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# Exploring LAB World from Engineering Viewpoints

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## Introduction

Lactic acid bacteria (LAB) are most familiar microorganisms as producers of dairy foods such as cheese, yogurt and widely utilized as a safety microorganism. Recently lactate production by LAB has been interested in as an intermediate raw material for biodegradable polymer, poly-lactate production, which will contribute to the global environment improvement. Of course most of intestinal LAB as a diary food are interested in from viewpoint of healthy life. And many bacteriocins are found in various LAB and will be utilized practically such as a food preservatives. Nowadays LAB have been widely used common microorganisms.

So called LAB research world will be divided into two from viewpoint of application. One area is on upstream problem such as strain breeding and another is on reactor operation problem. Molecular breeding based on the metabolic engineering is now rather far from practical application of LAB in food industry because of public acceptance of GMO. However the research focused on molecular breeding will be necessary because to know the mechanism of metabolic pathway control will be beneficial for getting an excellent strain based on traditional breeding using mutation techniques. And the system of the self - cloning, will be available soon for developing the genetic manipulation. The research activity should be kept high for that period.

In this article, research direction and activity are explored in the LAB world of my laboratory. Pathway recruiting based on metabolic engineering was attained by genetic manipulation for efficient production using LAB. And the enzyme-displaying system on the surface of LAB has been established for direct fermentation from cheap carbon source. For the reactor operation, co-culture system with yeast has been proposed and nowadays we have two practical example of the co-culture. One is nisin production and another is kefiran production. In both cases, yeast was used for the role of assimilation of lactate in order to eliminate of pH decrease as well as product inhibition.

## Upstream Problem

***Pathway recruiting for better production; Metabolic analysis of lactate dehydrogenase deficient***

***Lactococcus lactis***

Lactic acid bacteria produce large amount of lactic acid and have been related to human life from old times. Lactic acid bacteria are generally recognized as a safe microorganism and are expected to be the host to produce useful materials such as bacteriocin. But the growth of *L. lactis* is inhibited by the decrease of pH, which is caused by an accumulation of lactate during cultivation. Therefore, the lactate dehydrogenase deficient mutant of *L. lactis* was constructed, and we tried to analyze the effect of disruption of lactate dehydrogenase on metabolic flux and the change of NADH redox balance.

For constructing the *ldh* deficient strain, the integration vector pUC19A2B2ery was introduced into *L. lactis* IL1403. Erythromycin resistant clones were analyzed by southern analysis. As a result, IL1403 integrating pUC19A2B2ery were obtained. This integrant was cultured under absence of erythromycin and two erythromycin sensitive clones were obtained. These clones were confirmed to be the *ldh* deficient strain by PCR. As a result of fermentation of *ldh* deficient strain, the decrease of pH is suppressed and the growth of LDH deficient strain is better than that of wild type. As can be seen in Fig. 1, under aerobic conditions, the *ldh* deficient strain produced less lactate than wild type, but the acetoin and acetate production were increased. The *ldh* deficient strain of *L. lactis* allowed redistribution of the pyruvate toward products other than lactate.

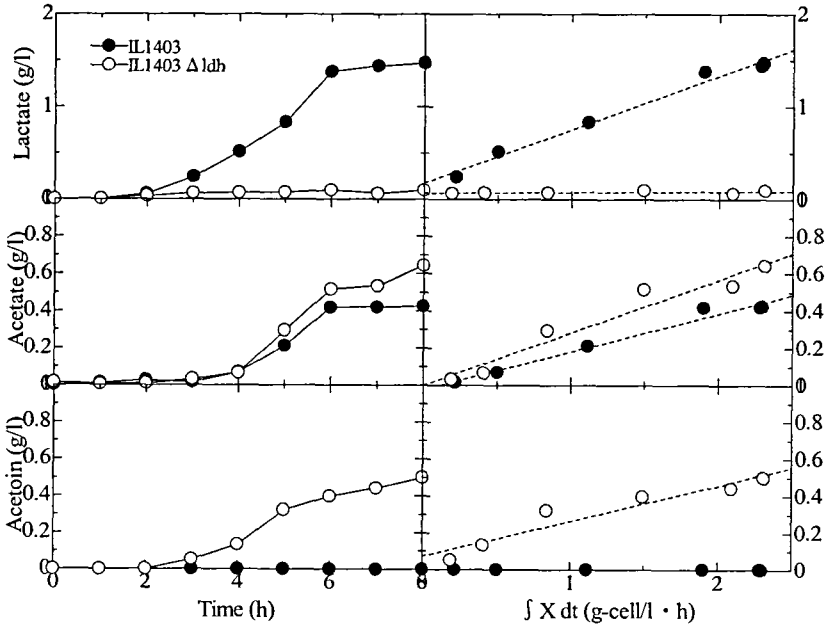


**Fig. 1 Comparison of metabolites production at *ldh* deficient strain with normal one.**

And the specific enzyme activity of NADH oxidase (r8 in Fig.2) of *ldh* deficient strain is larger than that of wild type. It is suggested that the wild strain perform the large part of NADH oxidation by LDH, but LDH deficient strain adapt to perform the large part of NADH oxidation by NADH oxidase.

Now our research target goes to the construct the rerouting system from lactate production to produce the ethanol by introducing the *adh* (alcohol dehydrogenase) and *pdc* (pyruvate decarboxylase) genes to the *ldh*

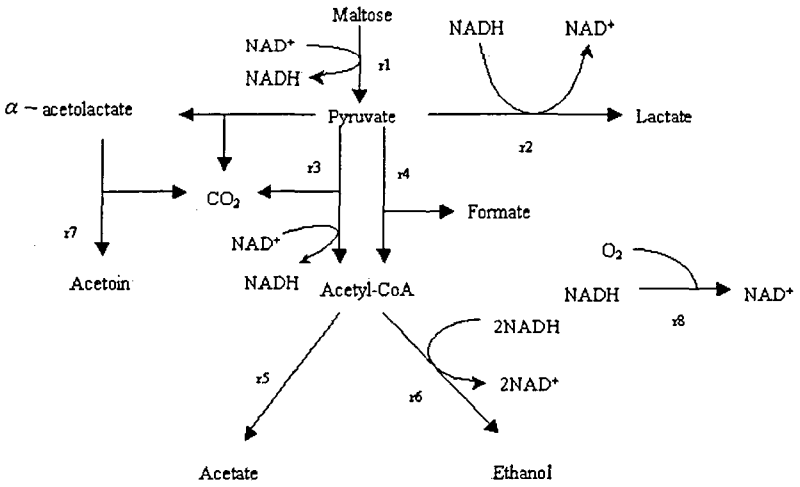


**Fig. 2 NADH-NAD balance in *ldh* deficient strain**

deficient strain as already explained or to alanine by introducing *ala*D(alanine dehydrogenase) to the *ldh* deficient strain. The growth of LAB is inhibited by low pH due to the production of lactate. And also the main product by this LAB is also inhibited because most of production by LAB is growth associated. So the final goal of this rerouting study is in crease the useful product by rerouting the pyruvate pathway from lactate to ethanol or alanine. Our research is now on going.

**Surface display system**

The surface display sys tem is the latest strategy adding the function of an exotic protein to microorganisms as yeast and lactic acid bacteria by fusing the exotic protein to anchor protein in cell surface of the host strain. Almost all anchor proteins previously reported in LAB, however, are able to display exotic proteins only at those N-terminals and these expression (displaying) levels have not been estimated. Therefore it is impossible that an arbitrary protein of use at the desired ratio. So in the design sense, the tool or system at C-terminal fused protein expression system and under controlled expression of those proteins is desirable. We are going to develop such a system.

## Reactor Operation

**Nisin production by co-culture of *L. lactis* with *K. marxianus***

Nisin is an anti-microbial peptide produced by certain *Lactococcus* species. Nisin has been accepted as a safe and natural preservative in more than 50 countries. However, the most serious problem in nisin production is the inhibition of growth due to the increase in lactate concentration and the decrease in pH. In order to avoid growth inhibition by the decrease in pH due to the accumulation of lactate in LAB fermentation processes, pH control methods by the addition of alkali or by the extraction of lactate using organic solvents have been employed. Continuous culture with mechanical separation system, such as electrodialyzer or membrane filter, is also applicable.

In this study, a new pH control strategy without alkali addition was developed. The nisin producer *L. lactis* assimilates maltose and produces nisin and lactate. *Kluyveromyces marxianus*, which was isolated from kefir grains, does not have ability to assimilate maltose but has ability to assimilate lactate. Since the consumption rate of lactate is affected by dissolved oxygen (DO) concentration, lactate concentration, that is, pH would be controlled by manipulation of DO concentration[1].

Without pH control, pH decreased below 5.0 within 3 h in the batch pure culture. The cell growth was completely terminated after 6 h and the concentration of nisin was 7.4 mg/L. Figure 3 shows time courses for high production of nisin under co-culture with yeast, in which yeast cell growth, assimilation rate of lactate for keeping pH level, was controlled by DO concentration through the agitation speed automatic-manipulation.

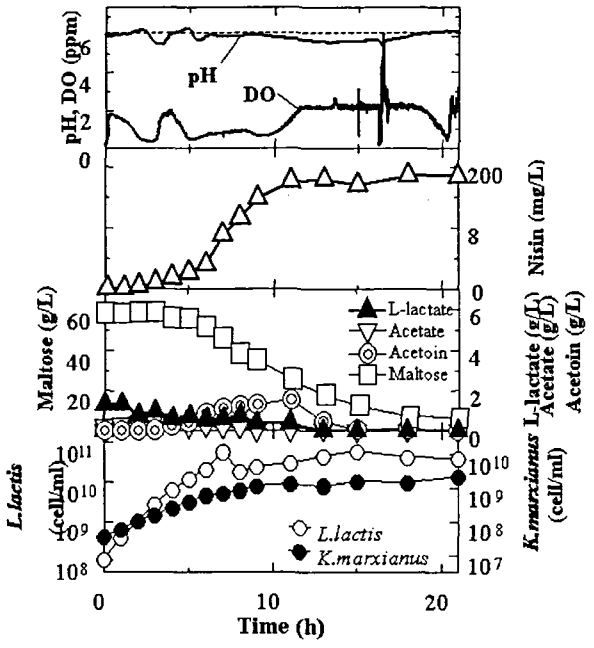


**Fig. 3 Nisin production in a batch co-culture with yeast**

Concentrations of maltose, yeast extract, and peptone were set 60, 40, and 40 g/L, respectively. pH was well controlled at 6.0 in this case as well. Nisin concentration finally reached to 200 mg/L and it was twice as much as nisin production in normal operation with pH control by alkali addition. In this case, surprisingly not only lactate but also acetate was removed. This fact would be due to that yeast assimilated not only lactate but also acetate.

Now the research interest moves to investigate a mechanism of the bacteriosin production through the signal recognition. And application side, improvement of ceiling level in nisin biosynthesis of *Lactococcus lactis,* subsp. *lactis* is aimed.

**Kefiran production by mixed culture of *L. kefiranofaciens* with *S. cerevisiae***

In the kefiran production process, *Lactobacillus kefiranofaciens* and *Saccharomyces cerevisiae,* are

co-cultured for enhancement of the productivity. *L. kefiranofaciens* produces an exopolysaccharide called kefiran, which contains approximately equal amounts of glucose and galactose. Bacterial polysaccharides are usually associated with the outer surface of the bacterium. They may either form an amorphous layer of polysaccharides called capsular kefiran in the form of a capsule surrounding the cell, or be excreted as extracellular kefiran to the medium. To investigate what factors affect the kefiran production is objective of this research. As a result, it was found that the direct contact of yeast with *L. kefiranofaciens* enhances the CPS kefiran production as seen in Fig.4[2].

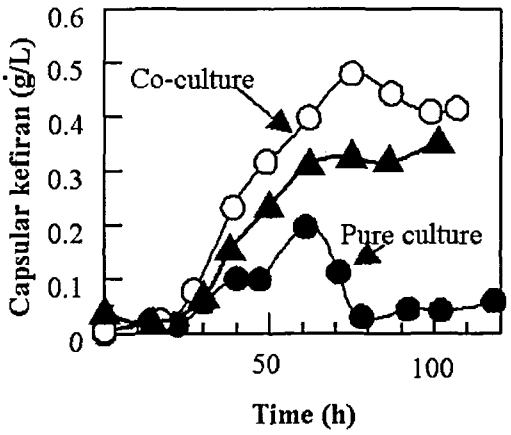


**Fig. 4 Capsular kefiran production in anaerobic pure culture (closed circles) and anaerobic co-culture (Open circle) compared with that in a mixed culture with *S. cerevisiae* inactivated by heat treatmen (closed triangle).**

**Database Network**

In order to utilize the information on LAB more effectively, we are going to organize the consortium for database of LAB resources as well as researchers related to LAB. That is, the aim of the organization is to investigate the bio-resources of LAB and to construct the database of LAB. LAB are interesting to be investigated from the viewpoint of not only lactate production itself but also for probiotics and functional materials production such as polysaccharides. We have huge variety of bio-resources in Asian region but most of those are not yet utilized for our life. For connecting Needs and Seeds we need to establish a researcher-oriented database and to collaborate each other based on the database, for Asian LAB researchers in academia and industries.

So, now we are asking all LAB researchers in Asian countries to collaborate for this activity. Of course, the information is not opened without permission and the established network you can freely use. Contact mail address is : LABQ@agr.kyushu-u.ac.jp.

## Conclusion

In this article, research activity in our laboratory has been explored focusing on LAB world. Most of researches are now ongoing. In that sense I hope to get fruitful results in those researches in near future and

have a chance to report the results. And I would like to give my sincere thanks to all of my colleagues and students have been contributed to those researches.

## References

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