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OPTIMIZATION OF ACTIVE ACIDITY FOR PEPTIDES DERIVATION IN THE PRODUCTION OF FERMENTED MILKS

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Abstract

There are three parameters that influences hydrolyses on milk and fermented milk products. Active acidity (pH) is one of that factors that determines speed of reaction and can influence on specific enzyme substrate. In production of yogurt, hydrolyses is measured by amount of peptides on different pH value. Maximum pH is determined to obtain maximum amount of peptides in yogurt. In order to stop the change in pH value during hydrolysis, the reaction it should be lead out in buffer system. An important parameter for hydrolysis of proteins is the distribution of the weight of the molecule obtained by hydrolysis of peptidite. For milk fermentation were used three different mixture (or combination) of microbiological starter cultures: Lactobacillus delbrueckii subs. bulgaricus and Streptococcus thermophilus; Lactobacillus acidophilus, Bifidobacterium bifidus and Streptococcus thermophilus; Lactococcus lactic subsp. cremoris and Lactococcus lactis subsp. lactis, production by Chr. Hansen's Laboratories. The task was subsequent measurement of pH value on different temperatures and time incubation of milk by different cultures. Quantity of amino group released in the process of microbiological growing in milk can be easy followed with OPA method, reaction on o-phtaldialdehid in β-merkaptoetanol. Specimens of yogurt were prepared from cow, sheep and goat milk. The measurement of optimal pH with usage three culture were: first mixture on the 43℃, fermentation of some pH= 4,3 for 4 hours; second on 37℃ fermentation some pH= 4,5 for 5 hours and third mixture on 22℃ and 30℃ temperature fermentation of some pH= 5,45 for 12 and 8 hours. Amount of total peptides in same type of milk and same pH, same temperature with different mixture of starter culture were different.

Key words: *hydrolysis, peptide, condition, microbiological culture*

1. INTRODUCTION

After we choose combinations of protein/protease (including possible before treatment of protein) we should define the conditions of hydrolysis. The main variable that depend the result of reaction are temperature, pH, relation enzyme/substrate, and the time of reaction. The first of the three factors determine speed on reaction and can influence on specific of enzyme substrate. The time of reaction is determining only by the dimension of the hydrolysis. Interactive effects between parameters of hydrolysis also influence on structure of hydrolysators. If we do not take a control of

process of hydrolysis, pH on the solution quickly after the start of hydrolysis will change as a result of creating new amino and carbon group which depending of pH on hydrolysis are capable to give or to receive proton's. At low pH value all amino groups are proton lyses until carboxyl group are no proton lyses, which result's a total consumption of protons with any interruption on the peptide link causing pH growing. At neutral or basic pH value, hydrolysis results with reducing of pH value because of no predomination of carboxyl group and proteinization on some small parts of amino group. As aim to stop changing on the pH value during hydrolysis, the reaction it should be lead out in buffer system or in pH-stat system where value of pH is keeping on the necessary level with adding acid or base(Adler-Nissen, J.).

Characteristics of hydrolysis

Because of hydrolysis, molecule's outstanding of proteins have been changed, such as reducing of molecules weight , growing of abundance, exposing on hydrophobic group and revealing of reactive amino acid's side rows (Nielsen, P.M) . These molecules changing can be detected with different analytic methods which reflects at one or more outstanding of molecules. As a result of molecule's changing, the functional characteristics of proteins have been changed. The expression functional characteristic is using frequently to point on technical-functional characteristic it should be included also and bio-functional characteristic that can be separate on nutritive and physiologic, more exactly biologic functional. Nutritive characteristic of hydrolysis's determined their expanding digestion, and reducing of allergy in comparison with starting proteins (Modler, H.W.). Physiological characteristics including a potential bioactivity of the hydrolysis, which origins from released bioactive peptides. At the end, technical-functional characteristic determines technological function as a dissolving, emulsion characteristic, foaming, and e.t.c.

Molecule's characteristic

Parameter that is often used for describing results from process of hydrolysis is the level of hydrolysis that indicates the volume of hydrolysis. Another important parameter for hydrolysis of proteins is distribution of molecule's weight on acquired with hydrolysis peptides (Adler-Nissen, J). Degree of hydrolysis present relation between number of hydrolyzed peptide link, and total number of peptide link:

$$DH=h/h\ tot.100 \qquad (1)$$

Where with *h* is marked the number of hydrolyzed peptide links, and with *h*tot total number of peptide links present in the starting protein. *h* and *h*tot are expressed in meq/g. *h*tot is determined from amino acid structure of protein. In the process of

hydrolysis with every interrupted peptide link, have been released new carboxyl and amino group (table 2). As a result of this, the number of hydrolyzed peptide links can be deduce from determining of number on newly created C- and/or N terminal groups in the hydrolysis's. As we mention before, in dependence of pH of liquid, amino and carboxyl group after hydrolysis are less

or more (de)protease. If the process of hydrolysis is occurring in pH-stat condition's, quantity of added acid or base can directly be used for accounting of DH, because adding of acid or base is proportional with released amino and carboxyl groups. However, this method is useful only in process of hydrolysis in neutral/alkali pH (pH>7), or acid pH (pH<3). On value of pH 5-6 there's not total releasing or accepting protons, because the process of proton lyses and deproton lyses of acid and base groups are in similarity. The pH value that are used in converting of dissociation degree of acid and base groups are not constant during the hydrolysis, because they depend of length of peptide link and side group of terminal amino acid (Mahmoud, M.I.). There's difficulty in determining value of DH from pH-stat result's, because of that there's included another methods to determine released amino groups. The quantity of released α-amino group can be determined with application of reagents who react specifically with amino groups giving a derivate who can be determined on spectrophotometer.

The most used reagents that are applying for this purpose are ninhidrin, o-phtaldialdehid (OPA) and trinitrobenzen sulfonic acid (TNBS). Determining of DH with these three reagents shows that the results received with OPA and TNBS are in good correlation , while the result for DH with ninhidrin are much lower. OPA method is better from TNBS because of he is rapid and more confidently.

2. MATERIALS AND METHODS

For milk acid fermentation was used microbiological cultures (taken from firm Chr. Hansen's Laboratories) and mixtures of cultures are shown.

System	Cultures
a	*Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus.*
b	*Lactobacillus acidophilus, Bifidobacterium and Streptococcus thermophilus.*
c	*Lactococcus lactic subsp.cremoris and Lactococcus lactic subs. Lactic*

Preparation of specimens from milk and milk products, preparation of yogurt specimens

Yogurt specimens were prepared from cow, sheep and goat milk. In case when milk was sterile, because cultures were not sterile, dishes for preparation was not necessary to be put on sterilization. For sterile and homogeny milk it was not necessary to warm up at high temperature. Goat's and sheep's milk were raw and because that reason they were warm up at temperature on 80-90°C in time of 5-10 minutes, after what they were made cold to temperature of 35-40°C and at the same temperature were added microbiological cultures for milk fermentation.

Tretament for starter preparation

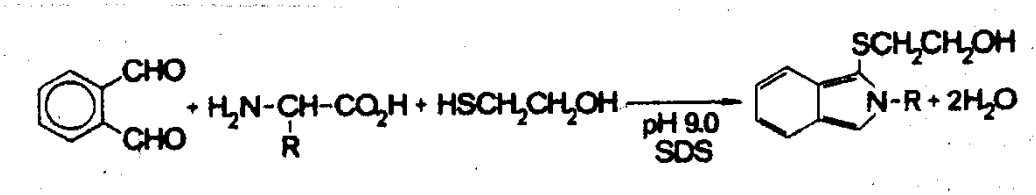
In spite of preparing starter for bigger safely to exclude the influence from external microorganisms, well cleaned Erlenmeyer's with volume of 300 ml are closed with aluminum foil and had been put on sterilization in autoclave warmed up before on 58-68 °C. Then we put the Erlenmeyer's in autoclave where the temperature is increasing to 121°C, for time of 10-15 minutes and after reaching the temperature Erlenmeyer's are leaved in autoclave for 15 minutes more. It must not to be put on milk in Erlenmeyer's before sterilization, because will become denaturizing of proteins on the high temperature. Remain part of microbiological cultures it must be kept well closed on -18°C. At next usage microbiological cultures are taking out on temperature of +4°C and are leaved on room temperature for 10-15 minutes. We weigh 0.1 g from starter culture and we put in Erlenmeyer's where is 100 ml milk. We shake Erlenmeyer's for 10-15 minutes, until whole cultures are dissolved. Then we closed with aluminum foil and we put in thermostatic dish or water bathroom for 24 hours at temperature of 37-38 °C. After that the cultures had been cold at room temperature for 15 minutes and before usage they must be kept for 24 hours on +4°C.

Treatment for yogurt preparation

In sterile Erlenmeyer's we put 200 ml milk and 2-3% starter (20-30ml), we shake 10 minutes to homogenize mixture, than incubated on 38°C or at the other temperature in dependence of starter. In time of fermentation which amount from 4 to 6 hours, in dependence from starter variety. Process of fermentation is following with pH meter; fermentation is considered for over when pH enlarge the value of 4.6. Then we put mixture to cold and mixed on low temperature, with what the fermentation is over. Product of coagulation becomes cold on 5-22°C, in dependence of kind of product. Then the products we put in refrigerator on 5°C to slow down physical, chemical and microbiological degradation. Process of fermentation was detailed kept out for cultures a, b and c. Change results in process of fermentation are presented in picture 3-5. Conditions of fermentation on the other cultures are shown in table 3-5.

OPA method for determining of total peptides

The method (Church F.C.) is based on the reaction of orto-phtaldialdehid (OPA) and β-merkaptoetanol with primary amines, which result in 1-alkiltio-2-alkilizoindol:



*Reaction on o-phtaldialdehid (OPA) with β-merkaptoetanol

In this equation R represent reactive amino acid, peptide or protein which contents primary amine. OPA adduct has strong, powerful absorption on 340 nm, which make possible spectrophotometer to determine value on amino acid's and following peptide hydrolysis .OPA method is fast, simply and

sensitive. Reaction is happen in sodium tetraborat buffer (pH 9,0) and in presence of sodiumdodecilsulfat (SDS). The main problem in this reaction during following the degree of hydrolysis on total peptides in milk and its products is solubility of protease and inactivating of proteolysis enzyme's in convenient time. With adding of 1% solution of SDS in OPA reagents, we are not only attain denaturizing to protein substrate which make possible entire accessible to amino group, but also is interrupt proteolyses activity. It had been concluded that absorption of OPA adduct is linear and is enlarging by adding of amino acids and other peptides. It is determined the value of molar absorption on α-amino group by different amino acids and peptides. (Table 2). Obviously, the nature of side row on amino acids does not influence significantly on results of absorption on OPA adducts. Also the present's of peptide link does not influence significantly on molar absorption (ε) on peptide OPA adducts. Medium value and standard deviation of ten specimens is 5992±144.

Table2. Molar absorption on o-phtaldialdehid adducts of different amino acids and peptides measured on 340nm

Amino acid or peptides	ε340 nm
Gly	5880
Ala	6090
Ser	6000
Leu	6020
Phe	5830
Glu	6120
Glu-Leu	6050
Leu-Glu	6103
Ala-Ser	6130
Glu-Leu-tyr	5700

Because of applicable on OPA spectrophotometer method for proteolysis measurement depends of measure ability on released α-amino group in present of protein, it had been measured absorption dependence to mixture of amino acids with adding β-globulin. In spite of that it had been concluded that constant adding of β-globulin does not influence at absorption linear, more exactly in present or in absent with protein linear is been protect. OPA reagent reacts also with ε-amino group (amino group that not become from amino acids), what means that this absorption it will disturb proteolysis process. The result shows that absorption of α and ε-amino adducts are similar and additive and because of irrelevant number of ε-amino adducts it not influence significantly on result's, what means that quantity released of α-amino group is not changing as a result of ε-amino adduct absorption. The advantage of OPA method is opportunity of determining number of peptide links divided in the process of hydrolysis. Proteins in milk are very distinctive, relatively easy can be prescribed the number of hydrolyzed peptide links. The number of released α-amino groups (n) can

be prescribing with next equation: $n = \frac{\Delta A_{340nm}}{\varepsilon \cdot M \cdot F}$ Where ε=6000, M-molar concentration of protein, F-factor on dissolving, ΔA340nm experimental change of absorption

Percent of hydrolysis can be calculated from amino acid structure (total number of residue) at milk protein substrate. Quantity of amino group released in the process of microbiological growing in milk can be easy followed with OPA method. Separation of low molecular proteins and peptides is obtained with centrifuge IEC Micromax RCF=20000 g. Measurement of fermentation process flow was done with pH meter Hanna instruments. Spectrophotometer determining on total peptides was done with spectrophotometer Specol 1200.

3. RESULTS AND DISCUSSION

Process of fermentation was detailed kept out for cultures a, b and c. Change results in process of fermentation are presented in figure 1-3. Conditions of fermentation on the other cultures are shown in table 3-5. Examination were deduce on main parameters that have influence on speed of enzyme reaction (temperature, pH, and time which define volume of hydrolysis).

Process of hydrolysis dependence from interactive effects between parameters with influence on hydrolysis's structure, also like milky acids bacteria that are producing proteases and peptidases from whom become enzyme hydrolysis. pH on environment was reducing from beginning on hydrolysis, so that was at the same time with another examination, where it was supposed that all of that it is result of creating a new amino and carboxyl groups. Starting from 6,7 (pH value of milk), she was reducing, and in some case attain to pH=4,0 what lead on facts that on low pH value all carboxyl groups are deprotonlyses, but small part of amino groups are proton lyses which result's with reduced consumption of protons in every peptide link. The end of the fermentation is at level under pH=4,6.

Table 3. pH on fermentation milk with Lactobacillus delbrueckii subsp. Bulgaricus and Streptococcus thermophilus.

Time (hours)	pH		
	35°C	41°C	43°C
0	6,52	6,5	6,5
1	6,5	6,45	6,45
2	6,4	6,05	6,0
3	6,25	5,5	5,2
4	5,75	4,75	4,4
5	5,45	4,5	4,3
6	5,0	4,4	4,2
7	4,75	4,3	4,15
8	4,55	4,1	4,05

Table 3. pH on fermentation milk with Lactobacillus delbrueckii subsp. Bulgaricus and Streptococcus thermophilus

9	4,45	4,1	4,0
10	4,4	4,0	4,0
11	4,35	4,0	4,0
12	4,3	4,0	4,0
13	4,25	4,0	4,0
14	4,2	4,0	4,0
15	4,15	4,0	4,0
16	4,1	4,0	4,0

On figure 1 is shown change of pH in the process of fermentation produced with system of cultures (a) on different temperatures 35°C, 41°C, and 43°C from where can be seen that on pH=4,6 (isoelectrical point on protein ground) fermentation is not over. As a result of ours examination fermentation is over on the temperature on 35°C at level on pH 4,55 for time of 8 hours; on temperature on 41 °C fermentation is over on pH=4,5 for 5 hours; and on the 43°C we think that the fermentation is over on pH=4,3 for 4 hours. Observing this examination we can conclude that on the higher temperatures, process of hydrolysis occurrence faster. Hydrolysis dependence from variety microbiological culture. In this system take part bacteria from thermophilus yogurt culture (CH1) *Lactobacillus delbrueckii subs. Bulgaricus* and *Streptococcus thermophilus* where they are acting in symbiosis. In dependence of their correlation (pillar, and cocas bacteria), and from different temperature of incubation it has been produced different quantity of milk acid. Producing yogurt with larger acidity shows and producing bigger quantity of amino acids and as a result of that larger proteolysis activity.

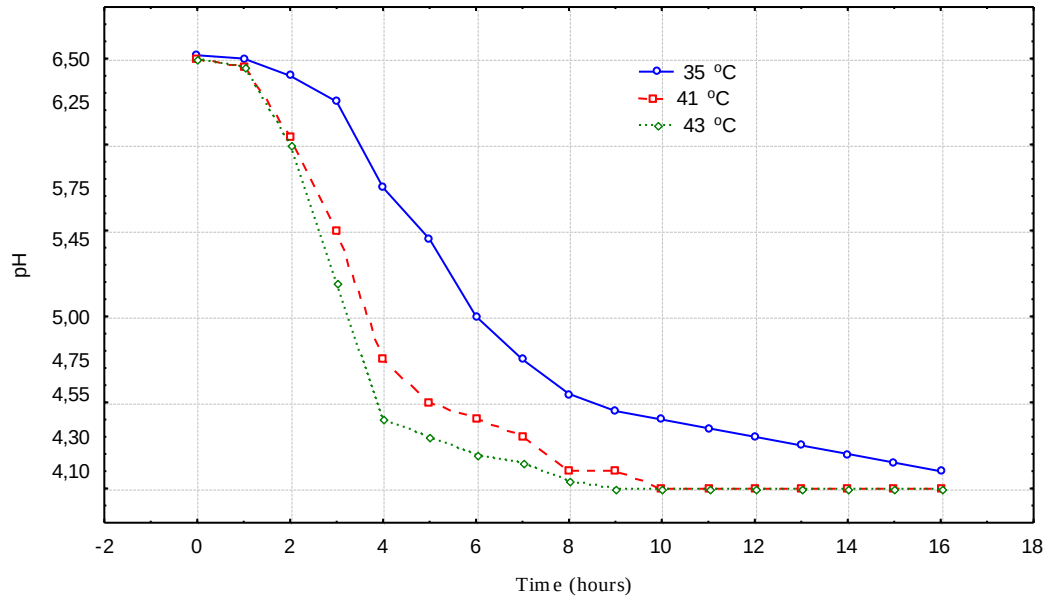


Figure 1. pH value at different temperatures with system of cultures (a) Lactobacillus delbrueckii subsp. Bulgaricus and Streptoccocus thermophilus;

Table 4. pH value on fermentation milk with Lactobacillus acidophilus, Bifidobacterium and Streptococcus thermophilus.

Time (hours)	pH		
	37°C	40°C	43°C
0	6,5	6,5	6,5
1	6,5	6,45	6,4
2	6,4	6,0	5,75
3	5,65	5,25	5,0
4	5,2	4,95	4,75
5	4,75	4,65	4,55
6	4,65	4,6	4,5
7	4,55	4,5	4,45
8	4,5	4,45	4,4
9	4,45	4,4	4,35
10	4,4	4,35	4,3
11	4,35	4,3	4,25
12	4,3	4,25	4,2
13	4,25	4,2	4,2
14	4,2	4,2	4,2
15	4,2	4,2	4,2
16	4,2	4,2	4,2

On figure 2. is shown pH change in the process of fermentation produced from system of cultures (b) at different temperatures 37 °C, 40 °C, and 43°C, where from we can see that on 37°C fermentation is overed on pH=4,5 for 5 hours, or on temperature of 40°C and 43°C fermentation is over at pH=4,65 same for 5 hours.

This hydrolysis is produced from culture system in whom except microorganism useful for producing of yogurt (ABT5), *Lactobacillus delbrueckii subsp. Bulgaricus* and *Streptoccocus thermophilus*, contain and intestinal bacteria *Bifidobacterium* whose role is in creating bioyogurt, acidophilus yogurt, kefir. *Bifidobacterium* is saharolityc bacteria which besides milk and vinegar acids produced nitrogen that is useful for bacteria like a resource of food. This system of cultures for the three temperatures gives similar pH on hydrolysis and fermentation is over for about 5 hours. This talk that the last system is more adequate for milk acid production and fast fermentation.

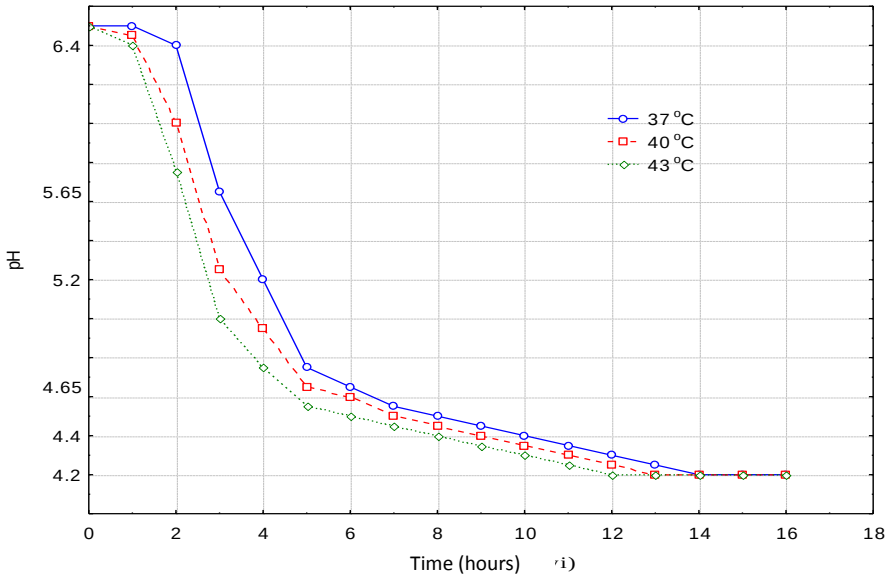


Figure 2. pH value at different temperatures with system of cultures (b) Lactobacillus acidophilus, Bifidobacterium and Streptococcus thermophilus

Table 5. pH value on fermented milk with Lactococcus lactis subsp.cremoris and Lactococcus lactis subsp. Lactis

Time (hours)	pH			
	22°C	30°C	37°C	40°C
0	6,5	6,5	6,5	6,5
1	6,5	6,48	6,46	6,45
2	6,5	6,46	6,43	6,43
3	6,44	6,43	6,4	6,4
4	6,4	6,38	6,3	6,2
5	6,35	6,25	6,18	5,7
6	6,3	5,7	5,7	5,3
7	6,28	5,5	5,3	5,0
8	6,25	5,28	4,9	4,8
9	6,1	5,15	4,75	4,7
10	5,9	5	4,6	4,6
11	5,45	4,9	4,5	4,5
12	5,25	4,8	4,4	4,45
13	5	4,76	4,35	4,4
14	4,85	4,7	4,3	4,35
15	4,55	4,5	4,3	4,3
16	4,5	4,4	4,3	4,3

In figure 3 is shown pH changes in the process of fermentation produced from system of cultures (c) on different temperatures 22°C, 30°C, 37°C and 40°C where from we can see that on 22°C and

30°C temperature fermentation is turn over at pH=5,45 for time of 12 and 8 hours, otherwise on 37°C and 40°C fermentation is turn over on pH=4,85 for 8 hours. In this process are included next bacteria *Lactococcus lactis subsp.cremoris and Lactococcus lactis subsp. lactis(*R704) which belong to mesophilus culture and mostly are used for production of hard cheese (cheddar). Best process of hydrolysis we have on temperature of 22°C, and 30 °C, at pH=5,45, which pH is appropriate to optimal temperature for growing to this bacteria in range at pH(5,3-5,6). We can conclude that this tip of bacteria gave to us the best enzyme hydrolysis at low temperature, and then we have faster growing of microorganism but for longer time for 12 hours. These changes on pH are result from different system of microbiological cultures, from producing milk acid in the hydrolysis on milk sugar, and from variety and quantity of proteasys and peptidasis who produced amino acids. In dependence of kind of microbiological cultures we watched temperature at which they attained their optimal growing, and as a result of that time what we needed for production of whole enzyme hydrolysis. Milky acid fermentation was happen at pH, temperature and time of incubation adequate to purpose given from producer.

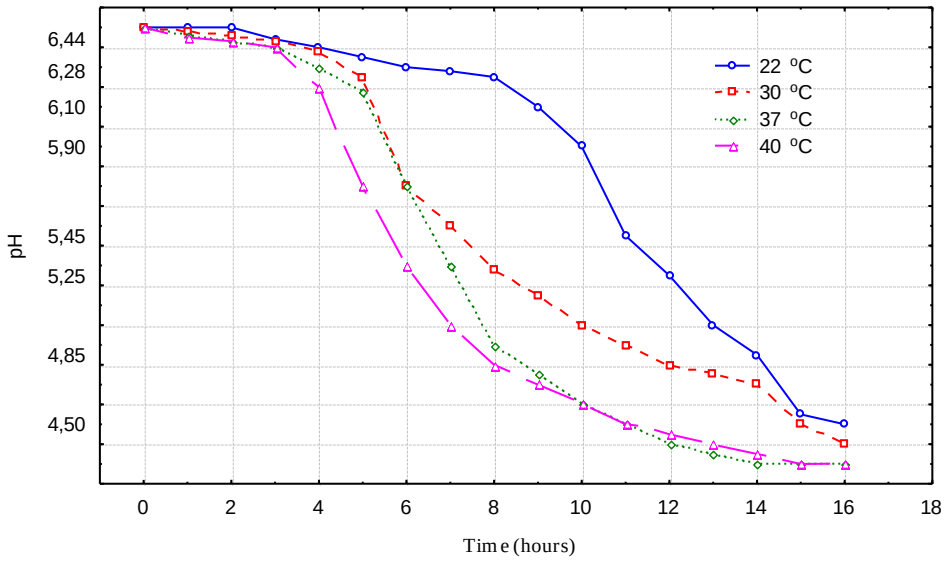


Figure 3. pH value at different temperatures with system of cultures (c) Lactococcus lactis subsp.cremoris and Lactococcus lactis subsp. Lactis.

Table 6. Conditions for Lactic-acid fermentation to mixture of cultures from production by Chr. Hansen's Laboratories

System microbiological culture	Temperature (°C)	Time of incubation (hours)	pH
a	38	4-6	4,6
b	38	5	4,6
c	22-30	6	5,3-5,6

Our examination were not compatibility with setup conditions. We have small variety on temperature and time on hydrolyses.

Table 7. Results for peptides in laboratory prepared jogurt with 3 system microbiologica culture

Combination of microbiological culture	Type of milk	Total peptides (micro mol)	pH
a	cow's milk	21,24	4,6
b	cow's milk	35,17	4,6
c	cow's milk	30,40	5,3-5,8
a	goat's milk	23,37	4,6
b	goat's milk	9,87	4,6
c	goat's milk	9,65	5,3-5,8
a	sheep's milk	11,25	4,6
b	sheep's milk	10,04	4,6
c	sheep's milk	15,12	5,3-5,8

4. CONCLUSIONS

- Determinated pH at process hydrolysis is one of important conditions to accurate measurement on hydrolisates such as peptides.
- Use microbiological cultures: *Lactobacillus delbrueckii subsp. Bulgaricus* and *Streptococcus thermophilus; Lactobacillus acidophilus, Bifidobacterium and Streptococcus thermophilus; Lactococcus lactis subsp.cremoris and Lactococcus lactis subsp. Lactis*, production by Chr. Hansen's Laboratories, in same milk derivation different amount of peptides on same temperature.
- Optimum on peptides with first system culture are on the 43°C we think that the fermentation is over on pH= 4,6 for 4 hours.
- Optimum on peptides with second system culture are on 38°C fermentation is overed on pH= 4,6 for 5 hours.
- Optimum on peptides with second system culture are on 22°C fermentation is overed on pH= 4,6 for longer time12 hours.
- Peptides by cow's milk is max.30,40 :mol at pH=4,6 with b culture
- Peptides by goat's milk is max.23,37 :mol at pH=4,6 with a culture.
- Peptides by sheep's milk is max.15,12 :mol at pH=5,3-5,8 with c culture.

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