Biotechnological studies concerning the obtaining of biomass with probiotic role from yeasts and bacteria

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Abstract

Two approaches for obtaining microbial biomass with probiotic action have been examined. Firstly, a mixture of three different yeast strains belonging to Saccharomyces cerevisiae, Candida robusta and Hansenula polymorpha was cultivated at micropilot level and the bioprocess flow sheet for obtaining the viable biomass was established. Secondly, the optimal conditions for cultivation of Lactobacillus plantarum CMG.B –3 in a wine spirits based medium were presented. The lactobacilli biomass obtained under these conditions could be used as inoculant for silage, for improving the quality of fodders.

Keywords: biomass, *Saccharomyces cerevisiae, Candida robusta* and *Hansenula polymorpha, Lactobacillus plantarum*, probiotic

Introduction

For the probiotic industry, it is important to be able to demonstrate the safety and beneficial effects of its products. A large number of products already exists today, but their impact on human or animal health warrants better documentation and better reasons. Intensive studies were carried out in different laboratories in several directions:

- to elucidate the direct and indirect way to action for probiotics;
- a more profound knowledge of survival of probiotics within the gastrointestinal tract, their translocation and colonization properties and the fate of their active components need to be known to predict their positive as well as their side effects;
- the study of the receptor sites nature for different probiotic microorganisms;
- to find the conditions in which the probiotics' alternative is optimum as a substitute for antibiotics and if these could also be complementary;
- to understand the better results obtained when the tests were performed on young animals, under stress conditions or under difficult sanitary conditions;

- to clarify the interactions between probiotic microbial strains when they are cultivated in mixture that gives better results than the use of a unique strain [1, 2].

Among the microorganisms utilised as probiotics, the yeasts (especially those from genus *Saccharomyces, Candida, Hansenula* or *Pichia)* have an important role as probiotic organisms and they are frequently used as additives in animal food [3].

It has been observed that the total weight efficiency and the specific fodder consumption were significantly improved when in the given ratio a combination of yeast and lactobacillus biomass was used [4].

In ensiled crops, lactic acid bacteria convert low molecular weight carbohydrates into lactic acid, which is the main preservative in ensilage. *Lactobacillus plantarum* is used as inoculum in grass silage, it produce lactic acid from low molecular sugars, thereby lowering the pH and allowing long-term preservation of these products [5, 6, 7].

The aim of our expriments was to establish the optimal conditions for cultivation of some probiotic microorganisms (three yeasts an a bacterium) for biomass production and to characterize the products obtained.

Materials and Methods

Microbial strains

Five microbial strains were used in our expriments.Three of them belong to *Saccharomyces cerevisiae, Candida robusta* and *Hansenula polymorpha* and two are bacteria: *Lactobacillus plantarum* CMG.B – 3 and *Lb. plantarum* ATCC 8014 (used as control strain).

Cultivation conditions

The bioprocess flow sheet at micropilot level involved the following stages:

- the cultivation of yeasts strains in lab conditions;
- obtaining the inoculum in shaken flasks;
- consortium cultivation of the three selected yeast strains in a microfermenter, LKB type with 8L working capacity;
- biomass separation from culture medium and drying this at 30^0C;
- physical-chemical analysis of the biomass.

The culture medium for inoculum and fermentation consists of: 10% glucose, 0,2% KH_2PO_4, 0,2% $(NH_4)_2SO_4$, 0,2% corn steep liquor, 0,05% $MgSO_4 \times 7H_2O$, 0,005% $FeSO_4 \times 7H_2O$, 0,1% microelements.

The bioprocess was performed under the following conditions: temperature of cultivation 30^0C, agitation speed 400 – 800 rpm, air flow rate 0,8 – 1 l/m.

The strains of *Lb.plantarum* were maintained on an MRS medium and cultivated under various exprimental conditions:

- in MRS as control;
- in washing solution of wine spirits, supplemented with 3,6% glucose and 3,6% yeasts extract (M_1);
- in washing solution of wine spirits supplemented with 1% peptone (M_2);
- in a mixture of equal volumes of washing solution of wine spirits and a solution containing 1,8% glucose and 1,8% yeasts extract (M_3).

Analytical methods

During the cultivation of the yeasts strains different parameters were determined: growth was measured as optical density (OD) at 570 nm, by wet cell weight (WCW, g/l) and by cell dry weight (CDW, g/l); the culture purity was checked microscopically; the level of

dissolved oxygen in culture medium was determined with a special electrode; the evolution of pH value during fermentations.

The evolution of the accumulation of the lactic acid produced by *Lb.plantarum* CMB.G-3 in fermentation conditions was also monitored and the transformation yield of the carbon sources was calculated.

Acidity was determinated by titration with sodium hydroxide 0,1N, considering that 1ml NaOH 0,1N = 0,009008 g lactic acid.

Results and Discussions

Dynamics of yeasts biomass production

A batch fermentation profile of yeast growth and biomass acumulation at controlled pH is presented in **Table 1**.

Table 1. Fermentation of the yeast mixture in LKB bioreactor.

Cultivation hours	**pH**	**O_2 (%)**	**OD_{570nm}**	**WCW g/l**	**CDW g/l**
0	4.6	100	0.240	ND	ND
4	4.2	40	0.240	ND	ND
8	4.2	48	0.290	ND	ND
12	4.0	22	0.485	75.66	16.02
16	4.0	10	0.610	111.32	23.10
20	4.2	30	0.980	148.87	29.52
24	4.0	10	1.340	178.22	36.4

ND = not determined

Exponential growth took place during a period of about 16 h, at a specific growth rate of 0,21h^{-1}. Maximum cell density was reached after 24 h and amounted to 178,22 g WCW per litre of medium, corresponding to 36.4 g/l CDW.

At the end of the fermentation, the microbial biomass was recovered by centrifugation (at 3200 rpm for 20 min), washed two times with sterile distiled water, dried at 30^oC for 8 h and subjected to further determinations. Different aspects of biomass as colour, smell and chemical composition were considered (**Table 2**).

Table 2. The analysis of yeast biomass obtained by batch fermentation.

No crt.	**Parameter**	**Value**
1	Aspect	Amorphous dried biomass
2	Colour	White
3	Smell	Distinctive, not musty
4	Loss by drying [%]	4,67
5	Sulphatated residuum [%]	4,97
6	Heavy metals [%]	0,001
7	Total nitrogen [%]	6,913
8	Ammoniacal nitrogen [%]	0,313
9	Crude protein [%, on dry weight basis]	43,27
10	Cell viability [CFU/g]	$4,5\times10^{11}$

No contaminants were detected and the viability of probiotic microbial strains was maintained during the downstream processing.

The results obtained permit the following bioprocess flow sheet in order to recover large quantity of (**Figure 1**).

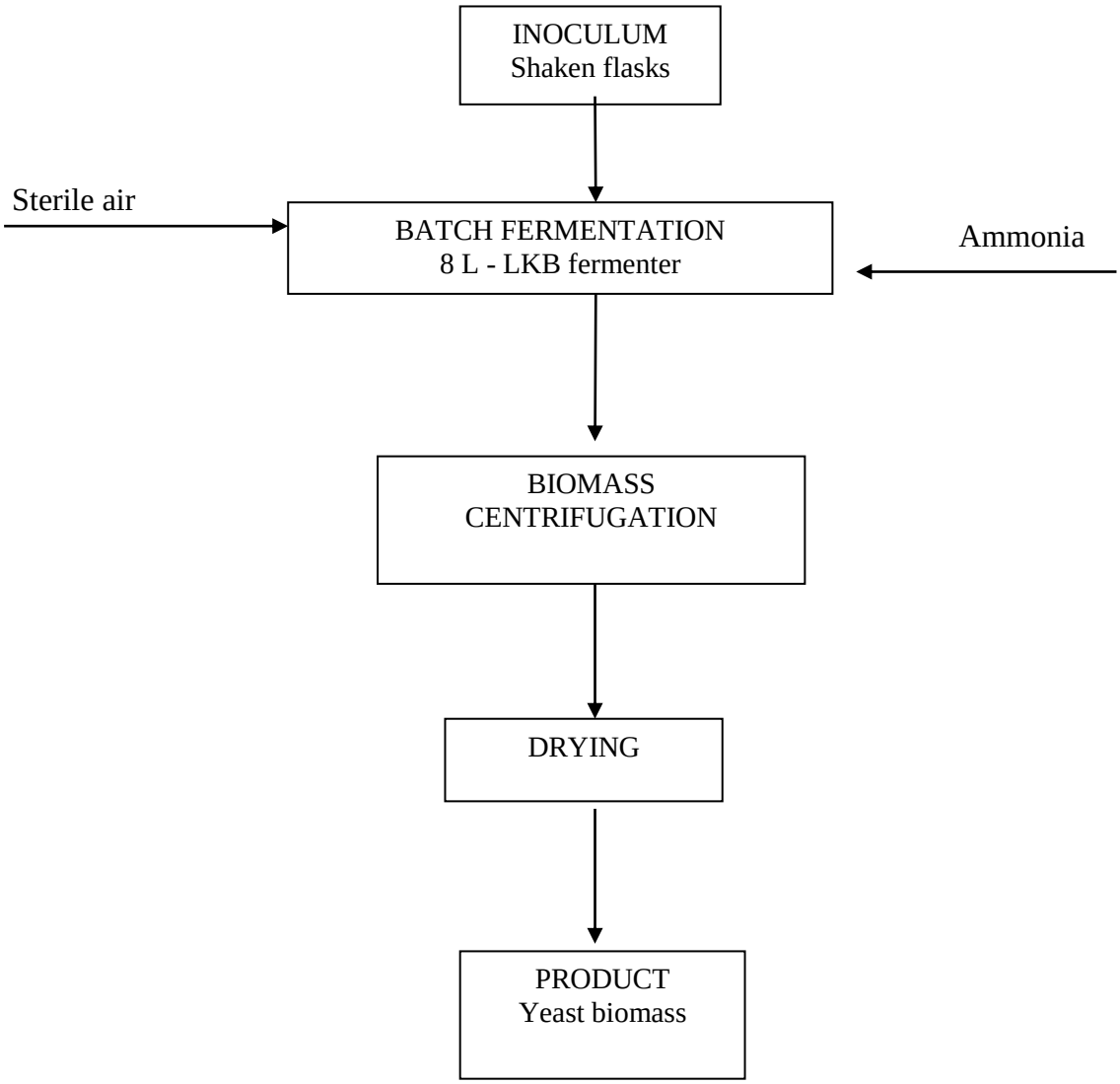

Figure 1. Bioprocess flow sheet for proteic biomass obtained with yeasts, at micropilot level.

The quality of viable yeast biomass obtained by this protocol is good and it could be used as probiotic product for animals nutrition.

Dynamics of *Lactobacillus plantarum* CMG.B – 3 biomass production

To obtain *Lb.plantarum* CMG.B – 3 biomass, fermentations were performed in microanaerobiosis conditions, at 37^0C the optimal temperature for this bacteria. The evolution of biomass acumulation and of lactic acid biosynthesis during fermentation in MRS were monitorized (**Figure 2**).

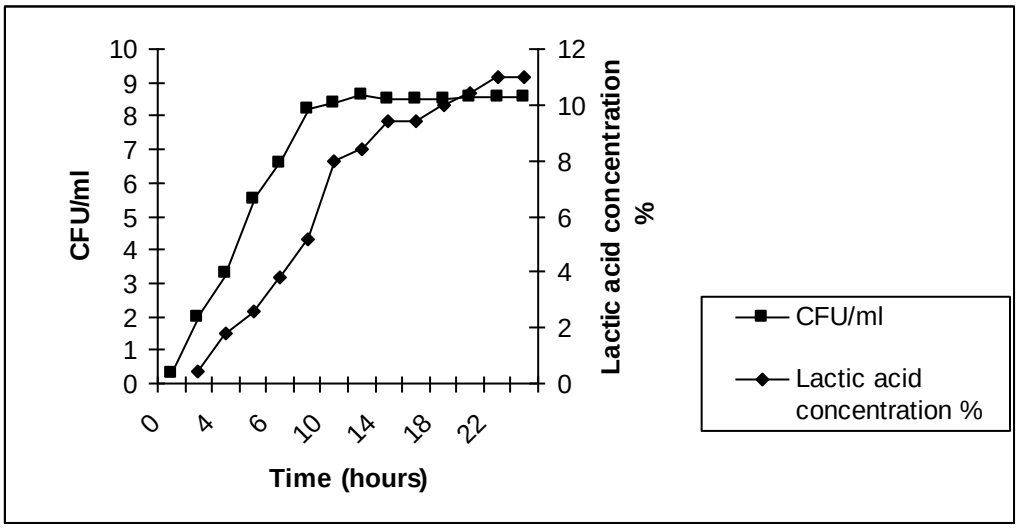

Figure 2. Profile of growth curve and lactic acid accumulation at *Lactobacillus plantarum* CMG.B – 3 strain on control medium (MRS).

In standard conditions (in an MRS medium) the dynamic of biomass accumulation was typical for *Lb.plantarum*, being observed an exponential stage of 6 h, then a slowing down of growth rate for 2 hours followed by stationary phase. Lactic acid production appeared to parallel that of biomass, slow at the beginning of the fermentation and accelerated starting with the middle of logarithmic phase. The highest lactic acid level was reached after 22 h of cultivation and contiune during stationary phase (fig. 2).

In order to establish an optimal low cost medium for maximum biomass accumulation, different media were tested.

When the bacteria was cultivated in M1 medium based on diffusion water from wine spirits (with 3,6 glucose) and 3,6% yeast extract, differences in growth rate and lactic acid production were observed (**Figure 3**). The exponential growth phase was extended to 18 hours, lower values of cell density were determined and the maximum lactic acid accumulation was reached at the end of the fermentation.

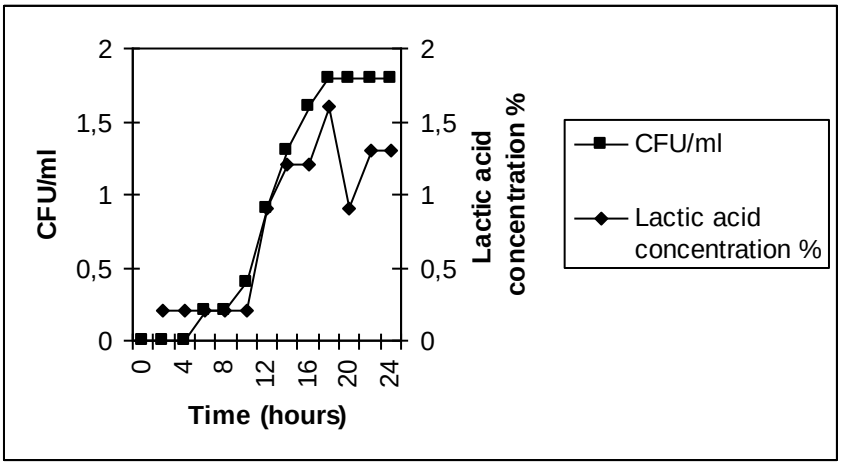

Figure 3. *Lactobacillus plantarum* CMG.B – 3 growth profile and lactic acid accumulation when cultivated in M1 medium (CFU/ml = number of colonies × 10^8 cells/ml).

In M2 medium based on wine spirits solution enriched with 1% peptone, biomass accumulation presented higher values comparing with those obtained in MRS medium. A 7 fold increase of CFU/ml of *Lb.plantarum* CMG.B–3 after cultivation in M2 medium was

observed: from 2.2×10^8 CFU/ml in MRS medium to 14.5×10^8 CFU/ml in M2 (**Figure 4**). Contrary, the maximum level of lactic acid was detected after 15 h of cultivation but a drastic decrease of the production of this aced apeared after 21 h of cultivation.

The better results in biomass accumulation obtained when the bacteria were cultivated in M2 medium could be explained by possible growth factors existing in wine spirit which particularly stimulates cell growth rather than lactic acid formation. The extension of exponential phase to 16-18 h observed in M1 and M2 media could also explain the increase of biomass production. Lactic acid was gradually accumulated till the end of fermentation (22 – 24 hours).

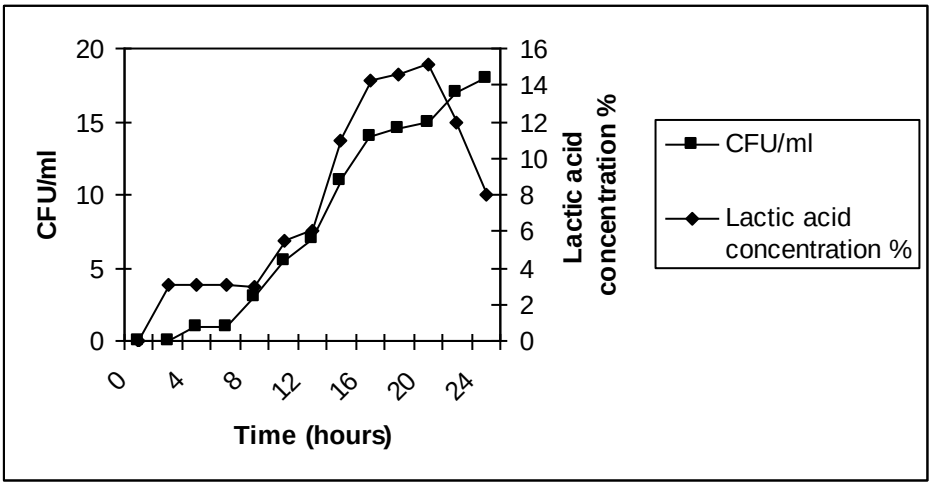

Figure 4. *Lb.plantarum* CMG.B – 3 growth profile and lactic acid accumulation when the bacteria were cultivated in M2 medium (CFU/ml = number of colonies × 10^8 cells/ml).

On the basis of the data obtained in these experiments, continous and semicontinnous fermentation technologies were developed which could be in particular advantageous for the bioprocess. In this respect, low cost cultivation conditions were examined. When incomplete yeast hydrolysate replaced peptone in culture medium, the level of polypeptides found in this substrate was insufficient for optimal growth of bacteria: it determined a low biomass accumulation and the extension of logarithmic growth phase up to 20 hours (**Figures 5** and **6**). Lactic acid accumulation was gradually produced till the end of fermentation.

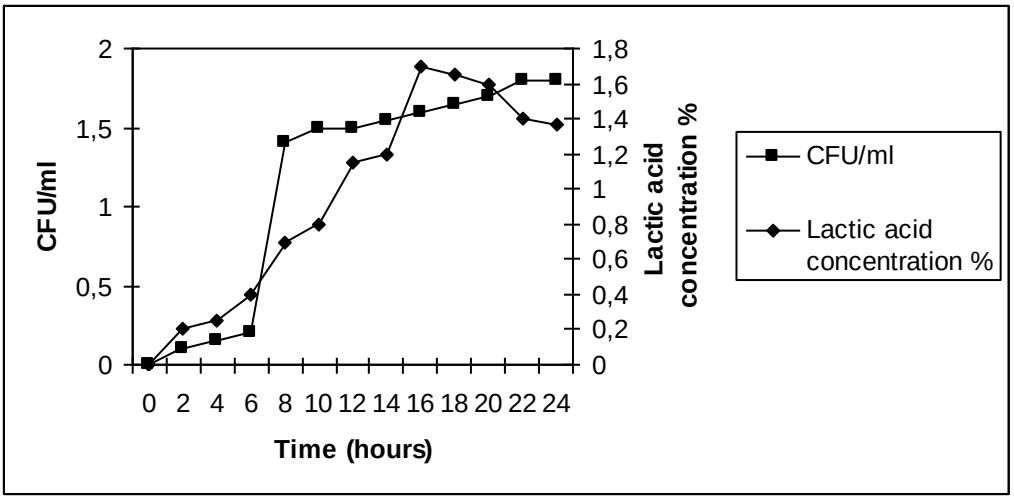

Figure 5. Profile of growth curve and lactic acid accumulation in *Lb.plantarum* CMG.B – 3 on medium of wine spirits washing solution with 1,8% yeasts extract (CFU/ml = number of colonies × 10^8 cells/ml).

Similar results were obtained when a mixture of 3:1 (vol/vol) wine spirits washing solution and 1,8% yeast extract solution was used as a fermentation medium (**Figure 6**).

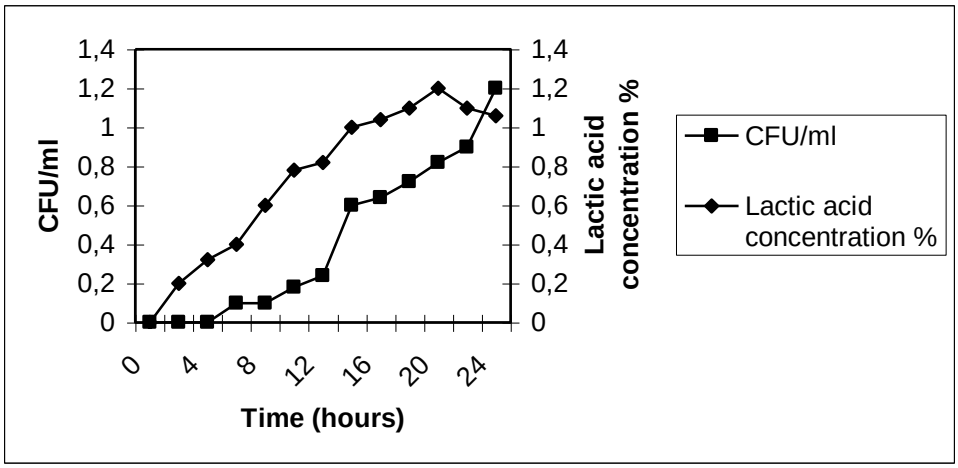

Figure 6. *Lb.plantarum* CMG.B – 3 biomass production and lactic acid accumulation in wine spirits washing solution with 1,8% yeasts extract solution (3:1, vol/vol) (CFU/ml = number of colonies × 10^8 cells/ml).

The above data clearly indicate that the fermentation medium containing wine spirits infusion enriched with 1% peptone gave the best results concerning both high cell yields and lactic acid accumulation. This medium is economically advantageous comparing with media proposed by different authors. The recommended culture media mentioned in literature derived from MRS standard medium and contain peptone, mineral salts, tensioactive agents, aminoacids, vitamines, etc, being expensive at micropilot level. The medium proposed in this work contains all the substances required by lactic acid bacteria which are provided by wine spirits solution (known to be rich in mineral salts and various growth factors).

Conclusions

It was established the bioprocess flowsheet at micropilot level in order to obtain a viable proteic biomass with yeast mixed culture (*Saccharomyces cerevisiae, Candida robusta* and *Hansenula polymorpha).*

The quality of yeast biomass produced in the mentioned conditions is high and allow its use as additive in animal fodder.

A simplified and economically medium for *Lb.plantarum* cultivation, based on wine spirits washing solution and enriched with 1% peptone, was proposed. It allows high cell yield and lactic acid accumulation so that the product obtained could be used as inoculant for silage.

Acknowledgments

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