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Biotechnological Approach for the Production of Prebiotics and Search for New Probiotics and their Application in the Food Industry

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Abstract

Background and objective: Prebiotics and probiotics intake have been widely recognized in past recent years due to possessing multiple health benefits. Prebiotics are non-digestible carbohydrates that promote the growth and/or activity of beneficial bacteria in the colon which improves the health. Moreover, the incorporation of probiotics in food has also been a growing practice due to its immunomodulatory effect, the production of organic acids and other compounds that promotes the absorption of nutrients and the general health of the digestive system.

Results and conclusion: Biotechnological strategies have been proposed for prebiotic production and purification in order to meet the demand to be included as ingredients in functional food formulation. Different aspects related to the substrates and different fermentation systems for their production as well as the purification and characterization processes are addressed. Also, we will present the benefits promoted by probiotics, the methods of isolation and characterization, as well as the evaluation of these attributes, so that they can be used in the food industry. With the technological developments in prebiotics and probiotics, it will be possible to deliver foods that respond to consumer demand with low cost and with pleasant sensory characteristics as well as providing beneficial health effects.

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1. Introduction

Currently, prebiotics are defined as non-digestible compounds that, through their metabolism by micro-organisms in the intestine, modulates the composition and/or activity of the intestinal microbiota, thus conferring a beneficial physiological effect on the host [1]. In the next section, we will focus on the substrates used in fermentation systems for the production of main polysaccharides.

1.1. Prebiotics and substrates used for production

In a fermentation system, the source of carbon and energy for the microorganism is very important for its rapid and adequate growth. For example, in a study, a bi-enzymatic system based on the combined use of levansucrase from *Bacillus* (*B.*) *amyloliquefaciens* and endo-inulinase from *Aspergillus* (*A.*) *niger* in a one-step reaction was investigated for the production of fructo-oligosaccharides (FOS) and oligolevans using sucrose as the sole substrate. One of the main conclusions of the authors was that the application of response surface methodology showed that the concentration of sucrose as substrate and reaction time were the most significant independent variables [2]. On the other hand, the production of the functional trisaccharide 1-kestose, O-β-D-fructofuranosyl-(2-1)-β-D-fructofuranosyl α-D-gluco-pyranoside, by β-fructofuranosidase from *A. japonicus* using sugar cane molasses as substrate was evaluated. This substrate is a byproduct of the sugar industry, but it contains a high amount of carbohydrates, it is considered to be of low value and is often treated as waste. They concluded that bioprocess demonstrated here increases the value of substrate and raises the prospect that sugar cane molasses will find utility for the production of other novel sugars [3]. In another research, the production of fructosyl transferase by *A. flavus* NFCCI 2364 was exploited in solid state fermentation (SSF) using low-cost agricultural wastes for their ability to generate FOS. Among sixteen screened agro wastes, sugar cane bagasse was remarked as the most promising substrate suited for excellent growth and adequate production of fructosyltransferase in 96 h of fermentation. The results showed that the substrates sugarcane bagasse and banana peel have the highest FOS formation [4].

1.2. Production of prebiotics using different fermentation systems and microorganisms

Currently, alternative methods for the production of prebiotics are being sought; this section explains recent research related to some general aspects of solid and submerged fermentation systems, as well as factors that affect production. The production of fructosyltransferase by SSF using aguamiel (agave sap) as culture medium and *A. oryzae* DIA-MF as producer strain. Fermentation was carried out to evaluate inoculum rate, incubation temperature, initial pH and packing density to determine the most significant factors through Box-Hunter and Hunter design. The authors showed that the FOS production was possible using aguamiel as substrate and concentrated enzyme in the reaction [5]. There are few works where SSF systems are used for the production of prebiotics. For example, in a study, the impact of the solid substrates mixture on fructosyltransferases and FOS production was investigated. An augmented simplex lattice design was used to optimize a three-component mixture for enzyme production. The results showed that it was possible to obtain large amounts of enzyme and FOS using very low-cost substrates. Mixture design could be an effective method for selection of the best combination of a multi-substrate for improved production of microbial enzymes under SSF [6]. In another research, FOS obtained by fermentation of sucrose purified at large-scale by continuous chromatography were evaluated. The results showed that the best experimental condition corresponded to 20 g l^{-1} of $NaNO_3$, 6.0 g l^{-1} of KH_2PO_4 and fermentation time of 47.8 h; a maximum yield of 105.7 g l^{-1} of FOS was reached. The authors concluded that the final FOS mixture obtained in this work was enriched with these compounds, but also contains low amounts of sucrose and salts, which will greatly improve the efficiency of the next purification step [7]. On the other hand, fed-batch synthesis of galacto-oligosaccharides from lactose with β-galactosidase from *A. oryzae* was evaluated experimentally and reaction yield was maximized via optimal control technique. The results supported the idea that substrate and/or enzyme feeding rate control is a suitable strategy of reactor operation to work with poorly or moderately soluble substrates and to operate at very high concentrations of substrates and fed-batch operation allowed the validation of the predicted galacto-oligosaccharides production [8]. Endoinulinase production was achieved by heteroexpression of an endoinulinase-encoding gene from *A. ficuum* in *Escherichia* (*E.*) *coli* BL21. In this research, the results showed that temperature of 55°C, enzyme 5 U g^{-1} of substrate and 24 h of time reaction reached 94.41% of maximum yield of inulooligo-saccharides. The authors concluded that, endoinulinase secretory expression level in *E. coli* was improved and demonstrated that modification of signal peptide may be a suitable method for cost-effective production of endoinulinases [9].

There is a wide range of microorganisms used for the production of prebiotics. For example, twenty micro-organisms comprising of sixteen molds, two yeasts, and two bacteria were evaluated for their ability to produce fructosyltransferase and generate FOS from sucrose. The results showed that a good yield was reached which clearly signifies their vast ability for commercial application in food and feed industry. The chromatography analysis deduced *A. flavus* (NFCCI 2364), *A. niger* (SI 19) and *Penicillium islandicum* (MTCC 4926) as the most prosperous strains for synthesizing high amount of FOS to be captivated on an industrial level [10]. On the other hand, β-galactosidase are enzymes that are able to catalyze lactose hydrolysis and transfer reactions to produce lactose-based prebiotics with potential application in the pharmaceutical and food industry. In this research, *A. lacticoffeatus* was described, for the first time, as an effective β-galactosidase producer. The extracellular enzyme production was evaluated in synthetic and alternative media containing cheese whey and corn steep liquor. The authors concluded that the microorganism was able to grow and produce the enzyme using alternative fermentation media containing cheese whey and corn steep liquor and these results were very promising and can be further improved through the optimization of the reaction conditions and/or by using purified β-galactosidase [11].

1.3. Purification and characterization of prebiotics by chromatographic techniques for their use in food industry

The chromatographic techniques and other techniques of separation and purification are the preferred and most used methodologies for the characterization and identification of prebiotics using different extraction methodologies. Table 1 shows the use of these techniques in research.

Table 1. Research techniques for prebiotic characterization

Substrate	Characterization and/or identification	Extraction	Compounds	Reference
Stevia rebaudiana	GC-MS, NMR, and off-line ESI-MS	Aqueous and ethanolic hydrolysis	Inulin and fructooligosaccharides	[13]
Stevia rebaudiana	NMR and ESI-MS	Aqueous and ethanolic hydrolysis	Fructo-oligosaccharides	[14]
Agave sisalana	FT-IR, NMR, XRD, and MALDI-TOF-MS	Aqueous hydrolysis	Inulin	[15]
Deffated copra meal	FT-IR and NMR	Enzymatic hydrolysis	Mono-oligosaccharides	[16]
Sucrose	FTIR and NMR	Biosynthesis	Inulin	[17]
Sucrose	TLC, MS, and NMR	Fermentation	Fructo-oligosaccharides	[18]
Carbohydrates	HPLC	Synthesis	Galacto-oligosaccharides	[19]

2. Probiotics

The term "lactic bacteria" encompasses a heterogeneous group of microorganisms with the defining characteristic being the production of lactic acid from the fermentation of sugars [19]. They are Gram-positive cocci or bacilli, not sporulated, not mobile, anaerobic, microaerophilic or aerotolerant; oxidase, catalase, and benzidine negative. They lack cytochromes and do not reduce from nitrate to nitrite; their main product of carbohydrate fermentation is lactic acid, which is why they are acid-tolerant since they can grow at very low pH; however, other short chain fatty acids as acetic can be also produced [20]. Some bacteria known as facultative anaerobes and others as obligate anaerobes can transiently colonize the intestine and survive during intestinal transit; in addition to their adhesion to the epithelium, they modify the local immune response of the host [21].

For the multiplication of lactic acid bacteria (LAB), sugars such as glucose and lactose are required, as well as amino acids, vitamins, and other growth factors. Milk is the typical and satisfactory medium for the proliferation of LAB. However, other foods are also excellent growth media and production of metabolites of LAB; among them are the cereals, meat, fruit juices and fermented agricultural products such as cabbage, squash, green beans, and olives [22]. Therefore, these microorganisms are generally used as starter cultures in the production of dairy products, such as acidified milk, yogurt, butter, cream, and cheeses; as well as in the processing of meats, alcoholic beverages, and vegetables.

LAB are one of the most representative groups of probiotics. The term probiotic means "in favor of life" and is currently used to designate bacteria that have beneficial effects on humans and animals [23]. This concept was created at the beginning of the last century by Russian scientist and Nobel laureate Elie Metchnikoff, who hypothesized that eating fermented products can improve health and increase the longevity of Bulgarian farmers. He was the first to indicate that it would be possible to modify the intestinal flora replacing the harmful microorganisms with useful microorganisms [24]. Currently, the term probiotic refers to a preparation or a product that contains strains of viable microorganisms in sufficient quantity to alter the microflora in some compartment of the host (by implantation or colonization) and that produces beneficial effects in a said host [25].

Therefore, probiotics fulfill diverse functions: 1) reinforce the immune system, 2) displace microorganisms harmful to health and prevent their proliferation, 3) aid in food digestion and collaborate in the formation of essential nutrients such as vitamins, digestive enzymes, and short chain fatty acids, 4) stimulate the formation of lactic acid by decreasing the pH of the digestive tract, and 5) promote the absorption of calcium, magnesium, and iron [26]. By growing in the intestine and adhering to the intestinal mucosa, they prevent other harmful bacteria from implanting and exerting their negative functions, acting as a barrier that prevents the colonization of the intestine by pathogenic bacteria [27].

2.1. Classification and identification of probiotic strains

A first step for LAB identification is phenotypic methods to assess cell morphology, motility, gram staining, catalase, and oxidase reactions. Also, their physiological properties can be tested based on fermentation properties of bacteria. The specificity of these methods is ambiguous and could cause misidentification or misinterpreted results. For this reason, physiological testing seems to be appropriate for underlining results based on genomic methods [22]. Sequencing of 16S/23S-5S rRNA allows a precise assignment of lactobacilli to the level of genus or species and discrimination at this level can be also done using denaturing gradient gel electro-phoresis, RAPD, pulsed-field gel electrophoresis and REP-PCR. Both techniques for RAPD and rep-PCR, can distinguish between bacteria at both interspecies and interspecies level [28].

The European Union (EU) request to deposit the strains used as probiotic supplement to animal feed in the International Culture Collection and states under the EU Directive No. 93/113: “Article 7, Part B. For micro-rganisms and their preparations: (a) the identifications of the strain(s) according to a recognized International code of nomenclature and the deposit number of the strain(s); (b) the number of colony forming units (CFU g^{-1})” [29]. The importance of knowing the nomenclature of a probiotic strain lies in the fact that the health benefits of probiotics are specific for each species [30]. However, the benefits of probiotics must be studied in each species, which is the benefits offered by a certain type of probiotic cannot be extrapolated to others. Even within the same genus and species, the strains may have a different probiotic capacity and diverse functions [20]. Among the lactic acid bacteria considered as probiotics are bacteria of the order of Lactobacillales and Bifidobacteriales [31].

2.2. Bifidobacteriales and Lactobacillales

The *Bifidobacterium* (*B.*) sp. encompasses more than 30 species. Bifidobacteria are bacilli that usually have a protrusion at one end, like a cane or spatula [32]. This is one of the largest genera of saprophytic bacteria in the intestinal flora. That is why they help in digestion, and are associated with a lower epidemiological incidence of allergies and also prevent some forms of tumor growth [33]. Usually, bifidobacteria can be found on the market lyophilized, that is, in the form of the powder that is rehydrated before its ingestion and is of clinical use. *B. bifidum*, *B. longum*, *B. lactis*, *B. infantis*, *B. breve*, *B. bifidum*, *B. animalis*, *B. adolescencis*, *B. catenulatum* and *B. pseudocatenulatum* are well-known probiotics.

Lactobacillales is an order of Gram-positive bacteria, within the Firmicutes division. Representative genera are Enterococcus, Streptococcus, Lactococcus, Leuconostoc, and Lactobacillus. They are scattered on any surface, mainly on soil, water, animals, and plants. Some of their species are able to live in the absence of oxygen so they are widely used in the production of fermented products, such as yogurt, cheese, butter, and wine, among other. Some of Lactobacillales used as probiotics are *Lactobacillus* (*L.*) *acidophilus*, *L. amylovarus*, *L. casei*, *L. crispatus*, *L. delbrueckii* subsp. *bulgaricus*, *L. gallinarum*, *L. gasseri*, *L. johnsonii*, *L. paracasei*, *L. plantarum*, *L. reuteri* and *L. rhamnosus*, *Enterococcus* (*E.*) *fecaelis*, *E. faecium*, *Lactococcus lactis*, *S. thermophiles*, *Leuconostoc mesenteroides*, *Pedicococcus acidilactici* and *Sporolactobacillus inulinus.*

2.3. Benefits that probiotics promote

Probiotics can exert multiple health benefits. In the intestinal light, probiotics compete with other micro-organisms for light and nutrients, and inhibit their growth by producing antibiotic substances and decreasing the intestinal pH that induces a hostile environment for pathogenic microorganisms. The protective effect of these microorganisms is carried out through two mechanisms: the antagonism that prevents the multiplication of pathogens and the production of toxins that make their pathogenic action impossible. This antagonism is due to competition for nutrients or adhesion sites [34]. In the intestinal mucosa, since they adhere to the intestinal cells preventing undesirable microorganisms from doing so, improve the intestinal barrier function [35]. Probiotics also produce essential nutrients for the intestinal function such as short chain fatty acids (main source of energy for intestinal colon cells), vitamins (B vitamins and vitamin K) and other substances beneficial to the body. Also, they promote the function of the intestine as an immune barrier, since they stimulate the phagocytic capacity of leukocytes, stimulate T cells, activate macrophages, stimulate the production of immunoglobulins, etc. [31].

2.4. Technological aspects of probiotics

For a strain that has demonstrated probiotic attributes to be incorporated in the preparation of a food or a supplement, it is necessary that it complies with the following characteristics:

1- Contain an adequate number of viable strains that lead to the demonstrated beneficial effect. 2-Phage resistance: Resistance to those viruses that affect bacteria. 3-Stability in the product and during storage: Despite the beneficial effects of probiotics, there is the problem of their stability in food products. An alternative to the release of live bacteria directly into the gastrointestinal tract is through the use of microcapsules. Micro-encapsulation techniques have been developed and successfully applied using various matrices to protect bacterial cells from damage caused by external environments (environmental stress, oxygen, bile salts, acid, etc.), guaranteeing and improving their survival during gastrointestinal transit and increasing its stability. 4-Feasibility during processing: Viability is the ability of these microorganisms to remain alive, both in the food and in the intestine of the consumer for a certain time, in order to achieve the benefits of such foods.

2.5. Production of biogenic amines

Biogenics or biogenic amines are non-protein, low molecular weight nitrogen compounds that have important biological activity. They can be elaborated by the organism (endogenous) or they can be found in the food that we ingest (exogenous) elaborated by the cells or during its process or storage. The most common amines found in foods rich in probiotics include histamine, tyramine, tryptamine, and phenyl ethylamine [36]. However, most food poisoning is due to histamine and tyramine, the main biogenic amines detected in cheese. In some cases, they have been considered as substances of risk due to their capacity to react with nitrites and form nitrosamines that are potentially carcinogenic [37]. The ingestion of these foods can produce symptoms of toxicity (a headache, reddening of the skin, etc.), so they are an index of the degree of freshness and safety of the food [38,39].

Histamine is an organic nitrogenous compound capable of triggering an allergic response in humans and other mammals. It is believed that other biogenic amines (such as cadaverine and putrescine) enhance the toxic effect of histamine. Amines can excite the central nervous system, increase or decrease blood flow and can trigger headaches in people sensitive to the substance [39].

The toxic capacity of these substances depends on factors such as the sensitivity of the consumer or the taking of medicines. Therefore, establishing specific toxicity levels for food products is a complex task. In spite of everything, the EU has established maximum content limits for histamine, which stands at 100 mg kg^{-1}, while the US Food and Drug Administration sets the maximum limit at 50 mg kg^{-1}. For the rest of biogenic amines, there are no legal limits, since it must be taken into account that the amount of biogenic amines ingested is the sum of all the biogenic amines present in different foods and beverages that are consumed [40].

3. Results and discussion

Lactic acid bacteria have been used for centuries for the production of a large variety of fermented products. They are the most important bacteria in food fermentations, responsible for the fermentation of certain bread, beer, fermented milk and most fermented vegetables. The fermentation of food by LAB is a natural bioprocess that can improve the quality of food and drinks [41]. Historically, bacteria of the genus Lactobacillus, Leuconostoc, Pediococcus, and Streptococcus have been the main species involved in food fermentation. Among the main microorganisms involved in fermented foods are *L. acidophilus*, *L. bulgaricus*, *L. plantarum*, *L. casei*, *L. pentoaceticus*, *L. brevis* and *L. thermophilus*.

There are studies that indicate that some lactococci isolated from plant material have technological characteristics such as; 1-formation of different flavors from amino acids, 2-production of antimicrobial peptides or bacterioncins with the ability to inhibit the growth of other strains and be used as preservatives for food and pharmaceutical products and 3-show probiotic properties [29,36,42]. The use of lactic acid bacteria as a starter culture has been considered in the fermentation of fresh vegetables to give a uniform quality and health benefits. Different crops such as *L. mesenteroides*, *L. citreum* and *L. plantarum* have been applied in the fermentation of kimchi to develop a better quality of the product [19]. On the other hand, cultures of *L. delbrueckii* and *L. paracasei* have been used as mixed starter cultures to improve the characteristics of fermented cabbage [43]. *L. mesenteroides* is a bacterium associated with the fermentation of sauerkraut. It differs from other LAB for having the ability to tolerate high concentrations of salt and sugar (up to 50% sugar). *L. mesenteroides* begins its growth in plants faster in a range of temperatures and salt concentrations than any other LAB, produces carbon dioxide and acids that rapidly reduce pH and inhibit the development of undesirable microorganisms [44]. However, probiotics can be used in conjunction with prebiotics to increase the benefit they both provide. It is well known that prebiotics, such as oligosaccharides exert beneficial effects on the microbiota of the colon, such as lactobacilli and bifidobacteria, as they promote their growth and the formation of products such as short chain fatty acids which have a stimulating effect on the immune system; In addition, prebiotics have such stability that allows them to be incorporated into edible food-matrix. For example, in a study conducted by Rad [45], chocolate was used as a vehicle for the delivery of galacto-oligosaccharides and *L. paracasei*. These authors found that chocolate that included 2.5% galactooligosaccharides and 2.5% tagatose had the best physicochemical and sensory properties, at the same time that the probiotic cells remained viable (log 8.0 CFU g^{-1}) up to 6 months later. On the other hand, Sharma and Kanwar [46] evaluated the influence of prebiotics (lactulose, FOS and inulin) on the growth of *L. plantarum* and its activity on anaerobic intestinal pathogens (*Clostridium perfringens*, *Bacteroides fragilis* and *Peptoestreptococcus anaerobious*). In this study, they found that lactulose was the prebiotic that mainly promoted the growth of *L. plantarum* in addition to increasing the inhibition of pathogens, this was due to the production of metabolites such as short chain fatty acids. The production of large-scale probiotics using malt shoots, a by-product of the industry, added with 20% FOS was proposed by Cejas et al. [47] by checking that these shoots function as a support and substrate for the growth of *L. salivarius* and *L. plantarum*, where the FOS also play a cryoprotective role during storage in freezing so this strategy is innovative for the production of lactobacilli as well as for the preparation of foods containing prebiotics and probiotics. Foods that incorporate both prebiotics and probiotics have been called synbiotics, due to the benefits they offer as a whole being prebiotics an excellent substrate for the development of probiotics.

4. Conclusion

It is evident that the administration of probiotics promotes beneficial effects on human health. These have been used as complementary treatment in intestinal diseases since they are capable of competing with pathogenic microorganisms in addition to activating the cells of the immune system. It is also important to consider that the unbalance or lack of these microorganisms has been linked to the risk of suffering from obesity, diabetes and some types of cancer. Nowadays we know the effect of certain species of bacteria that have beneficial effects on human health and, the balance of these can influence the maintenance of homeostasis. This type of microorganisms, therefore, should be included in diets frequently to achieve high levels of population in the intestine and can thus exert their beneficial effects. To assist in the development of delivery vehicles for these microorganisms, prebiotics represent a promising strategy for the development of dietary matrices that favor the interaction between prebiotics and probiotics in order to enhance the benefits they can offer individually. For this reason, the development of strategies for the production, characterization and purification of oligosaccharides is of utmost importance. However, it is also necessary to study the interaction between these and the strains known as probiotics, as well as the search for new strains with probiotic activity. In this way it will be possible to design foods that respond to consumer demand for food with beneficial effects on health, low cost and with pleasant sensory characteristics.

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6. Conflict of Interest

The authors declare no conflict of interest.

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رویکرد زیست فناورانه در تولید کمک زیست‌یارها و جستجوی زیست‌یارهای جدید و کاربردشان در صنایع غذایی

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چکیده

سابقه و هدف: در سال‌های اخیر دریافت کمک زیست‌یارها (Prebiotics) و زیست‌یارها (Probiotics) به علت اثرات مفید بر سلامتی به طور گسترده‌ای تایید شده است. کمک زیست‌یارها کربوهیدرات‌های غیرقابل هضمی هستند که رشد و/ یا فعالیت باکتری‌های مفید روده بزرگ را افزایش داده و موجب بهبود سلامتی می‌شوند. علاوه بر این، افزودن زیست‌یارها به مواد غذایی، به علت اثر ایمن‌سازی و تولید اسیدهای آلی و سایر ترکیباتی که جذب مواد مغذی و سلامت کلی دستگاه گوارش را افزایش می‌دهند رو به افزایش است.

یافته‌ها و نتیجه‌گیری: راهبرد زیست‌فناورانه برای تولید کمک زیست‌یارها و خالص‌سازی آن‌ها به منظور تامین نیاز به مواد اولیه برای فرموله کردن غذاهای فراسودمند پیشنهاد شده است. انواع رشد مایه‌ها (Substrates) و روش‌های تخمیر، خالص‌سازی و تعیین ویژگی‌های آن‌ها بررسی شده‌اند. گذشته از این، ما مزایای زیست‌یارها، روش‌های جداسازی و تعیین و ارزیابی ویژگی‌های آنها که موجب استفاده از آنها در صنعت غذا شده است را نشان خواهیم داد. با پیشرفت‌های فناورانه انتقال کمک زیست‌یارها و زیست‌یارها به مواد غذایی به منظور تامین نیاز مصرف‌کننده، با هزینه پایین و داشتن ویژگی‌های حسی مطلوب و اثرات مفید سلامتی امکان‌پذیر شده است.

تعارض منافع: نویسندگان اعلام می‌کنند که هیچ تعارض منافعی وجود ندارد.

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واژگان کلیدی

- تعیین ویژگی‌ها
- تخمیر
- اولیگوساکاریدها
- کمک زیست‌یارها
- زیست‌یارها

***نویسنده مسئول**

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