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REVIEW APPLICATIONS OF SACCHAROMYCES IN THE INDUSTRY

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INTRODUCTION

Yeasts of the Saccharomyces genus are ascosporated yeasts belonging to the Ascomycota division and to the Saccharomycetes family and can bind as transient mucosal flora. They are outstanding in the industrial production of ethanol and in the elaboration of alcoholic beverages such as beer, the preparation of which depends on the grain used and the form of fermentation in which species of the *Saccharomyces pastorianus* and *Saccharomyces cerevisiae* type are frequently used. This one is also used in the production of table wine, sake, rum, whiskey and other alcoholic beverages. Other species of the same genus are also used to make this type of drink, *S. ellypsoideus* and *S. apiculatus* are examples of these. (Bonifaz, 2012; Zhang et al., 2015).

Yeasts of the Saccharomyces genus are used in many areas of science and the food industry, an example of the latter is fermentation, which is a process that consists of the degradation of carbon sources, usually carbohydrates, which results in the production of ethanol. In this context they play a relevant role because they are the microorganisms responsible for the fermentation process. *Saccharomycodes ludwigii* and *Saccharomyces cerevisiae* are used in special ferments such as kombucha together with other yeasts and with fermenting bacteria such as *Gluconobacter oxydans* and *Bacterium xylinum.* For the production of bread, it is also necessary to develop a fermentation process similar to that used in alcoholic beverages, and *S. cerevisiae* yeasts (mainly) and *Saccharomyces exiguus* are used too. They are also used as a food supplement where *S. cerevisiae* is once again mainly used, which been integrated into certain foods represents a source of proteins and vitamins, especially the B complex (except for vitamin B12).(Bonifaz, 2012; Moysés, Reis, Almeida, Moraes, & Torres, 2016; Pérez-Torrado & Querol, 2016; Popiel et al., 2015).

S. cerevisiae is the most representative organism of these yeasts, it has multiple uses in the food industry and also plays an important role in the health area as regeneration treatment/prophylaxis (probiotic, together with S. boulardii) of the normal microbiota of immunosuppressed patients who have been treated with antibiotics for a long time and who therefore have diarrhea; and in pilot studies in the search for cures to certain diseases, it was also the first eukaryotic organism to which its genomics were sequenced, thus providing great advances in science.(Bonifaz, 2012; Pérez-Torrado & Querol, 2016; Popiel et al., 2015; Zhang et al., 2015).

Despite all the beneficial contributions that Saccharomyces has provided to humanity throughout history, yeasts belonging to this genus can produce pathologies when the patient is immunosuppressed or in a critical state, causing systemic infections of the bloodstream, endocarditis, infections in essential organs, fungemias, among others. However, it has been shown that it can cause health problems in healthy patients, such as skin diseases and vaginitis.(Bonifaz, 2012; Pérez-Torrado & Querol, 2016; Zhang et al., 2015).

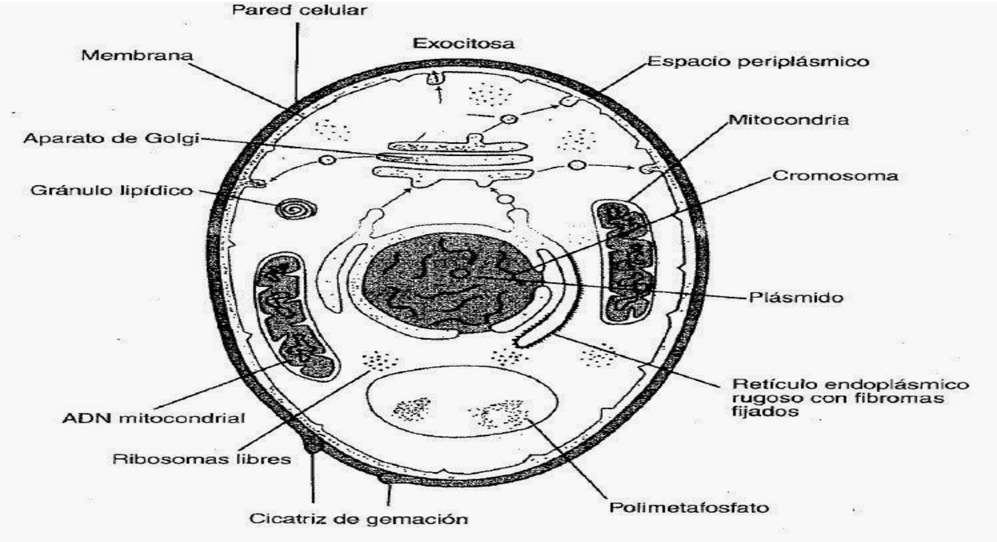
In recent years it has been considered as a yeast that can be opportunistic. Studies have reported that the term opportunistic sepa is not exclusive to those strains that have been isolated in clinical settings, since not all clinical strains of yeast cause infections, and it has been observed that many clinical strains of *S. cerevisiae* have less virulence. in models of infected mice that you know of *S. cerevisiae* isolated in food supplements. (Pérez-Torrado & Querol, 2016; Zhang et al., 2015).

Agent characteristics

Depending on the oxygen requirement, these fungi can be classified as optional fermenters, since they can grow in aerobic conditions but also in the absence of oxygen, fermenting sugars. (Carro, 2004; Cepero, Restrepo & Franco, 2012).

Saccharomyces cerevisiae has protein-like macromolecules such as tubulin, mannoprotein, and actin, lipids such as ergosterol, triglycerides, phospholipids, and free fatty acids. The MRNA, RRNA, TRNA that are ribonucleic acids, plus polysaccharides like chitin and glycogen. (Cáceres, J & Reyna, A., 2002).

The main constituents of the cell wall are glucan, which is estimated to be the determining compound in the wall structure, and mannoprotein, although both are found in the same proportion. Among the main organelles of *S. cerevisiae* are the vacuoles, which allow it to store metabolites, as part of the intermembrane system that includes the endoplasmic reticulum, whose main function is to secrete enzyme and is found in the growth region of cells.It also has mitochondrias that enable it respiratory for aerobic growth in a non-fermentable carbon source, and the nucleus that is surrounded by a nuclear membrane, which keeps the genetic material isolated from other cellular organelles. (Cáceres, J & Reyna, A., 2002).



Esquema de una célula de levadura. (P. Ribéreau-Gayon y Gaillardin-Heslot).

Figure 1. Diagram of a yeast cell.

Source: Ribéreau-Gayon, Glories, Maujean and Dubourdieu, 2006.

Most yeasts are produced by budding (asexually), giving rise to what is known as blastoconidium. They are classified according to the place where the yolk is generated, in the case of *Saccharomyces* it is of the multipolar type, since each time it sprouts, the production of the yolk occurs in different places on the surface of the stem cell. Furthermore, *Saccharomyces* is classified as simple, in terms of the number of outbreaks that can be observed under a microscope at one time in one cell, since only one daughter cell is observed, attached to the stem cell at any given time. (Cepero, Restrepo and Franco, 2012).

One of the main differences between filamentous fungi and *Saccharomyces cerevisiae* is that in high glucose concentrations this yeast prefers to carry out fermentation and repress genes that are associated with the tricarboxylic acid or citric acid cycle; as the first stage carried out in the mitochondria as part of aerobic respiration is known. (Cepero, Restrepo & Franco, 2012).

A fundamental physiological characteristic of this genus is the great capacity to ferment D-glucose, D-fructose and D-mannose, and to a lesser extent the case of D-galactose. But they can't use pentoses or polysaccharides as the carbon source, except for some strains capable of using starch. There are studies where the strains are modified to enable the yeast to ferment sugars such as pentoses, which would allow bioethanol to be obtained from by-products of papermaking. (Carro, 2004; Novy, Krahulec, Longus, Klimacek, & Nidetzky, 2013).

Saccharomyces is a eukaryotic unicellular organism, it has a haploid, asexual reproduction phase, in which somatic cells reproduce by budding as previously mentioned. It also presents a sexual reproduction phase, in which compatible somatic cells merge in pairs and form diploid somatic cells; that reproduce by budding forming more isolated cells, as shown in figure 1. Under certain conditions, diploid cells sporulate, meiosis and transform into asci, each with four haploid ascospores; 2 cells a and 2 α. Ascospores can be released and behave like haploid somatic cells. Thus, this life cycle contains generations of haploid and diploid cells, that is, with a haplophase and a diplophase, this phenomenon is known as haplodiplobionic, and this allows them to be kept in the laboratory in both states. (Carro, 2004; Ulloa, 2015; Nieto, & Maldonado, 2010).

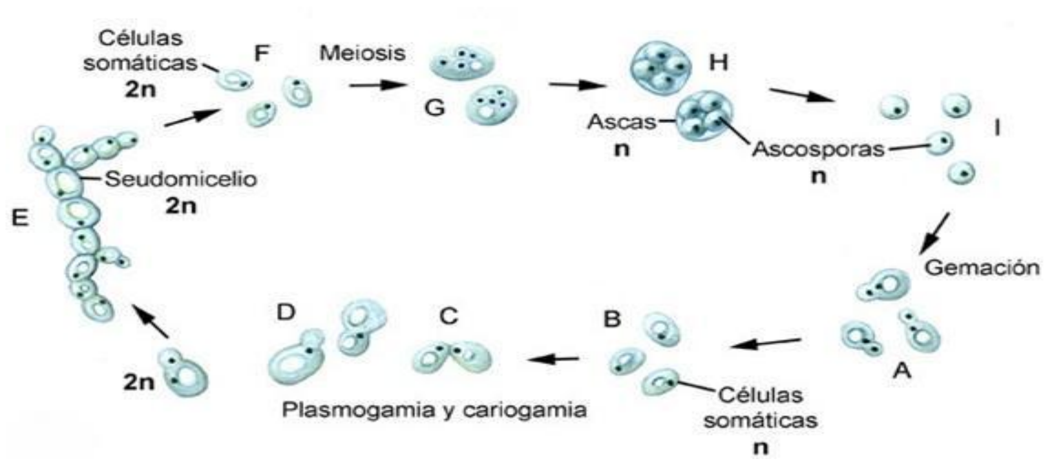


Figure 2. Saccharomyces cerevisiae life cycle.

Source: Ulloa, 2015.

The transformation of a fungus can be carried out with genetic material that comes from the same fungus, a technique known as homologous transformation, or with genetic material from other fungi including a very distant phylogenetically organism, this method is known as heterologous transformation, the yeast *Saccharomyces* is one of the most used for this type of genetic transformation and expression of introduced genes, maybe because it has a unifactorial type system and a haplo-diplomatic life cycle, that is alternation between haploid and diploid state, is the reason why it is one of the few fungi with alternating generations, for which great importance is attributed in industrial uses and for a great variety of studies. In addition to this, it was the first eukaryote whose genome was completely sequenced and contains more than 6000 ORFs (Open reading frame), which in Spanish means marco abierto de lectura, that is the RNA sequence comprised between a translation start codon and a stop codon. (Carro, 2004; Cepero, Restrepo & Franco, 2012)

It also has a fast cell cycle, a high growth rate, is easy to grow and does not require special security measures to be handled (Herrero-Romero, 2015).

It is also the organism that has the most highly characterized eukaryotic pRNAs (Ribonucleic Acid Polymerase), whose transcriptional machinery is very similar to that of human cells. Yeast pRNA I is composed of 14 subunits, pRNA II of 12 subunits and pRNA III of 17 subunits. SiRNAs I and III are not as well characterized as siRNA II at the subunit level. Human pRNA has 12 subunits that are conserved in sequence and function with a corresponding yeast subunit. For this reason, on many occasions, individual human pRNA subunits can function instead of their yeast counterparts in vivo. Therefore, the information obtained from studies using model systems that are easy to manipulate genetically and biochemically such as *Saccharomyces cervisiae* can be extrapolated to human pRNA. All the aforementioned characteristics and the knowledge of many of its molecular processes have made it one of the most widely used organisms in research. (Woychik, 2003; Carro, 2004; Cepero, Restrepo & Franco, 2012)

Food industry

-Beer production

Beer production is a biological process where two agricultural products, such as hops and barley, are transformed into beer through biochemical reactions that occur during various stages (Comi & Manzano, 2008; Taylor & Shellhammer, 2011b). The most important conversion of sweet must to an alcoholic beer occurs during the fermentation stage, which occurs as a result of the metabolism of simple sugars in an anaerobic process mediated by yeasts; the selection of yeasts is a determining step, since they have characteristics that affect the flavor of the beer and their effectiveness during the fermentation process directly influence the quality of the final product. The spices used in the brewing industry include only the genus *Saccharomyces*, particularly the strains belonging to the spices *S. cerevisiae* (yeasts that ferment at high temperatures) and *S. pastorianus* (also known as S. carlsbergensis, a mixture between *S. cerevisiae* and *S. bayanus* and fermenting at low temperatures). (Comi & Manzano, 2008; Taylor & Shellhammer, 2011a).

Brewing wort is a complex solution of sugars, amino acids, peptides, vitamins, minerals, and a long list of other dissolved substances; however, *Saccharomyces spp.* can only assimilate and metabolize small units of sugar such as sucrose, glucose, fructose, maltose, and maltotriose. Invertases hydrolyze sucrose into glucose and fructose outside the yeast cell, while all other sugars are transported in the cytoplasm for further treatment. Both maltose and maltotriose are hydrolyzed to glucose within the cell by α-glucosidases, with glucose and fructose being absorbed first from maltose and maltotriose. (Taylor & Shellhammer, 2011b).

Alcoholic fermentation begins with the breakdown of glucose in the cytoplasm in a series of reactions that ultimately results in two pyruvate molecules (glycolysis). The next step towards ethanol formation is pyruvate decarboxylation to form acetaldehyde and CO2 catalyzed by pyruvate decarboxylase (PDC). CDP activity depends on the help of coenzymes thiamine pyrophosphate (TPP) and magnesium. Ethanol is further formed by the reduction of acetaldehyde by alcohol dehydrogenases, it is a “make-accumulate-consume” strategy in which yeast recycles NADH in the conversion of acetaldehyde to ethanol and if oxygen is subsequently available, the accumulated ethanol is converted back to acetaldehyde (Figure 2). (Pires & Brányik, 2015; Piskur et al., 2006).

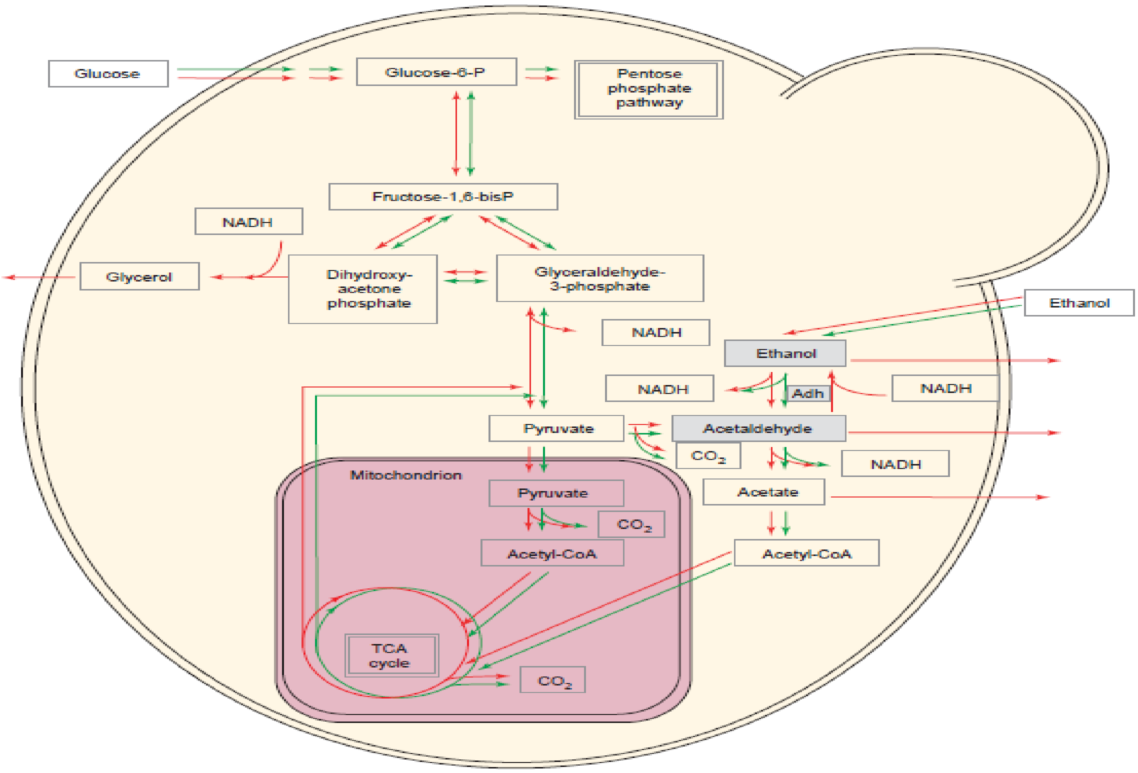


Figure 3: Diagram of the alcoholic fermentation carried out by *Saccharomyces sp.*
Source: Pires & Brányik, 2015.

Despite the fact that brewer's yeasts have the means to carry out aerobic respiration, *Saccharomyces spp.* Possessing the ability to degrade carbohydrates, such as glucose, into two-carbon components, particularly ethanol, without completely oxidizing to CO2, even in the presence of oxygen, as many other yeasts do, this event is known as the "Crabtree effect"; This effect is largely based on a glucose suppression circuit, in which the presence of glucose suppresses respiration. The great advantage of fermentation is the suppression of microorganisms that compete for the food source by producing ethanol. It is important to emphasize that not all microorganisms will feel as comfortable as *Saccharomyces spp.* in an alcoholic environment, that is, Saccharomyces kills its competitors by producing ethanol, but it can also consume the ethanol formed later, avoiding the expense generated in the production of antimicrobial molecules. This “efficient regulation” is probably the most unique invention of *Saccharomyces*, providing a 'key competitive advantage' when production strains are selected for industrial beer and wine fermentations. (Pires & Brányik, 2015; Piskur, Rozpedowska, Polakova, Merico, & Compagno, 2006).

-Wine production

The use of certain yeast species in wine production lies in their ability to convert the sugars present in the grapes into alcohols that assist in the extraction of pigments and tannins from the peel of these fruits during maceration, being these two substances which ultimately give the wine its color, body and final flavor. The mechanism involved in alcoholic fermentation follows the same basic process previously described for beer, counting the fact that the source of glucose is from the sugars present in the macerated grape. For years, the production of wines from alcoholic fermentation was done spontaneously, but this entailed the problem that the yeast species that participated in said fermentation, might not provide the best characteristics to the wine that was wanted to be produced. In the last 30 years, the trend of selecting specific yeast species was followed, in a controlled fermentation that guarantees the desired flavor, color and aroma of the wine. The yeast of choice for this type of fermentation has been *Saccharomyces cerevisiae* given its high capacity to carry it out. Thus, with the isolation of *Saccharomyces cerevisiae*, the problem of including other yeast species that may not provide good performance (in spontaneous fermentation) was solved. But not all the strains of this yeast are optimal to provide the desired organoleptic qualities in the wine and therefore the careful isolation of different strains, which offered that combination of optimal organoleptic characteristics, was essential in the production of high quality wines. Such isolation required a lot of time and resources; but it is from 1996, when this paradigm changes, with the publication of the *Saccharomyces cerevisiae* genome. At this point, the use of genetically modified *Saccharomyces cerevisiae* strains replaced the tedious and difficult selection of crop combinations to search for the desired characteristics. (Bordiga, 2015; Schuller & Casal, 2005)**.**

The difference between strain and strain lies in the demand for nitrogen and oxygen required by each one (fermentation performance); thus, those that require less oxygen and nitrogen in the process, will guarantee a lower production of organic acids (which improves the flavor), and a greater production of glycerol and volatile phenols (which improve fermentation). Based on this, the winemaker makes the correct choice of *Saccharomyces cerevisiae* strain that meets these requirements. (Bordiga, 2015).

What is sought in the genetic improvement of *Saccharomyces* strains is to achieve an improvement in the fermentation performance, a greater tolerance to ethanol, better utilization of sugar and nitrogen assimilation, apart from improved organoleptic properties. Commonly, all genetic materials applied for the genetic construction of microorganisms used in food fermentation must be derived from the host species (self-cloning) or GRAS organisms (generally considered to be safe) with a history of safe food use. Of course, the use of DNA sequences from species closely related taxonomically to pathogenic species should be avoided. The expression of heterologous genes is obtained for example from *Lactobacillus casei* (LDH), *Lactobacillus plantarum* (pdc), *Aspergillus sp.* (Egl1, abfB, xlnA, rhaA), *Fusarium solani* (pelA), while ATF1, GPD1 or PGU1 are obtained directly from *S. cerevisiae*. Regarding promoters and terminators, these are generally derived from glycolytic enzymes that are expressed constitutively under the fermentation conditions. Following is a more extensive list of these genetic modifiers (Schuller, D &, Casal, M, 1994):

Table I: Genetic modifiers used in *S. cerevisiae*.

Improvement	**Metabolism**	**Protein or necessary action**	**Gen producer**	**Source**
Sensory quality	Release enzymatic aroma	Endoglucanase	*egl1*	*Trichoderma longibrachiatum*
		Arabinofuranosidase	*abfB*	*Aspergillus niger*
	Acidity adjustment	Endoxylanase	*xlnA*	*Aspergillus nidulans*
		Ramnosidase	*rhaA*	*Aspergillus aculeatus*
		Malate permease	*mae1*	*Schizosaccharo-Myces pombe*
		Malic enzyme	*mae2*	
		Lactate dehydrogenase	*LDH*	*Lactobacillus casei*
		Acetaldehyde dehydrogenase	*ALD6* (por deleción)	*Saccharomyces cerevisiae*
	Glycerol production	Glycerol – 3-phosphate dehydrogenase	*GPD1*	
	Acetate ester production	Acetyltransferase alcohol	*ATF1*	
	Sulfide hydrogen production	Sulphite reductase	*MET10*	
	Volatile phenols formation	Phenolic acid decarboxylase	*pdc*	*Lactobacillus plantarum*
Health aspects and security	Resveratrol production	β-Glucosidase	*bglN*	*Candida molischiana*
		Resveratrol synthase	4CL216	Poplar hybrid
		Coenzyme-A ligase	vst1	Grapevine

Continuation of Table I. Genetic modifiers used in *S. cerevisiae.*

Improvement	Metabolism	Protein or necessary action	Gen producer	Source
Health aspects and security	Ethyl carbamate elimination	Lock urea discharge	*CAR1 (deleción)*	*Saccharomyces cerevisiae*
Bacteria from decomposition control	Antimicrobial enzymes production	Pediocin	*pedA*	*Pediococcus acidilactici*
		Chitinase	*CTS1–2*	*Saccharomycescerevisiae*
		Leukosin	*lcaB*	*Leuconostoc carnosum*
		Glucose oxidase	*gox*	*Aspergillus niger*
Performance in fermentation (complete conversion of sugar to alcohol and CO2 undeveloped secondary flavors)	Stress tolerance	Trehalose	*TPS1,TPS2, ATH1*	*Saccharomyces cerevisiae*
		Glycogen	*GSY1,GSY2*	
		Sterols	*SUT1, SUT2*	
	Sugar consume and assimilation	Hexose conveyors	*HXT1–1*	
		Hexose kinases	*HXK1,HXK2*	
	Nitrogen assimilation	Proline oxidase	*PUT1*	
		Pyrroline-5-carboxylate dehydrogenase	*PUT2*	
		PUT1 And PUT2 repressor	*Ure2*	
	Ethanol tolerance	Sterol accumulatión	*SUT1, SUT2*	
		Membrane ATPsa activity	*PMA1,PMA2*	
	Agrochemical resistance	Copper chelation	*CUP1*	

Continuation of Table I. Genetic modifiers used in *S. cerevisiae.*

Improvement	Metabolism	Protein or necessary action	Gen producer	Source
Process efficiency	Obstructing polysaccharides removal	Endopoli-galacturonase	*PGU1*	*Saccharomyces cerevisiae*
		Pectate lyase	*pelA*	*Fusarium solani*
	Flocculation time	Floculine	*FLO1, FLO11*	*Saccharomyces cerevisiae*

Source: Schuller, D &, Casal, M, 1994.

When these modifications are introduced, they should not change the essential characteristics of the host in the fermentation process. The genetic modifications must demonstrate that, apart from the metabolic change introduced, there are no significant differences between the wines produced with the commercial strain and the corresponding modified strain, this with respect to their oenological characteristics. (Schuller, D &, Casal, M, 1994)

-Bread production

Breads have been used as the basis of food for 7000 or 8000 years, originally they were flat, unfermented pasta made from ordinarily crushed grains, it is believed that it was in Egypt that the first fermented bread appeared, when it was observed that a dough made the next day produced air bubbles and increased its volume, which also improved its taste and texture. (Mesas & Alegre, 2002)**.**

In the 19th century, thanks to the work of Pasteur, a bounded industrial area was created for the production of yeasts, by 1920 a modern method of producing baker's yeasts (*Saccharomyces cerevisiae*) had been developed, innovated by the Danish Soren Sak and called "Zero Method" because it prevents the production of ethanol. (Legras, Merdinoglu, Cornuet, & Karst, 2007; Mesas & Alegre, 2002)**.**

The “lifting of the dough” is the name of the phenomenon in which the microbial component is added to the dough in order to make it ferment, it means that ethanol and CO2 are produced; in addition to being responsible for this alcoholic fermentation, these yeast microorganisms give the organoleptic characteristics such as acidity. Differences in production of metabolites by yeast such as ethanol, acetic acid and succinic acid during dough fermentation could influence its extensibility, and therefore affect the gas retention capacity in the dough. (Aslankoohi, Rezaei, Vervoort, Courtin, & Verstrepen, 2015; Mesas & Alegre, 2002; Rezaei et al., 2014)**.**

Natural, commercial or bakery yeasts and chemical yeasts or dough boosters can be used in the bread making industry. The first is prepared from the microbiota of the flour itself, in a process of about 4 successive stages, the second is prepared industrially from *Saccharomyces* *cerevisiae* cultures, and can be found in various presentations (pressed, liquid, dehydrated active or instantaneous, flakes, among others) and it is very commonly used to ferment doughs prepared mainly with wheat and barley flour, and lastly, chemical yeasts that are gasifying additives based on the addition of an acid and a base in the dough, which react with the heat at the time of cooking, generating CO2, but this is more used in the pastry area. The fermentation process takes place from the kneading where all the ingredients are mixed until the dough and in the oven reaches about 50 ºC. (Códon et al., 2003; Mesas & Alegre, 2002)**.**

Changes in lifestyle and the existence of refrigerators and microwave ovens have caused an increase in the demand for food that is more convenient to prepare and suitable for storage in freezers. Frozen dough production is increasing considerably in response to the wide-ranging distribution challenge and an increase in the consumption of rapidly prepared home products. Controlled fermentation is the technique that blocks fermentation with cold and allows it to be reactivated at the desired time. (Codon et al., 2003; Mesas & Alegre, 2002)**.**

Thus an important consideration to ensure the quality in the production of baker's yeast is in its storage stability. Yeasts must be able to ferment bread efficiently by producing considerable amounts of carbon dioxide, through a variety of sugars present in the dough despite the freezing process that affects both the yeast and the matrix of gluten. The stress caused by freezing affects the physiological state of the cells, the phases and the growth rate. In addition, technological factors such as the formulation of the mass, and the speed of freezing and thawing also affect the result of the final mass. (Codon et al., 2003; Stecchini, Maltini, Venir, Torre, & Prospero, 2002)**.**

Yeasts can survive freezing when quickly frozen at -80°C. However, for food preservation in commercial practice, the temperatures used are around -20°C, which is a relatively high temperature, so the survival rate is lower. Studies have shown that temperatures from -10 to -30 ° C decrease the vitality of the yeast. This is attributed to the formation of ice crystals that lead to membrane damage causing intracellular dehydration, so the integrity of the membrane is essential for the viability of the technique. During the cooling process that occurs in the presence of high levels of alcohol and high osmotic pressure, a high content of phospholipids is required to prevent the integrity of the membrane from being damaged.Furthermore ethanol is believed to produce a cryoprotective effect under fast freezing conditions, but a toxic one during slow freezing. (Codón et al., 2003; Stecchini et al., 2002)**.**

Therefore, the mixture of certain strains of yeast used for the production of wines is studied, as it is believed that their mitochondria increase tolerance to the temperatures of the freezing process, since they maintain metabolism, biosynthesis of phospholipids, ergosterol, and unsaturated fatty acids, and therefore the viability is high. Formation of this "heteroplasmon" type could result in yeasts more resistant to low temperatures and storage at variable temperatures. Hybridization techniques and recombinant DNA techniques have also been shown to increase the likelihood of greater freezing tolerance. (Codon et al., 2003; Stecchini et al., 2002)

Saccharomyces pathogenic activity

In the last hundred years, fungal infections have increased considerably, mainly in developed countries. This fact is associated with the appearance of situations, diseases and medical techniques such as transplants, use of intravenous catheters, Human Immunodeficiency Virus and the use of broad-spectrum antibiotics. Within yeasts, Saccharomyces cerevisiae has become one of the emerging fungal pathogens in the last two decades, this being an aspect to consider because it is found in many products of the food industry such as bread, wine and beer. (Pérez-Torrado & Querol, 2016; Zhang et al., 2015)

S. cervisiae can be found in nature in the soil and in plants. (Torriani & Rossi, 2009) It can cause invasive disease, and this disease can be caused by two main routes: translocation of the gastrointestinal lumen or by gaps in the skin barrier, especially in the context of vascular access devices. Consumption of S. cerevisiae in significant doses (between 107–1010 yeast cells / day) can lead to gastrointestinal involvement. The mechanism of infection of S. cerevisiae has been found to be physiologically close to that of Candida albicans which is based primarily on adhesion to the human tissue surface by proteins called adhesins; however, this process of tissue adhesion is stronger in Candida albicans. Because S. cerevisae has a low capacity for epithelial adhesion, it also has a poor ability to interrupt the integrity of the intestinal barrier or induce cytotoxicity, so it is suggested that infection of S. cerevisiae to tissue occurs only if the epithelial barrier is previously compromised or if the intestinal barrier presents some type of dysfunction. (Pérez-Torrado & Querol, 2016; Popiel et al., 2015).

The immune system reacts to the presence of a pathogen through the activation of macrophages, neutrophils and other cells with phagocytic capacity. These cells have the ability to release reactive oxygen species and reactive nitrogen species (ROS and RNS) that can be lethal for many fungal pathogens because they can cause damage to the pathogen's proteins, lipids, and DNA. Analysis in human blood exposed to an infection with Saccharomyces cervisiae have shown a specific transcription pattern (Yap1p) that generates a specific response to oxidative stress, in addition, there is an increase in amino acid synthesis, and a response to repair damaged DNA (see figure X). These yeasts show resistance to the oxidative stress that is generated after the encounter with cells of the immune system to the phagocytosis process of microorganisms and to the reactive oxygen and nitrogen species released by macrophages and neutrophils during the immune response. Two genes, TSA2 (thioredoxin peroxidase 2) and GPX2 (glutathione peroxidase 2) are induced in strains of S. cervisiae when it is exposed to neutrophils, producing a remarkable antioxidant response by yeast. (Pérez-Torrado & Querol, 2016).

Also, an increase in deoxyribonucleotide triphosphate (dNTP) is presented as a compensatory mechanism to repair DNA damaged by the oxidative processes generated by phagocytes immersed in the neutrophil. Another defense mechanism used by this yeast is an increase in amino acid synthesis and induction of the glyoxylate cycle after being phagocytized. It has been shown that in a murine model arginine and methionine biosynthesis genes are induced and it is also capable of obtaining and redirecting the little available nitrogen from different yet unknown sources, which constitutes a crucial strategy for the survival of yeast in an amino acid deficient environment. (Pérez-Torrado and Querol, 2016).

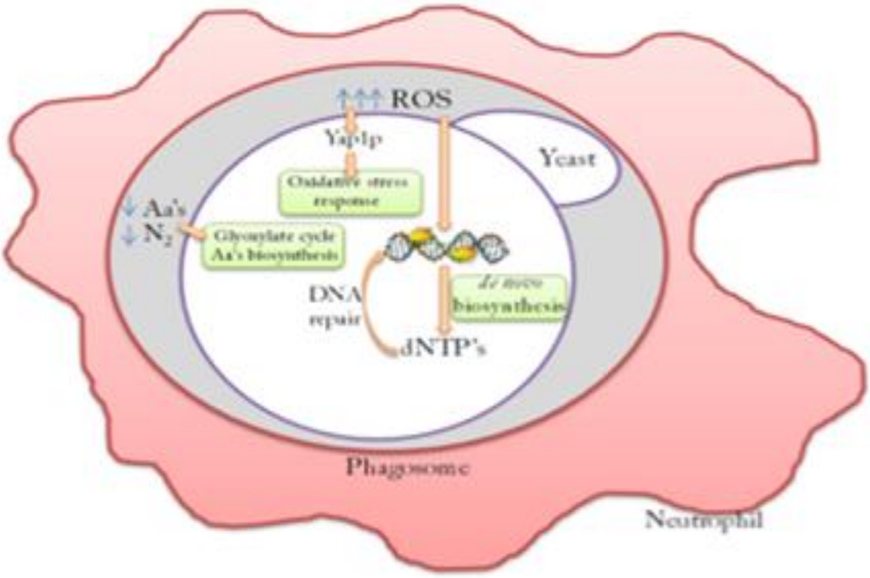


Figure 4. Molecular mechanism of persistence of Sacaromyces cervisiae cells after neutrophil embedding.
Source: Pérez-Torrado & Querol, 2016.

Saccharomyces applications in health

- Opiates from yeast

Morphinan alkaloids are narcotic pain relievers that are currently used for the treatment of acute pain and palliative care. They include codeine and morphine and semi-synthetic derivatives such as dihydromorphine and hydromorphone. The antagonists of opioids naloxone and naltrexone, which are used to treat opiate addiction and overdose, are derived from thebaine. (Fossati, E. et al., 2015; Hawkins & Smolke, 2008; Galanie, Thodey, Trenchar, Filsinger & Smolke, 2015).

Thebaine is a precursor of codeine biosynthesis and morphine in plant, and it is also the precursor for the chemical synthesis of pain relievers such as oxycodone and buprenorphine, which have more favorable side effect profiles than morphine. (Chacón, González-Esquinca, & Riley-Saldaña, 2012; Fossati, E. et al., 2015).

All natural and semisynthetic opiates are currently derived from the opium poppy Papaver somniferun, the crops of which reach about 800 tons to satisfy licit needs, but these crops are susceptible to environmental conditions, from pests and inclement weather, in fact, it is estimated, according to WHO data, some 5.5 million people have limited access to pain treatments, which has led to the search for alternative sources of morphine alkaloids, using microbial production systems that also contribute to the creation of new drugs. (Fossati, E. et al., 2015; Hawkins & Smolke, 2008; Galanie, Thodey, Trenchar, Filsinger & Smolke, 2015).

Morphinan alkaloids belong to a broader class of secondary metabolites of plants, known as benzylisoquinoline alkaloids (BIAs). BIAs are a large and structurally diverse family of tyrosine derivatives with a wide range of pharmaceutical properties including muscle relaxants such as papaverine, antimicrobials such as berberine and bloodroot, and the potential cancer drug noscapine. Other BIAs have been studied for their antioxidant properties, they have also been evaluated as acetylcholine receptor ligands and as anti-HIV therapeutic agents. (Fossati, E. et al. 2014, Fossati, E. et al., 2015; Hawkins & Smolke, 2008).

Despite the fact that the levels of alkaloids such as morphine and berberine are relatively high in plants, the molecular diversity is very wide (there are some 2,500 structures with a 1-benzyltetrahydroisoquinoline system; basic carbon skeleton that comes from a link between an isoquinolinic ring and another benzyl) and there is no mechanism for the production of these in significant quantities at the industry level, despite the attempts of chemistry to synthesize them, which has run into their high molecular complexity. In this way, efforts have been redirected towards the clarification of the biosynthetic pathways to try to characterize the activity of the enzymes linked to the process. (Chacón et al., 2012; Hawkins & Smolke, 2008).

The biosynthesis of BIAs can be divided into 3 stages, the first is the production of S-norcoclaurin, from two L-tyrosine molecules, the second is the transformation of this to S-reticulin, and finally different routes that diversify the products of BIAs. So far 43 enzymes have been elucidated in the biosynthesis of BIAs, grouped into 25 oxidoreductases, 15 transferases and 3 lyases, there are still 4 enzymes of which their catalytic activity is unknown. (Chacón, González-Esquinca, & Riley-Saldaña, 2012).

The first step in the synthesis of BIAs is the condensation of dopamine and 4-hydroxyphenylacetalaldehyde (4-HPA), catalyzed by norcoclaurin synthase to produce S-norcoclaurin, S-norcoclaurin undergoes a series of methylations catalyzed by norcoclaurin 6-O-methyltransferase (6-OMT), coclaurin-N-methyltranferase (CNMT) and (S)-3´-hydroxy-N-methylcoclaurin-4´-O-methyltranferase (4-OMT), in addition to a cytochrome P450 catalyzed hydroxylation to produce the intermediate (S)-reticulin, as shown in figure 4. (Chacón et al., 2012; Hawkins & Smolke, 2008).

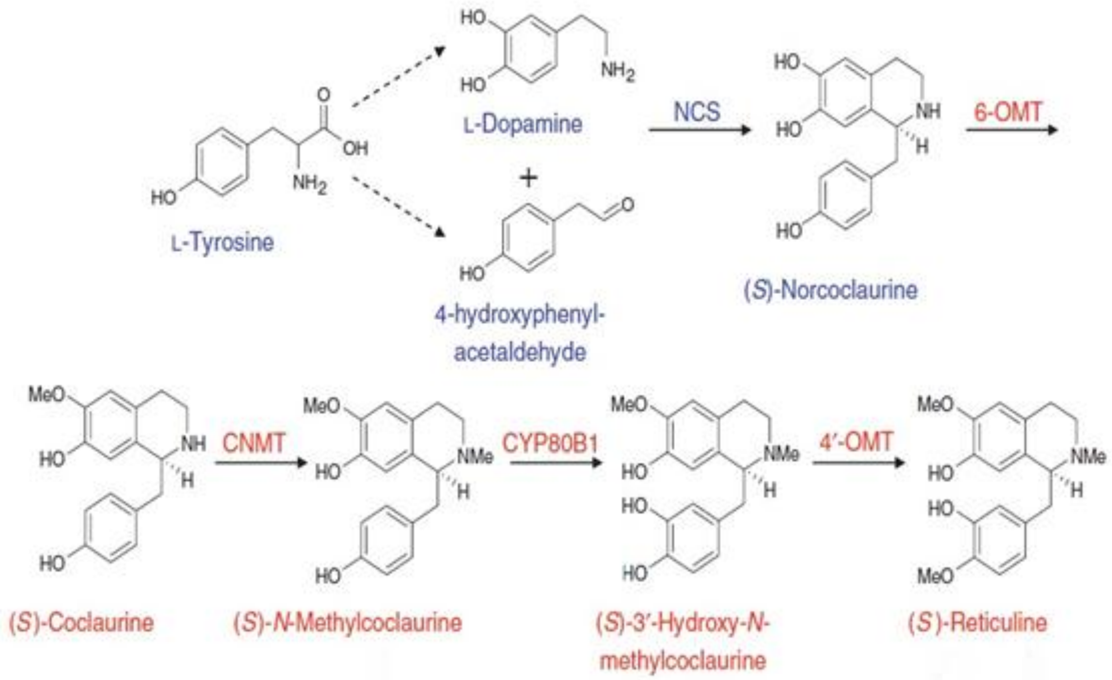


Figure 5: Scheme of synthesis of (S) -reticulin from L-tyrosine.
Source: Hawkins & Smolke, 2008.

Poppy has the unique ability to convert (S)-reticulin to (R)-reticulin which is the precursor for morphine and berberine for example. Many of the plant enzymes that are necessary for this transformation have not yet been isolated. As an alternative, a synthetic BIA pathway has been constructed using S. cerevisiae that converts the commercially available substrate (R, S)-norlaudanosoline to (R, S)-reticulin. (Fossati, Narcross, Ekins, Falgueyret, & Martin, 2015; Hawkins & Smolke, 2008, Galanie, Thodey, Trenchar, Filsinger & Smolke, 2015).

The epimerization of (S)-reticulin to (R)-reticulin occurs by dehydrogenation of (S)-reticulin to 1,2-dehydroreticulin by dehydroreticulin synthase (DRS) with the subsequent selective enantium reduction to (R)-reticulin by dehydroreticulin reductase (RRD). As shown in the following figure 5. But as mentioned before, the genes that encode these enzymes could not be fully characterized, making their cloning impossible. (Chacón, González-Esquinca, & Riley-Saldaña, 2012; Fossati, Narcross, Ekins, Falgueyret, & Martin, 2015; Hawkins & Smolke, 2008; Galanie, Thodey, Trenchar, Filsinger & Smolke, 2015).

Figure 6: Biosynthetic scheme for the DRS-RRD catalyzed reaction.

Source: Novy et al., 2013.

However, with the use of (R, S)-norlaudanosoline as a substrate, the synthesis of the two forms of reticulin is obtained, allowing to obtain racemic reticulin in a S. cerevisiae medium, achieving the metabolites that extend throughout the branch towards the production of morphine and codeine, such as thebaine that is converted to codeinone and morphinone prior to obtaining morphine and codeine, as shown below in figure 6. Despite the progress that this implies, there are several challenges for deal, including the complete reconstitution of BIA from a low-cost substrate and the optimization of yields. Thus, the chemical synthesis of most of these alkaloids is possible, but it is not economically feasible, except in the case of the sanguinary, which is obtained from (S)-reticulin, although its medical importance is less relevant. (Chacón, González-Esquinca, & Riley-Saldaña, 2012; Fossati, Narcross, Ekins, Falgueyret, & Martin, 2015; Hawkins & Smolke, 2008; Kempe, k. Et al. (2009).

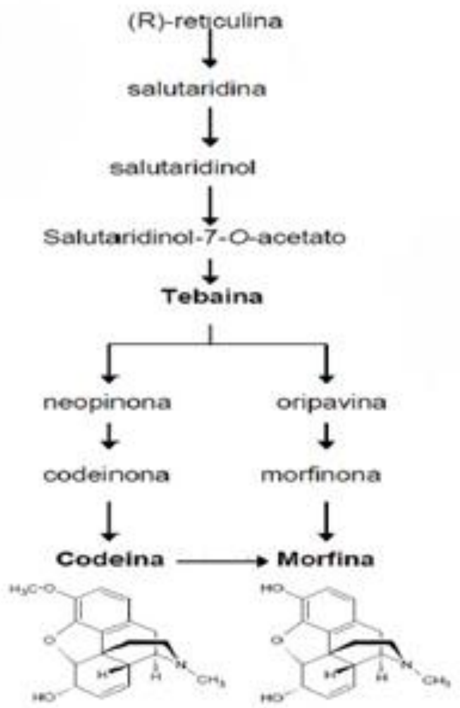


Figure 7. Scheme of synthesis of codeine and morphine from thebaine from (R)-reticulin.

Source: Chacón, González-Esquinca & Riley-Saldaña, 2012.

In general cultures of the treated yeast strains are grown in test tube in volumes ranging from 2 ml to 10 ml at a temperature of 30°C and 200 rpm in an appropriate medium with 2% dextrose (m /v) as a source of sugar. The cultures are diluted to 10% in 1 ml of fresh medium and incubated for an additional 6 hours. The cells are then harvested by centrifugation for 2 min. The supernatants are decanted and the cells are suspended at neutral pH, and then supplemented with the appropriate substrate for the desired metabolic pathway, generally norlaudanosoline or laudanosoline. Cultures are assayed 24 hours after substrate addition to observe maximum accumulation as cells reach in the stationary growth phase. They are then collected by centrifugation for 1 min, for the extraction of BIAs from the cells; the cell pellet is suspended in 300 µl of methanol and stirred for 30 min. The cell extracts were rinsed by centrifugation for 1 min. (Fossati, E. et al. 2015; Hawkins & Smolke, 2008).

Generally, the analysis of the growth media to detect the presence of BIAs molecules is performed by liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) that has greater sensitivity and precision besides a simplified preparation of samples, range dynamic quantitative and, most importantly, the possibility of practicing multiple processes. The reticulin produced by the yeast is isolated by reverse phase HPLC and analyzed by chiral LC-MS. As expected, the chiral analysis found that the reticulin produced is R and S enantiomer (Galanie, Thodey, Trenchar, Filsinger & Smolke, 2015).

The synthesized metabolites accumulate in the growth medium, which simplifies the measurement or estimation of production levels directly from the growth medium without rigorous extractions or multiple purification passes, which represents an advantage in the method. (Fossati, E. et al. 2015; Hawkins & Smolke, 2008)

Also, modifications have been made to different strains of yeast to obtain thebaine from sugars. In one of these processes, 21 enzymes will be used and the other 23 enzymes. There are no enzymes in the latter; the result was hydrocodone, a compound that has inhibitory properties of the pain, which are not natural products of the poppy, which opens the opportunity to the creation of new pain treatments. This study carried out in the United States had a negative result: a very low amount of synthesized will require approximately 17 liters of yeast to obtain a single dose of pain reliever. However, the techniques developed may need to broaden the range of affected anticancer or antibiotics. (Galanie, Thodey, Trenchar, Filsinger and Smolke, 2015).

Saccharomyces cerevisiae has been used for the biosynthesis of molecules derived from central metabolism. Advances in recombinant DNA technology make this yeast an interesting setting for the characterization of enzymes and the reconstitution of complex metabolic pathways, allowing the discovery and the production of diverse and complex natural products. This is the case previously described of benzylquinoline alkaloids or the production of artemisinic precursor artemisinic acid, used as an antimalarial. (Fossati, E. et al., 2014; Trenchard, I. et al. 2015).

-*Biopharmaceuticals from S. cerevisiae* (recombinant proteins)

The use of microscopic organisms as biopharmaceuticals is a multimillion-dollar industry, in which advantage is gained over controlling the expression of recombinant proteins in the cytoplasm or periplasm of microorganisms, as well as strategies for secreting the product into a culture medium. The use of recombinant proteins constitutes an advance in genetic engineering that allows the production of proteins that are identical or that differ slightly from native proteins of human origin, which significantly reduces adverse immunological reactions; This process is carried out using the following scheme: 1) treatment with restriction enzymes of a cloning vector and of the DNA that contains the gene that encodes the protein of interest, 2) ligation of the gene with the vector to obtain the recombinant DNA (RDNA), 3) internalization and replication (cloning) of the rDNA in a host cell and 4) expression of the gene in the protein. Cloning vehicles used in yeast often carry the PGK gene (3-phosphoglycerate kinase, 3-phosphoglycerate kinase), ADH1 (alcohol dehydrogenase 1), acid phosphatase, and the GAL (galactose) cluster genes as promoters (Berlec & Štrukelj, 2013; Drago Serrano & Del R. Sainz Espuñes, 2006)

In the specific case of S. cerevisiae, the use of leader sequences is a highly modified factor to obtain different products; the leader sequence determines, in part, the trafficking of a secreted protein. The presequence determines whether the cotraductional translocation or post-translational translocation is produced by entering the ER and the pro-sequence determines the classification mechanisms in the trans-Golgi network. Native S. cerevisiae leader sequences, external leader sequences and theory derived leader sequences have been used to direct secretion of heterologous proteins. (Hou, Tyo, Liu, Petranovic, & Nielsen, 2012)

During secretion, protein folding, disulfide bond formation, and glycosylation takes place. Correctly folded proteins are transported to the Golgi apparatus for further processing including additional glycosylation. Proteins leaving the Golgi can be secreted extracellularly or directed into storage or degradation vacuoles. Alternatively, the recombinant products are subjected to proteolytic degradation by various proteases located in cellular compartments such as the Golgi apparatus, cytosol, mitochondria, and cell wall (Fig 7). (Kim, Yoo, & Kang, 2015; Sudbery, 1996)

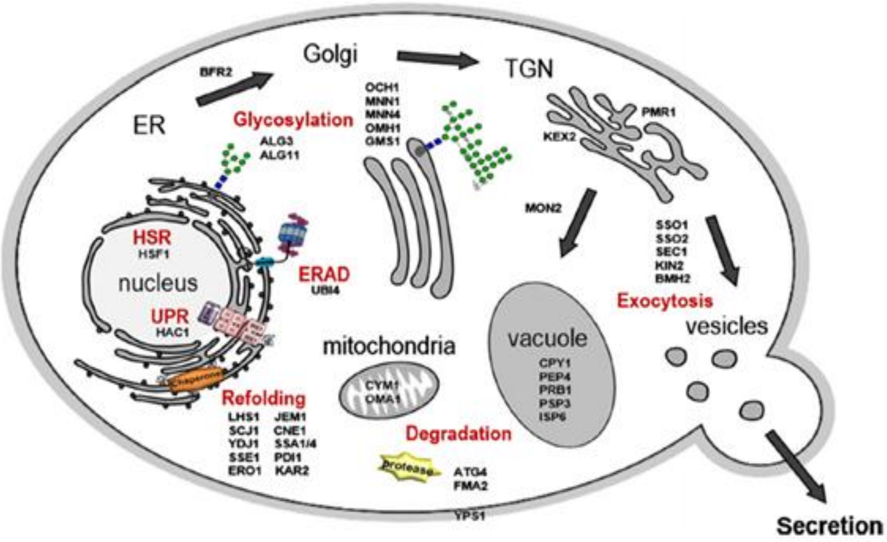


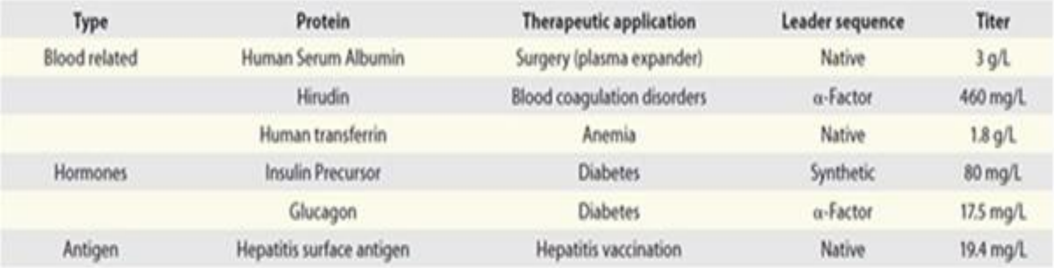
Figure 8: Schematic representation of the yeast secretory pathway.

Source: Kim, Yoo & Kang, 2015.

Although there are various sources for the production of biopharmaceuticals, Saccharomyces cerevisiae yeast is an important cell factory, since it does not produce toxic compounds and is used for the production of various high-volume products, for example Insulin and its analogues, human serum albumin, hepatitis vaccines, and virus-like particles used for vaccination against the human papilloma virus. In addition, yeast expression systems are highly valued since they can be performed in chemically defined media and also it is not required to remove bacterial endotoxins, both aspects reduce the cost of product production. (Berlec & Štrukelj, 2013; Drago Serrano & Del R. Sainz Espuñes, 2006; Nielsen, 2013).

The advantages of using S. cerevisiae as a cell factory for the production of biopharmaceutical products are that its eukaryotic system allows the adequate production of human proteins as well as its secretion into the extracellular medium, which facilitates subsequent purification. An additional advantage is that in many cases yeast can perform post-translational modifications of the protein, including proteolytic processing of signal peptides, disulfide bond formation, subunit assembly, acylation, and glycosylation. (Berlec & Štrukelj, 2013).

In general, biopharmaceutical products are used to compensate for the deficiency or lack of important body proteins for the normal functioning of the organism and can be mainly divided into the following categories: blood factors, thrombolytics and anticoagulants, hormones, enzymes, growth factors, interferons and interleukins, vaccines, and monoclonal antibodies, some of these are listed in Figure 1. (Berlec & Štrukelj, 2013; Nielsen, 2013).



Type	Protein	Therapeutic application	Leader sequence	Titer
Blood related	Human Serum Albumin	Surgery (plasma expander)	Native	3 g/L
	Hirudin	Blood coagulation disorders	α-Factor	460 mg/L
	Human transferrin	Anemia	Native	1.8 g/L
Hormones	Insulin Precursor	Diabetes	Synthetic	80 mg/L
	Glucagon	Diabetes	α-Factor	17.5 mg/L
Antigen	Hepatitis surface antigen	Hepatitis vaccination	Native	19.4 mg/L

Figure 9. Overview of some biopharmaceuticals produced by S. cerevisiae.

Source: Nielsen, 2013.

-Expression of insulin

Insulin is a hormone produced by the β cells of the Langerhans islets of the pancreas, and is synthesized as pro-insulin, which is then folded and processed; The enzymatic conversion of proinsulin to insulin occurs within acidifying secretory granules where the C-peptide is removed by cleavage by the action of endopeptidases PC3 and PC2 that are equivalent to the endoprotease Kex2 of S. cerevisiae. Insulin is essential to maintain normal blood glucose levels, and is an effective treatment in patients with Type I Diabetes. Obtaining insulin for this purpose has been carried out since 1920 from the extraction of the purified peptide from the bovine and porcine pancreas; however, the demand increases year by year and this generates the need to use new sources of insulin to meet current demand, so the development of molecular biology and biotechnology opened the doors to S. cerevisiae, a microorganism extremely efficient in the expression and secretion of insulin, and also offers high productivity. (Kjeldsen, 2000; Kjeldsen et al., 2001).

The effective obtaining of insulin from *S. cerevisiae*, is a product of an arduous work in which it is constantly trying to optimize the process of expression and secretion of the peptide by this yeast. The *S. cerevisiae* secretory pathway has a structure and function very similar to that of the mammalian secretory system, with the ability of protein folding, proteolytic processing, glycosylation and secretion, and consisting of translocation through the membrane of the endoplasmic reticulum (RE), folding in the lumen of the RE, transport of the RE to the Golgi apparatus, post-translational modification in the Golgi apparatus, transport by secretory granules to the cell membrane and exit to the extracellular space. The α-type haploid reproduction cells of *S. cerevisiae* secrete a pheromone (α factor) essential for mating between cells of the type to which it is used as a guide sequence to facilitate the secretion of numerous heterologous proteins including insulin, since it mediates the introduction and stabilization of the insulin precursor in the ER. This leading α factor is composed of a prepropeptide followed by a dibasic site for Kex2 endoprotease maturation and a spacer peptide consisting of 2 or 3 pairs of Glu and Ala (Fig 9). (Kjeldsen, 2000; Kjeldsen eral., 2001).

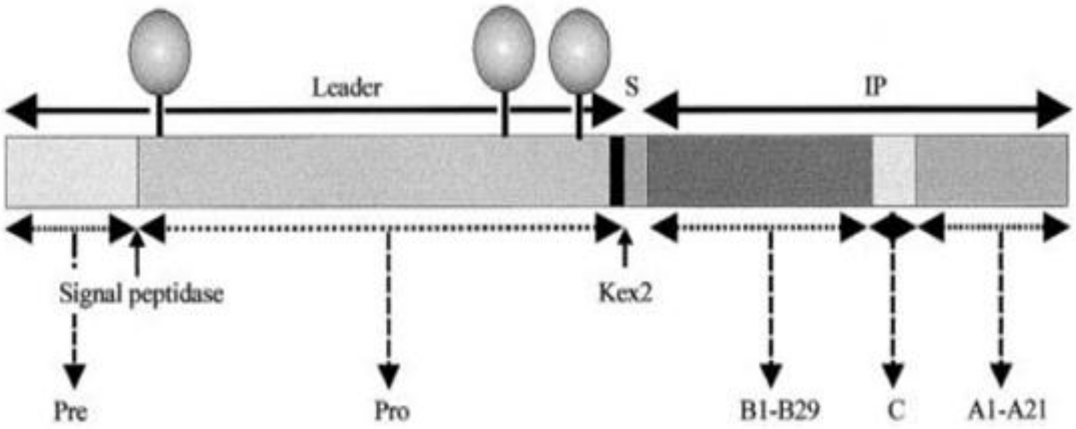


Fig 10: Schematic representation of the insulin precursor fusion protein guide factor.
Source: Kjeldsen, 2000.

For the efficient expression and secretion of recombinant proinsulin in yeast, the insulin peptide was modified by genetic engineering to contain as A chain and a B chain that lacks threonine B30 at the C-terminus, either directly fused or linked through a short synthetic C peptide (Baeshen et al., 2014). The cDNA sequence coding for this construct was fused with the α-factor signal sequence of Saccharomyces cerevisiae for efficient secretion to the supernatant culture of the individual chain of proinsulin-like molecules (Fig 10), which can be purified and subsequently converted to human insulin by transpeptidation in an organic-aqueous medium in the presence of a threonine ester, with which a yield of up to 80 mg/mL of insulin was obtained. (Kjeldsen, 2000; Kjeldsen et al., 2001)

In addition to recombinant insulin, various insulin analogous are also being produced in S. cerevisiae. Insulin Aspart is another type of analog fast-acting insulin, which was produced in S. cerevisiae, developed by Novo Nordisk and approved by the U.S. FDA. in 2001 for therapeutic use in humans. Insulin Aspart was generated by replacing the proline residue at position 28 with aspartic acid on chain B. This genetic modification has resulted in an increase of the inter-chain loan repulsion, a decrease of the self-association, and thus causing a fast entry into the blood from the subcutaneous injection site.

Insulin Detemir is another long-acting recombinant insulin analog that is commercially produced in S. cerevisiae, developed by Novo Nordisk and approved for therapeutic use in humans in 2004 by Europeans regulatory authorities. Detemir recombinant has been generated by removing the threonine residue at position 30 of the B chain, and a C14 fatty acid chain covalently linked to the lysine residue at position 29 of the B chain. These genetic alterations result in the bindings of insulin to albumin in the plasma, which ensured the slow and constant liberation of insulin and therefore a prolonged duration of action of up 24 hours. (Baeshen et al., 2014).

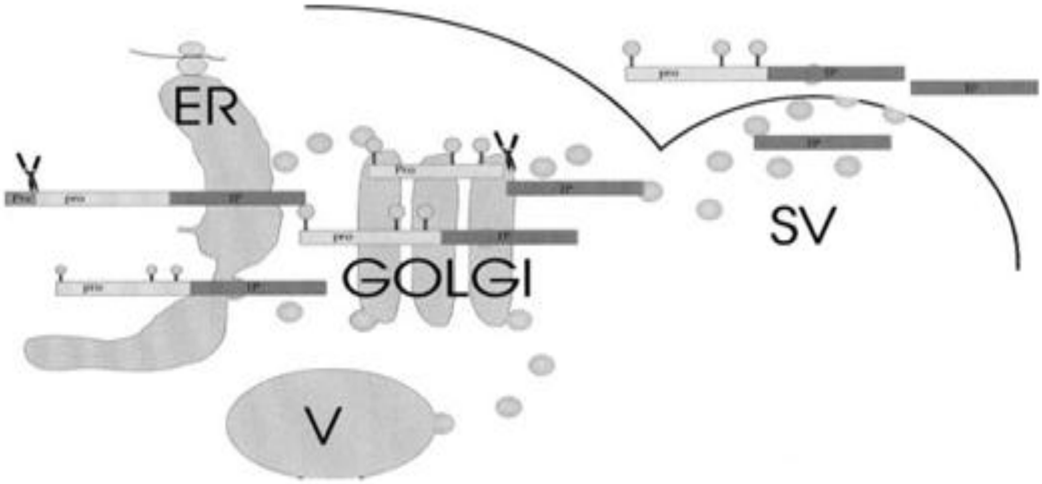


Figure 11. Schematic representation of the secretory pathway of *Saccharomyces cerevisiae*, showing the transport, processing and export of factor α.
Source: Kjeldsen, 2000.

-S. cerevisiae in virology studies.

Saccharomyces can to perform post-translational modification such as myristylation, proteolytic processing, and acetylation at amino-terminal ends that are critical to the proper performance of many proteins derivatives. Another feature that gives Saccharomyces advantage in virology studies is that 30% of genes involved in human diseases have orthologs in Saccharomyces; furthermore, its genetic manipulation makes it possible to use to find therapeutic targets. (Herrero-Romero, 2015)

This yeast also is widely used to study possible interactions between proteins or protein-DNA by using the Two hybrid system technique (Herrero-Romero, 2015), which is a genetic assay based in yeast to detect protein-protein interactions, and it can use to identify the proteins that bind to a protein of interest or to delineate critical domains or residues for an interaction.

Variations about this methodology have been developed to clone genes that encode DNA-bindings proteins to identify peptides that bind to a protein, and one of its highlight uses is the selection of drugs. (Fields & Sternglanz, 1994).

Saccharomyces cervisiae has been used successfully as a eukaryote model in the study of effects on the cell, in the search of possible viral protein inhibitors, in antigenicity, in the production of virus-like particles (VLPs) for the analysis of the role and expression of certain viral proteins, and the development of viral antigens required for the vaccine production.

Table II. Use *Saccharomyces cerevisiae* in virology studies.

Type of study	**Virus**	**Proteins**
Production of VLPs for serological and cellular studies.	HPV Mouse Polyomavirus HIV-1 HIV-2	L1 VP1 Gag Gag
Recombinant vaccines.	BHP	HBsAg
Phenotypic studies of viral proteins and search for inhibitors	Poliovirus Influenza HIV-1	2BC M2 Nef Proteasa Vpu Vpr Integrasa
Interactions between proteins with Two hybrid system technique.	HIV-1 Bunyamwera HCV HPV	Vpr /14-3-3/Cdc25C Nef/CD4 NSs /MED8 NS4A/NS2 E5/Bap31

Source: Modified from Herrero-Romero, 2015

-Production of vaccines against Human Immunodeficiency Virus:

HIV has been one of the viruses in which Saccharomyces was used in the study of viral antigens for the production of vaccines. The viral proteins Vpr and Vpu of HIV-1 have been expressed with this yeast, and amino acids of the Vpr protein that are essential for the inhibition of the growth of Saccharomyces have been found. Vipirinin (whose inhibitor has a physical interaction with the Vpr protein) and fumagillin were found to be able to reverse the inhibition of cell growth that Vpr produces. The degradation effect produced by the viral protein Vpu on CD4 lymphocytes has also been studied, and it has been shown that the damage can be redone in yeast through the co-expression of Vpu and the CD4 cellular receptor. Although the phosphorylation of the Vpu protein is essential for the degradation of CD4 lymphocytes, it does not intervene in the binding between the CD4 receptor and Vpu. Furthermore, it has been determined that Vpu causes ubiquitination of the CD4 receptor before its proteolysis. (Herrero-Romero, 2015)

This type of yeast has been used in pilot studies to observe its role in the development of a serum against Human Immunodeficiency Virus type 1. It has been found that the glycan shield on the HIV glycoprotein could function as a suitable target for an effective immune design. In these studies, a triple mutant strain of Saccharomyces cerevisiae is used that strictly expresses the Man8GlcNac2 form of N-glycans, which is the largest form of glycans in the epitope recognized by neutralizing antibodies 2G12 and PGT. Antibodies generated with individual yeast glycoproteins were shown to have the ability to capture virions, however, these lack neutralizing activity unless such virions exclusively harbor N-linked glycans with high mannose content. This represents a breakthrough as a basis for future research in the area. (Zhang et al., 2015).

-S. cerevisiae and Human Papillomavirus

Human papillomavirus (HPV) is an enveloped DNA virus that infects skin or epithelial mucosa tissues, and represents the main cause of cervical cancer when the infection is by high-risk types of HPV. (types 16, 18, 45, 31, 52, 58). The Human Papillomavirus contains two proteins encoded by the virus (L1 and L2). These two proteins form an icosahedral capsid of approximately 60 nm in diameter, which encloses a circular double-stranded DNA. The L1 protein is the main protein of the HPV capsid which can self-assemble into virus-like particles (VLP); these particles are the main component of prophylactic vaccines in the prevention of HPV, since they have neutralizing epitopes on their surface, and are structurally and antigenically similar to native virions but don't contain the virus genome and therefore don't have the capacity of replicate, cause infection, or cause cancer. (Castellsagué & Bosch, 2007; Kwag, Kim, Chang, & Kim, 2012; Lowy & Schiller, 2006; Park et al., 2002).

The production of VLP is done by cloning the cDNA of the L1 protein, these particles are produced by the expression of the L1 gene that codes for the largest protein in the capsid, in eukaryotic systems such as yeast. This gene is expressed as a recombinant protein, in eukaryotic cells it self-assembles into VLPs. By performing PCR, the L1 protein is inserted into an S. cerevisiae expression vector system which is relatively safe and highly efficient in the production of recombinant proteins (Fig 11). The VPLs are purified by ultracentrifugation and, to validate the efficacy of the vaccine, the ELISA test is carried out to measure the levels of antibodies against VPL of HPV in a model with laboratory mice. (Roldão, Mellado, Castilho, Carrondo, & Alves, 2010; Sasnauskas et al., 2002; Woo, An, Kim, Park, & Kim, 2008).

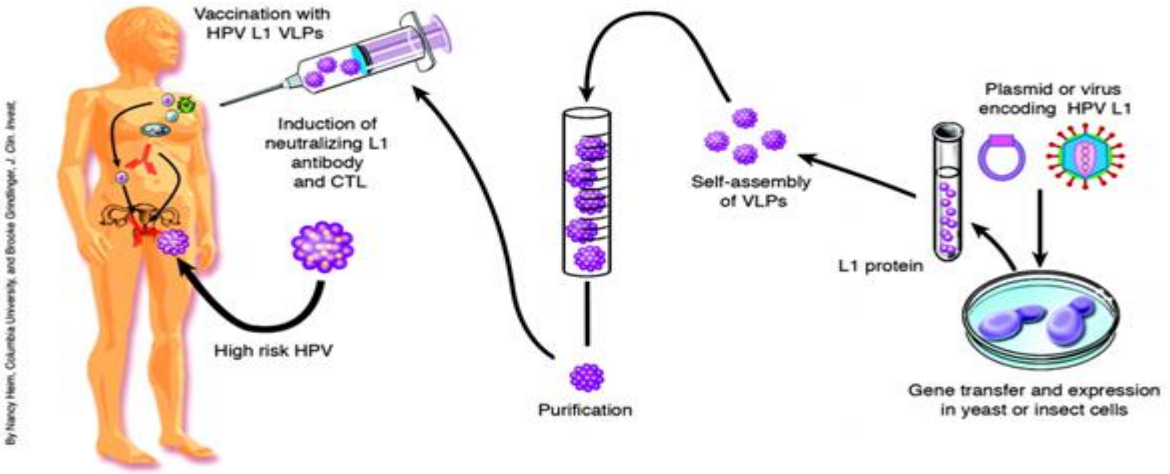


Figure 12: Obtaining VPL for the preparation of vaccines against the Human Papillomavirus.

Source: Berzofsky et al., 2004.

For each type of virus, a recombinant yeast strain has been constructed and used to generate a cell bank. The L1 proteins of each virus type are produced in separate fermentation batches that use chemically defined culture media, followed by cell harvest and a cell lysis step to release accumulated intracellular L1. After debris removal, VLP purification is carried out by chromatography techniques. The adsorption of each VLP in the adjuvant of choice is carried out before the final formulation stage, where the individual HPV packages are combined and formulated. (Castellsagué & Bosch, 2007; Lowy & Schiller, 2006).

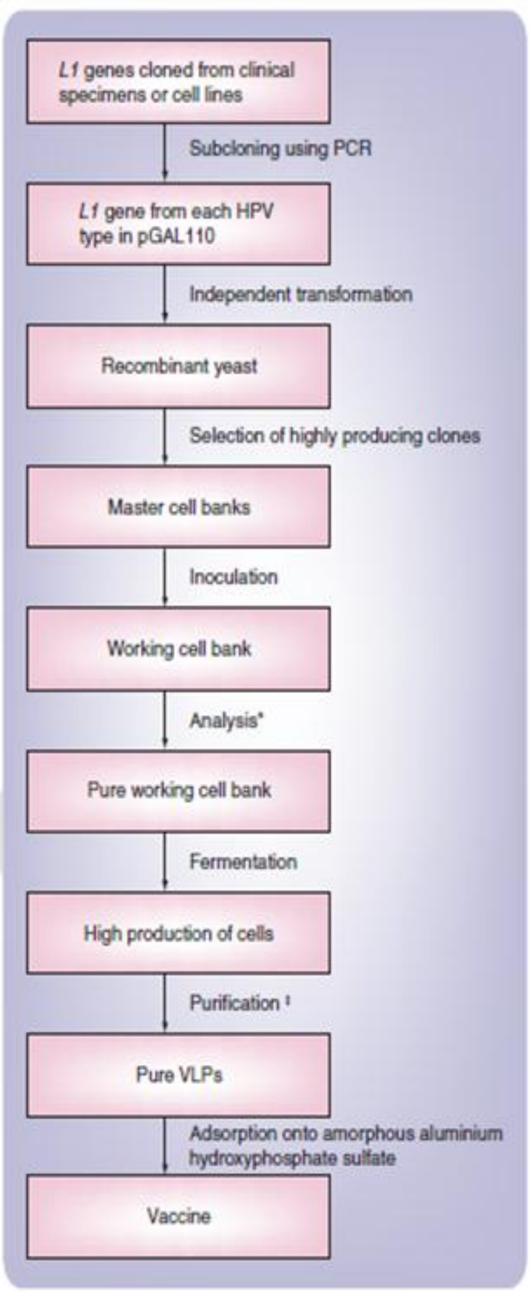
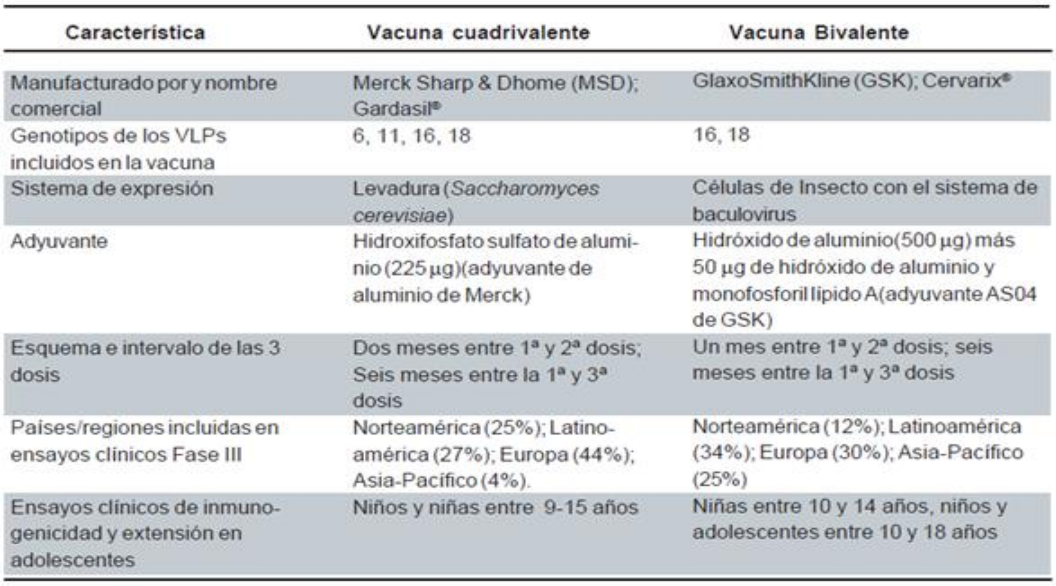


Figure 13. Schematic representation of the process to obtain HPV vaccine from yeast.

Source: García & Galván, 2007

There are currently vaccines that use the S. cerevisiae expression system that contains virus-like particles of HPV for types 16, 18.6 and 11 (Fig 13), which offer advantages for the development of vaccines concerning cost-effectiveness and are easily adaptable to large-scale growth; besides, its potential for contamination by toxins or infectious viruses is low compared to bacteria and mammalian expression systems. On the other hand, VLPs produced from S. cerevisiae are known to have superior structural integrity, while their cost of production is less than in insect cells or mammalian cell expression systems. Furthermore, the safety and efficacy of biopharmaceuticals products made from S. cerevisiae are better defined than in other yeast expression systems such as *Pichia pastoris* or *Hansenula polymorpha.* (Kwag et al., 2012; Park et al., 2002)



Característica	Vacuna cuadrivalente	Vacuna Bivalente
Manufacturado por y nombre comercial	Merck Sharp & Dhome (MSD); Gardasil®	GlaxoSmithKline (GSK); Cervarix®
Genotipos de los VLPs incluidos en la vacuna	6, 11, 16, 18	16, 18
Sistema de expresión	Levadura (*Saccharomyces cerevisiae*)	Células de Insecto con el sistema de baculovirus
Adyuvante	Hidroxifosfato sulfato de aluminio (225 µg)(adyuvante de aluminio de Merck)	Hidróxido de aluminio(500 µg) más 50 µg de hidróxido de aluminio y monofosforil lipido A(adyuvante AS04 de GSK)
Esquema e intervalo de las 3 dosis	Dos meses entre 1ª y 2ª dosis; Seis meses entre la 1ª y 3ª dosis	Un mes entre 1ª y 2ª dosis; seis meses entre la 1ª y 3ª dosis
Países/regiones incluidas en ensayos clínicos Fase III	Norteamérica (25%); Latinoamérica (27%); Europa (44%); Asia-Pacífico (4%).	Norteamérica (12%); Latinoamérica (34%); Europa (30%); Asia-Pacífico (25%)
Ensayos clínicos de inmunogenicidad y extensión en adolescentes	Niños y niñas entre 9-15 años	Niñas entre 10 y 14 años, niños y adolescentes entre 10 y 18 años

Figure 14. Characteristic of prophylactic vaccine against Human Papillomavirus.

Source: Baechen et al., 2014

-Hepatitis B vaccine production by microorganisms

Hepatitis B (HBV) has infected billions of people worldwide, and currently, around 370 million people serve as chronic carriers of HBV. Chronic HBV infection can cause severe liver damage, which can eventually progress to cirrhosis and liver cancer. The virus is made up of a part of a double-stranded DNA genome of approximately 3,200 bases, which encodes the HBV nucleus antigen (HBsAg), the HBV surface antigen (S-HBsAg), DNA polymerase (reverse transcriptase), and the HBV e antigen (HBeAg). (Parolin, Corso, Alberghina, Porro, & Branduardi, 2005; Yong & Tan, 2015).

Since the 1980s, recombinant HBV vaccines have been used to prevent HBV infection; they use HBsAg synthesized in yeast cells into which the HBsAg gene has been introduced by plasmids. The transformed cells are grown in large containers and the expressed HBsAg is self-assembled into spherical particles of approximately 20nm (called virus-like particles), immunogenic, and exposing the high immunogenicity α antigen. Recombinant particles differ from natural ones only in glycosylation of HBsAg. In mammalian cells, HBsAg proteins are co-translationally inserted into the membrane of the endoplasmic reticulum (ER) by the signal peptide within the NH2-terminal region of the S protein. In S. cerevisiae the HBsAg proteins are initially translated through the ER membrane, to then assemble into 20 nm particles in the yeast secretory apparatus. Saccharomyces cerevisiae was the first yeast expression system used in the production of the recombinant HBV vaccine based on the HBV surface antigen (HBsAg). (Yong & Tan, 2015).

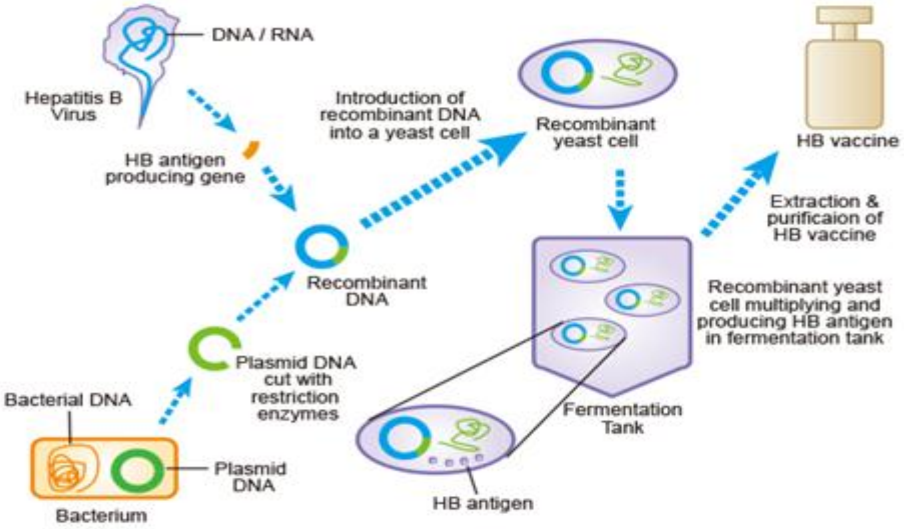


Figure 15: Preparation process of the recombinant Hepatitis vaccine from a yeast.

Source: Yong & Tan, 2015.

The fact of choosing yeasts instead of mammalian cells for the production of this surface protein lies in several advantages of the yeast over the mammalian cells. These include, among them, cell characterization is less tedious, and the production requirements are less expensive. Additionally, yeasts can grow in fully synthetic media unlike mammalian cells, and produce higher amounts of protein per unit volume. (Stephenne, J., 1990).

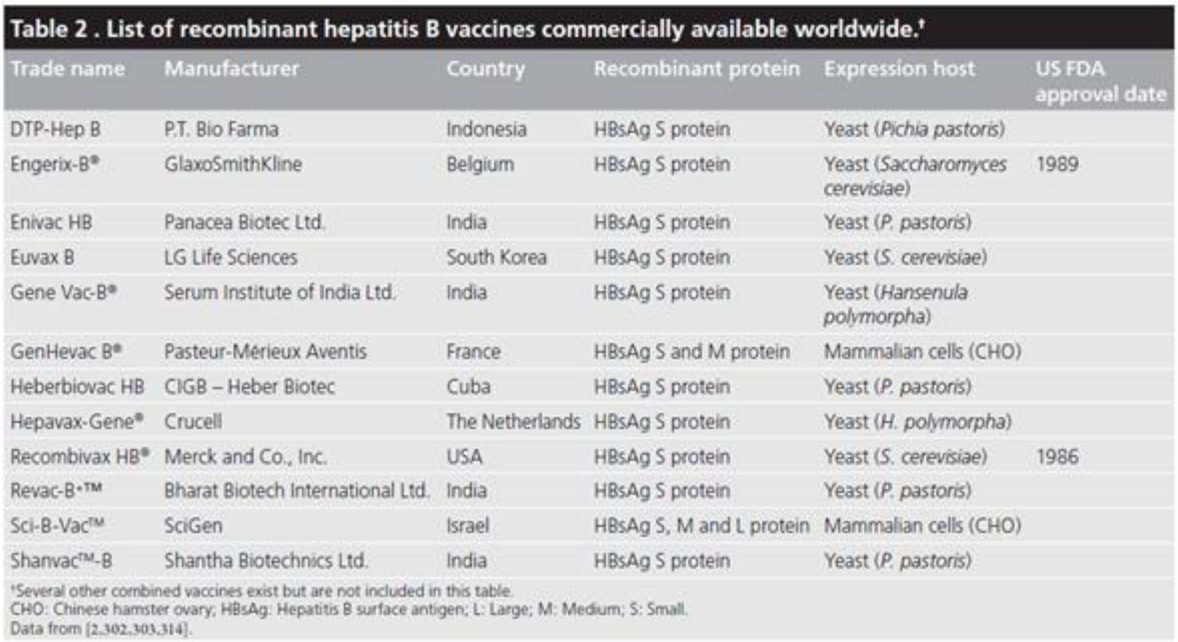


Table 2 . List of recombinant hepatitis B vaccines commercially available worldwide.†

Trade name	Manufacturer	Country	Recombinant protein	Expression host	US FDA approval date
DTP-Hep B	P.T. Bio Farma	Indonesia	HBsAg S protein	Yeast (*Pichia pastoris*)	
Engerix-B®	GlaxoSmithKline	Belgium	HBsAg S protein	Yeast (*Saccharomyces cerevisiae*)	1989
Enivac HB	Panacea Biotec Ltd.	India	HBsAg S protein	Yeast (*P. pastoris*)	
Euvax B	LG Life Sciences	South Korea	HBsAg S protein	Yeast (*S. cerevisiae*)	
Gene Vac-B®	Serum Institute of India Ltd.	India	HBsAg S protein	Yeast (*Hansenula polymorpha*)	
GenHevac B®	Pasteur-Mérieux Aventis	France	HBsAg S and M protein	Mammalian cells (CHO)	
Heberbiovac HB	CIGB – Heber Biotec	Cuba	HBsAg S protein	Yeast (*P. pastoris*)	
Hepavax-Gene®	Crucell	The Netherlands	HBsAg S protein	Yeast (*H. polymorpha*)	
Recombivax HB®	Merck and Co., Inc.	USA	HBsAg S protein	Yeast (*S. cerevisiae*)	1986
Revac-B+™	Bharat Biotech International Ltd.	India	HBsAg S protein	Yeast (*P. pastoris*)	
Sci-B-Vac™	SciGen	Israel	HBsAg S, M and L protein	Mammalian cells (CHO)	
Shanvac™-B	Shantha Biotechnics Ltd.	India	HBsAg S protein	Yeast (*P. pastoris*)	

†Several other combined vaccines exist but are not included in this table.
CHO: Chinese hamster ovary; HBsAg: Hepatitis B surface antigen; L: Large; M: Medium; S: Small.
Data from [2,302,303,314].

Figure 16. Commercially available recombinant hepatitis B vaccine.
Source: Roldão et al., 2010.

The characterization of the purified protein is usually done with electron microscopy, to check the distribution of size and shape, which, as mentioned before, is spherical. (Hadiji-Abbes, N. et al., 2013).

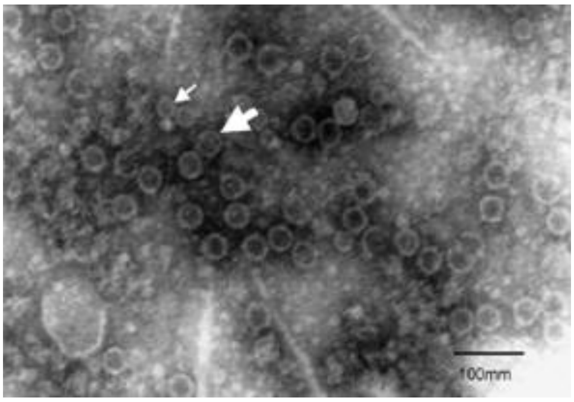


Figure 17. HBsAg protein in its spherical form seen in electron microscopy.
Source: Chen et al., 2004

The M-P31c gene is in charge of producing the HBsAg protein, whose nomenclature *is LM-P31C*. Its sequence has been clarified from the nucleotides of that gene, and it is the follows:

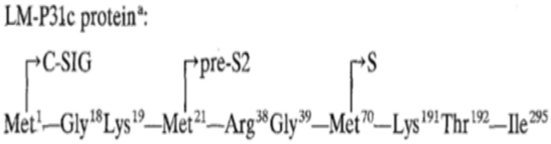


Figure 18: LM-P31c protein sequence.
Source: Kuroda, Miyasaki & Otaka, 1993.

-*Saccharomyces boulardii* in diarrhea treatment

Diarrhea occurs when there is an electrolyte imbalance in the intestine that leads to water losses due to the effect of "entrainment" of the ions that are leaving the lumen in an unbalanced way, or in other words, due to the effect of osmosis, water diffuses the medium that is more concentrated in ions, which in this case is the intestinal lumen, which, due to the release of electrolytes by channel changes, ion pumps, among other effects, has a higher concentration of these than normal. Several factors can lead to diarrhea: decrease in the intestinal flora that normally protects against the colonization of pathogenic bacteria (diarrhea associated with antibiotics), infection with a bacteria "foreign" to the intestine, or exposure to bacteria that don't belong to the normal intestinal flora by the introduction of instruments as the patients with parenteral nutrition. In antibiotic-associated diarrhea (AAD), when antibiotics are administered to kill certain pathogens, an imbalance of the normal intestinal flora occurs, because its microorganisms are also being targeted for its antimicrobial effects. This colony of endogenous microorganisms are responsible for exerting a resistance effect against the colonization of other (pathogenic) bacteria and the fermentation capacity of the colon. Due to this imbalance and the amount of microorganisms in the intestinal flora decreases, it allows colonization of pathogenic bacteria such as Clostridium difficile, which through toxins in specific receptors of the intestinal epithelium, cause diarrhea due to an imbalance electrolytic. (Bleichner et al., 1997; Marteau, Vrese, & Cellier, 2001).

In cases of bacterial diarrhea, the use of a non-pathogenic yeast from Saccharomyces boulardii has been used, in the form of a harmless powder, which is highly beneficial in the control of bacterial diarrhea. Saccharomyces boulardii exerts a trophic effect on the intestine, producing an increase in the secretory activity of the intestinal mucosa, resulting in increased IgA secretion that creates a more intense immune response against bacterial antigens. Also, in that same trophic effect, S. boulardii stimulates intestinal enzymes such as hydrolase, which prevent the growth of colonies. Additionally, it has been reported that it induces the luminal release of polyamines, an antagonistic effect against bacterial growth in the intestine, and that added to the competition against other microorganisms for available resources, makes this non-pathogenic yeast control the action of the "foreign" bacteria in the intestine. (Bleichner et al., 1997; Marteau et al., 2001; Surawicz et al., 2000).

In most cases of bacterial diarrhea, the most common microorganism involved is Clostridium difficile, either due to weakening of the intestinal flora, or high exposure to this pathogenic organism. Clostridium difficile is usually treated with antibiotics, but it usually shows recurrence because enough spores remain to produce new growth of the bacteria, no matter how much antibiotic is used, some spores always remain, which are invulnerable to antibiotics. The use of Saccharomyces boulardii added to antibiotic treatment, a decrease in the frequency of such recurrences has been demonstrated, with greater effect in high doses of antibiotic. Colony regrowth of Clostridium difficile is seen in both, high and low doses of the antibiotic. At low doses (or suspension of the antibiotic) the recurrence is because there are still active forms of the bacteria that reproduce and restore the growth of the colony, since the amount of antibiotic was not enough to eliminate each bacterium. In the case of high doses, recurrence occurs due to the spores mentioned above; although all *Clostridium difficile* bacteria are eliminated, it leaves spores that, as is known, resist the affection of antibiotics; thus, when all the *C. difficile* bacteria have been eliminated, the spores are responsible for reestablishing the colony of this microorganism. The positive effect in reducing the recurrence of this pathogenic bacterium by *Saccharomyces boulardii* is due, apart from the aforementioned effects for the other pathogenic microorganisms, to the fact that this yeast produces a protease that specifically inactivates *Clostridium difficile* toxin receptors. Once this inactivation is exerted, even the recurrence of *Clostridium difficile* is not capable of producing symptoms because the toxins cannot bind to the receptors since they are inactivated, and thus don't cause an electrolyte imbalance. However, this protease cannot inactivate the receptors if the toxins are already bound to them, which explains why at low doses of antibiotic, the incorporation of *Saccharomyces boulardii* does not decrease the frequency of recurrence of *Clostridium difficile*, since at low doses *Clostridium difficile* is not eliminated, some receptors with attached toxins remain. (Bleichner et al., 1997; McFarland et al., 1995).

-Uses in the diagnosis of Crohn's Disease

Crohn's disease is an inflammatory bowel disease (IBD) just like ulcerative colitis. Both are heterogeneous chronic inflammatory bowel disorders that have been associated with genetically predisposed individuals to an immune response by the host against normal intestinal flora. In the case of Crohn's disease, the entire digestive system can be affected, from the mouth to the anus. In contrast, ulcerative colitis is usually limited to the colon and rectum. Otherwise, both diseases have very similar characteristics in their course, which makes them difficult to differentiate. (Bertin et al., 2013; Laass, Roggenbuck, & Conrad, 2014).

For years, the biggest problem in these interstitial disorders has been the difficulty in differentiating (and subsequent diagnosis) between Crohn's disease and ulcerative colitis, especially when the problem is located in the colon, since either of them can act there. In fact, in 10% of IBD cases, discrimination between one disease and another is reduced to the diagnosis of “undetermined colitis”. As a solution to this differentiation problem, it was found that one intestinal disease could be differentiated from the other through the use of serum markers; one of them, anti-saccharomyces cerevisiae antibodies (ASCA). These anti-glycan antibodies were first described for IBD, and although they can also be found in autoimmune diseases such as systemic lupus erythematosus or antiphospholipid syndrome, they present a particular affinity in cases of Crohn's disease compared to the previous diseases and the ulcerative colitis, which is where this difference in affinity is most useful. (Bertin et al., 2013; Laass et al., 2014; Mow et al., 2004).

When comparing percentages, it can find that in cases of Crohn's disease, ASCA are found in 39-68% of patients, while in ulcerative colitis it occurs only in 0-14% of patients, which is a fairly reliable determinant in case you need to differentiate the two diseases. Anti-saccharomyces cerevisiae antibodies are commonly detected with the use of techniques such as ELISA or, indirect immunofluorescence, and their presence can be used for the diagnosis of Crohn's disease, rule out ulcerative colitis. (Klebl et al., 2004).

The determination by the ELISA method is carried out by diluting the plasma sample to an appropriate concentration. This is added to plates where the Saccharomyces cerevisiae antigen has previously been incorporated. Conjugated ASCA is detected by incubation and incorporation of tetramethylbenzidine chromogen. Both, sample and negative control are added to each plate. The reactivity of ASCA is determined with the ASCA binding index (ASCA BI), by the formula optical density of the sample (ODm) / optical density of the control (ODcont) (Teml, A et al., 2003; Walker et al., 2004). This is the type of results obtained.

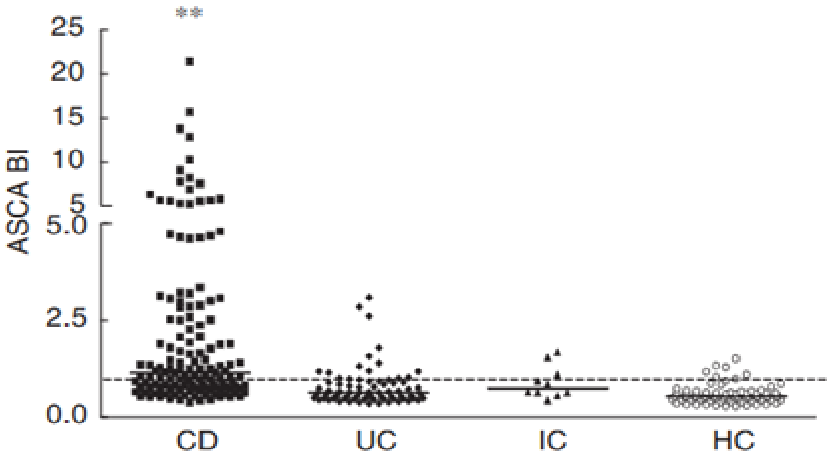


Figure 19: Result obtained for ASCA by an ELISA, where ASCA BI = ODm / OD$_{con.}$

Source: Walker et al., 2004

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