attempts to expand the substrate utilization properties of 2,3-BD-producing strains are very limited (Zheng et al. 2008).

In bacterial metabolism, the monosaccharides are first converted to pyruvate via a combination of Embden–Mayerhof and pentose phosphate pathways. Then, two molecules of pyruvate are converted to one molecule of α-acetolactate, subsequently reduced to acetoin. The acetoin is reduced to 2,3-BD by a reversible reaction (Ji et al. 2011). Among all

44. Tsvetanova F., Petrov K. (2014) "Influence of pH and aeration on 2,3-butanediol production from glucose by *Klebsiella pneumoniae* G31" *Bulgarian Chemical Communication* (in press) (IF 0.320)

Influence of pH and aeration on 2,3-butanediol production from glucose by *Klebsiella pneumoniae* G31

Flora Tsvetanova, Kaloyan Petrov

Abstract

The strain *Klebsiella pneumoniae* G31, an extra-producer of 2,3-butanediol (2,3-BD) from glycerol, was tested in glucose fermentation. Key factors affecting the fermentation, such as pH and aeration regime were optimized. The best conditions favorable for 2,3-BD production were found to be pH 6.0, airflow 1.45 vvm and agitation speed of 500 rpm. Thereby, after 18 h of fermentation, the concentration of 2,3-BD reached maximum of 30.4 g/l with productivity of 1.69 g/lh and yield of 0.32 g/g. The efficiency of the process demonstrates the potential industrial application of *K. pneumoniae* G31 as 2,3-BD producer from glucose - containing feedstock.

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(57) Изобретението се отнася до метод за биотехнологично получаване на млечна киселина, която е метаболитен продукт на Lactobacillus rhamnosus в чист вид, отделен от биомасата. Млечната киселина е приложима както в хранителната промишленост, така и при производство на деградиращи опаковки (полиестери на млечната киселина).

1 претенция, 6 фигури

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46. Калоян Петров, Пенка Петрова, Венко Бешков (2014) “Метод за получаване на 2,3-бутандиол”, патент №66411, Бюлетин на патентното ведомство, (01.2014)

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(54) МЕТОД ЗА ПОЛУЧАВАНЕ НА 2,3-БУТАНДИОЛ

(57) Изобретението се отнася до метод за получаване на 2,3-бутандиол, ценен продукт, намиращ широко приложение в химическата, фармацевтичната и хранителната промишленост като реагент в синтезата на 1,3-бутадиен (градивен елемент на синтетичния каучук), на полибутилен-терефталатни гуми, гама-бутиролактон, спандекс и техните прекурсори и др. Изобретението представлява метод, при който глицеролът се подлага на ферментация, като по време на ферментацията pH на средата изкуствено се променя през определен интервал от време чрез добавяне на основа или киселина. По този начин pH на средата циклично се променя, в едната посока - изкуствено чрез контролиране (водене на процеса), в другата - вследствие на естествения метаболизъм на клетките. Изкуствените флуктуации могат да са с различната амплитуда (Делта pH) и да бъдат осъществявани през различен интервал от време.

1 претенция, 4 фигури

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