SUMMARY

This doctoral thesis, by proving and developing a new concept on the use of molecular biology techniques for the production of genetically modified yeast, the concept of Genomic Evolution assisted by Molecular Design, proposes an alternative to the current situation of social rejection against the use of GMOs in the food industry. The basic idea of this new concept is simple. It is based on mimicking nature in its constant evolution, but hastening and directing the evolutionary process in order to select, in a short period of time, those genetic changes that we have specifically designed at molecular scale. Yeasts are not an invariable-in-time entity. On the contrary, like all living organisms, evolve over time. The new methodology we have developed can speed up the evolutionary process, since we have introduced genetic modifications that could have been made spontaneously by yeasts in their natural environment as a result of genetic elements recombination, duplication or deletion processes along of their reproductive cycle. However, these changes would have been lost without the presence of certain selection conditions and, given the low probability of these events, it would have required an immense period of time.

The concept of Genomic Evolution assisted by Molecular Design has been proved by the construction and chromosomal integration of a selection cassette based on *YAP1* gene overexpression, which gives rise to a dominant marker conferring the yeast with resistance to various toxic compounds such as cycloheximide and cerulenin. For the development of the selection cassette the following conditions have been imposed and respected:

1) Use of the homologous recombination ability of *Saccharomyces* genus yeast.

2) Use of genetic material exclusively of the own yeast strain that we're going to evolve.

3) The genetic material can't pass through any intermediate process of cloning or expression in other biological systems.

With all these conditions fulfilled, the resulting strains should not fit in the European official definition of GMO: "organisms in which the genetic material (DNA) has been altered in a way that does not occur naturally by mating and/or natural recombination".

To prove this concept we have first worked with a laboratory yeast strain as a model to follow with the same experimental approach but working with an industrial yeast used in the production of lager beer. The experimental methodology consisted in obtaining, only through PCR reactions, a construction which, subsequent to its chromosomal insertion by homologous recombination, will replace the original promoter of the gene

YAP1 by a strong promoter, *PGK1*, whose gene is involved in the glycolysis path. It was determined whether overexpression of *YAP1* gene in the beer strain evolved was able to decrease the presence of spontaneous respiration-deficient mutants ("petites") during the fermentation of beer wort. The continued industrial reuse of brewing yeast is associated with an increase in frequency of appearance of "petites", with consequent negative effect on the fermentation process. According to our results, the evolved strain clearly showed a decrease in the "petites" formation during 10 successive microfermentations with respect to the parental strain.

Once the feasibility of the concept of Genomic Evolution assisted by Molecular Design has been proven, the next step was the application of the methodology of this concept to the development of yeast with industrial interest. Cromosomic reorganization over 3 different industrial yeasts has been designed:

a) A lager beer yeast evolved to achieve low production of diacetyl. Its phenotype was obtained by overexpression of *ILV5* gene, that encodes for a reductoisomaerase-converting the α-acetolactate into β-dihydroxyvalerate. This helps to prevent the α-acetolactate accumulation in worts and significantly reduces the maturation process because of the reduction in diacetyl production. In the evolved strain the lagering time is reduced in more than 10 days with respect to the wild one.

b) An industrial yeast with high pectinolytic activity for its use in the industrial processing of pectin rich materials. This phenotype was obtained by overexpression of *PGU1* gene that encodes for an endopolygalacturonase. We have obtained 2 new variants of the wild type strain, one containing the selection cassette and the other one in which the selection cassette was removed by a processed called “pop out” allowing the re-use of this cassette in a new genomic reorganizations of the same strain.

Both evolved strains show a remarkable increase in the hydrolytic activity over pectin with respect to the wild type strain, not affecting its ability for the production of ethanol or its fermentative capacity.

c) A wine yeast evolved seeking for a better tolerance to low temperatures. We have tried to obtain the desired phenotype by inactivation of the gene *INP51* that encodes for an inositol polyphosphate 5-phosphatase, involved in phosphatidylinositol 4,5-bisphosphate homeostasis. This function lost in the enzyme induces the accumulation of the metabolite and, according to literature, confer cold-resistance properties to the phenotype.

We have obtained new strains; a transformant which has one copy of *INP51* gene inactivated and another transformant which has two copies of the same gene inactivated. Nevertheless, contrary to what happen with the parental strains, the gene inactivation does not increase the cold resistance of the evolved strain.

All the applications of the developed methodology described above show the adventages of using the concept of Genomic Evolution assisted by Molecular Design in order to improve the performance of industrial yeast strains of the *Saccharomyces* genus.