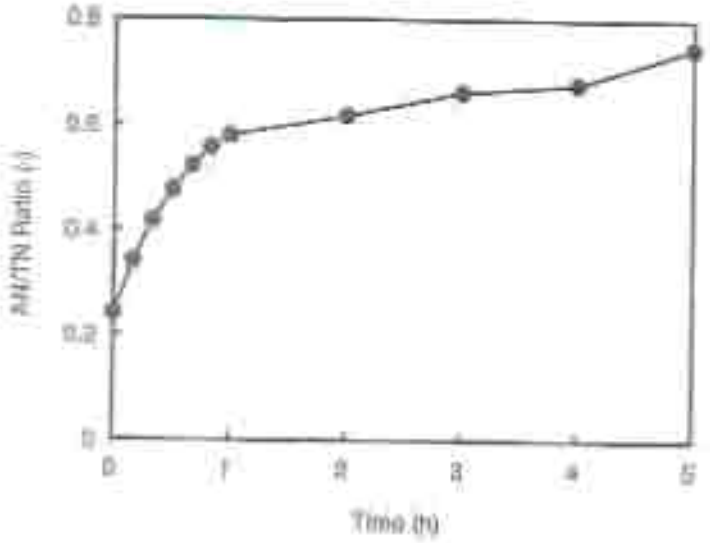


Fig. 12a. Time course of AN/TN ratio in digestion of fish soluble with HCl.

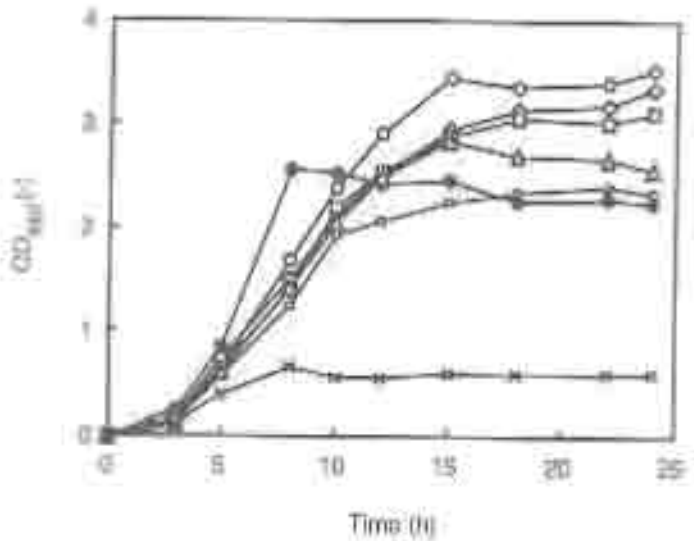
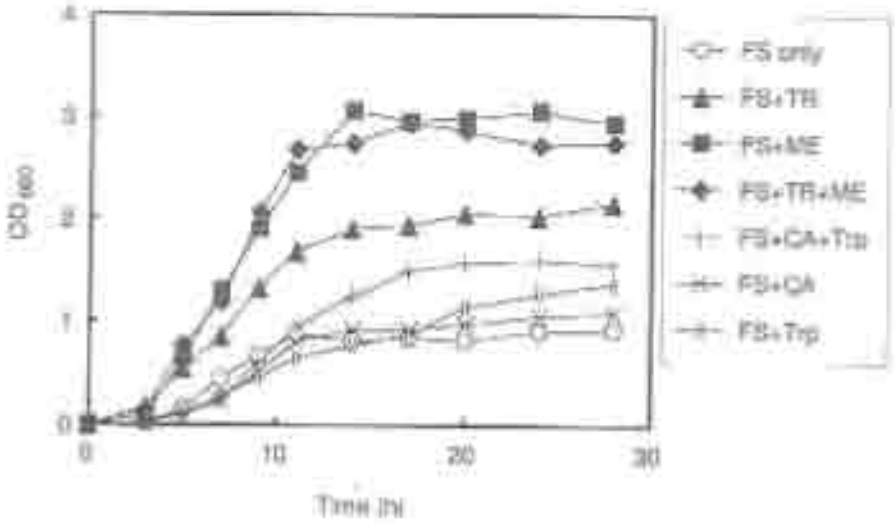


Fig. 12b. Growth of *L. acidophilus* in fish soluble digested for various time. Symbols: ●, 2% (w/v) yeast extract; ×, non-digested fish soluble; ◇ ; ○ ; □ ; △ ; ▫ ; fish soluble digested at 10 min., 30 min., 1 h., 3h. and 6 h respectively. All medium was adjusted to have equal total nitrogen content.



Note: The medium in all experiment was prepared at equal total nitrogen.
Abbreviations; FS:Fish solubile; TR:Tryptone; ME:Meat extract; CA:casamino acids; Trp:Tryptophan.

Fig. 13. Comparison of growth of *L. acidophilus* using fish solubile as a sole protein source and with supplement of various sources of protein.

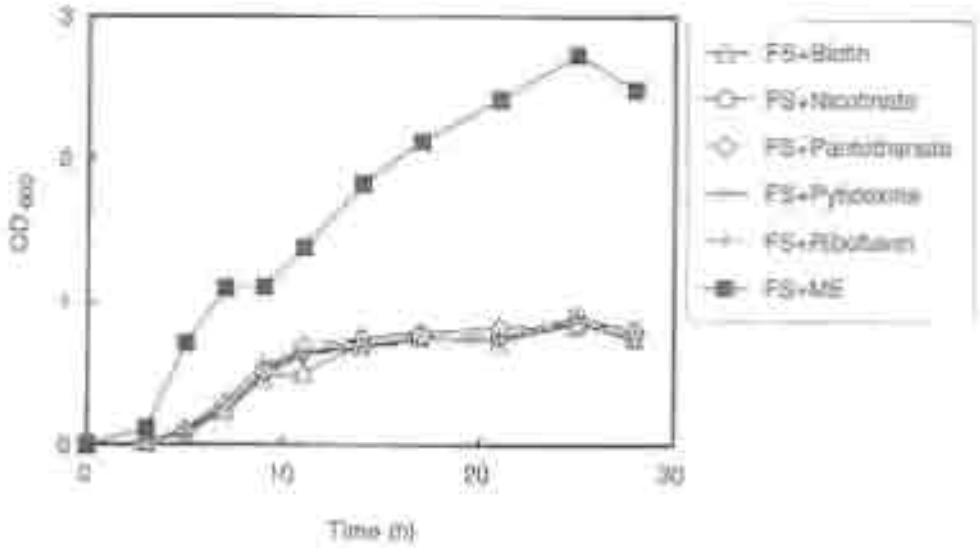


Fig. 14. Effect of vitamins addition on growth of *L. acidophilus*.

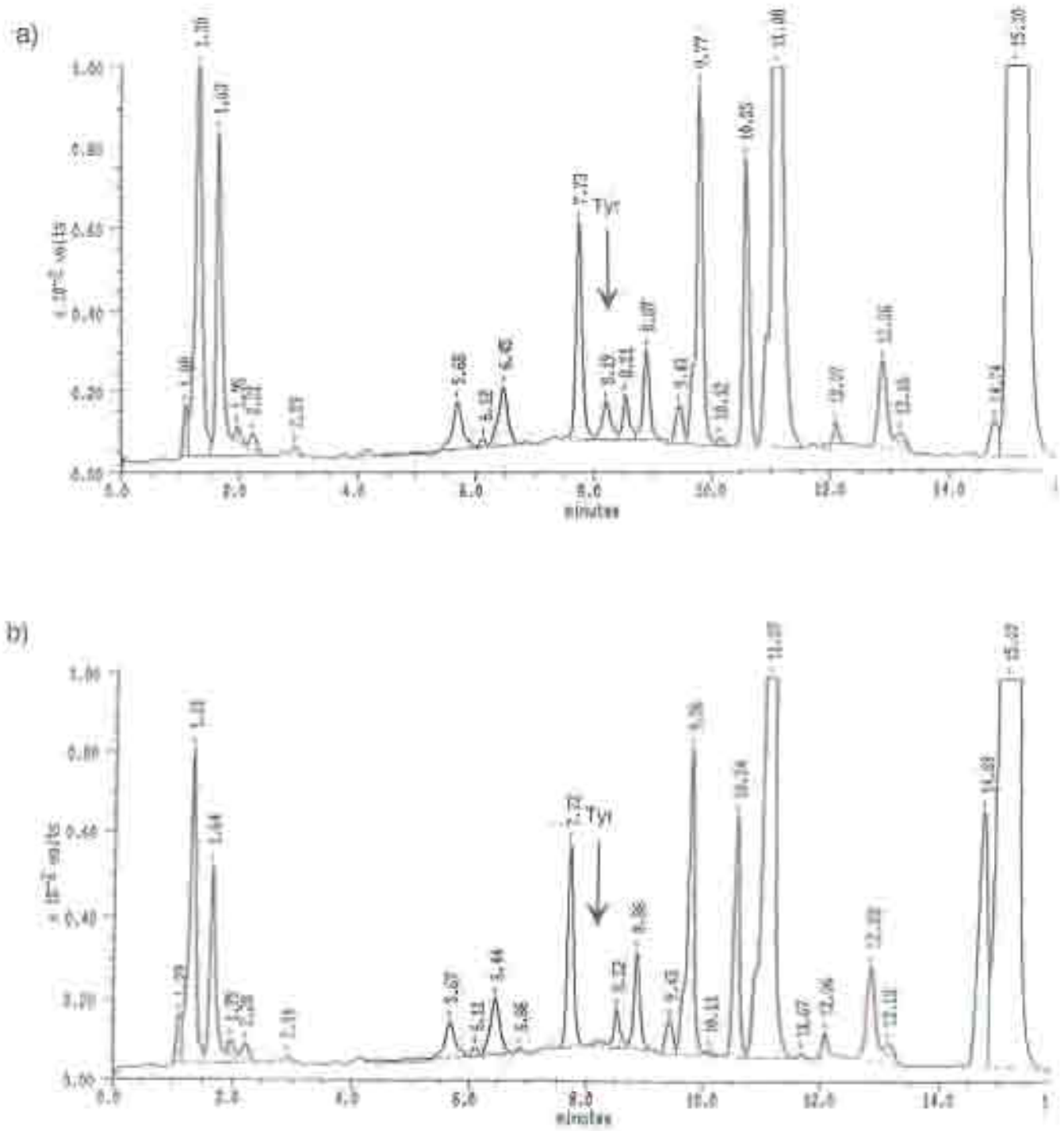


Fig. 15. Analysis of amino acids content in broth during growth of *L. acidophilus* at a)0 hr and b) 8hr.

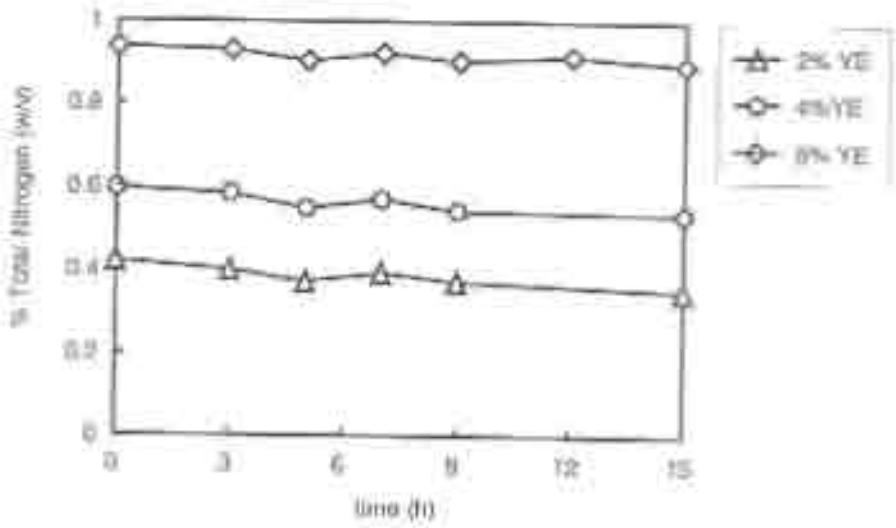


Fig. 16. Time course of total nitrogen remained in supernatant of culture broth using yeast extract at different concentration as complex carbon source instead of glucose.

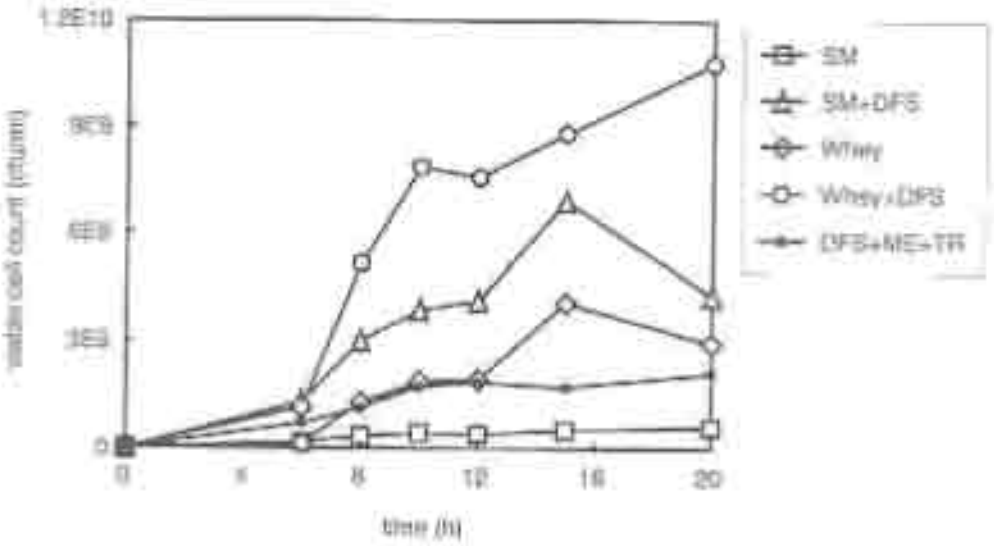


Fig. 17. Growth of *L. acidophilus* on various sources of milk base protein. The protein content of medium in all experiment was adjusted to be equivalent in total nitrogen (TN = 0.46 %)

Abbreviation: SM, skim milk; DFS, digested fish solution; ME, meat extract; TR, tryptone

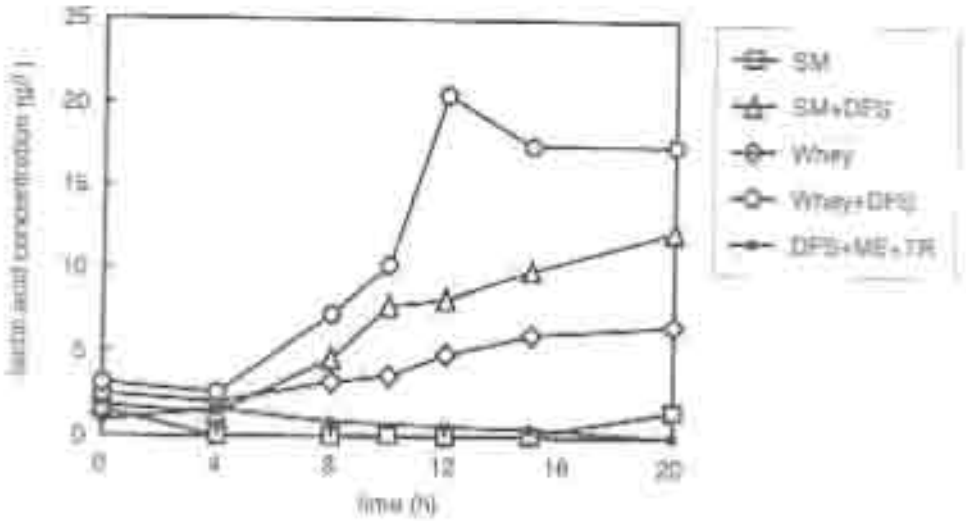


Fig. 18 Lactic acid formation of *L. acidophilus* cultured on various sources of milk base protein. Calcium carbonate was added to buffer the system in order to reduce the inhibitory affect of lactic acid.

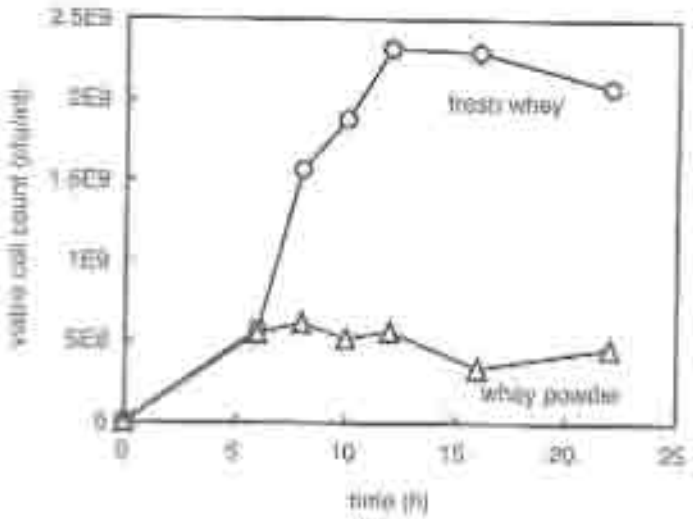


Fig. 19. Comparison of growth of L. acidophilus on medium contained fresh whey and whey powder as a protein source. Total nitrogen content for both medium was adjusted to be equivalent.

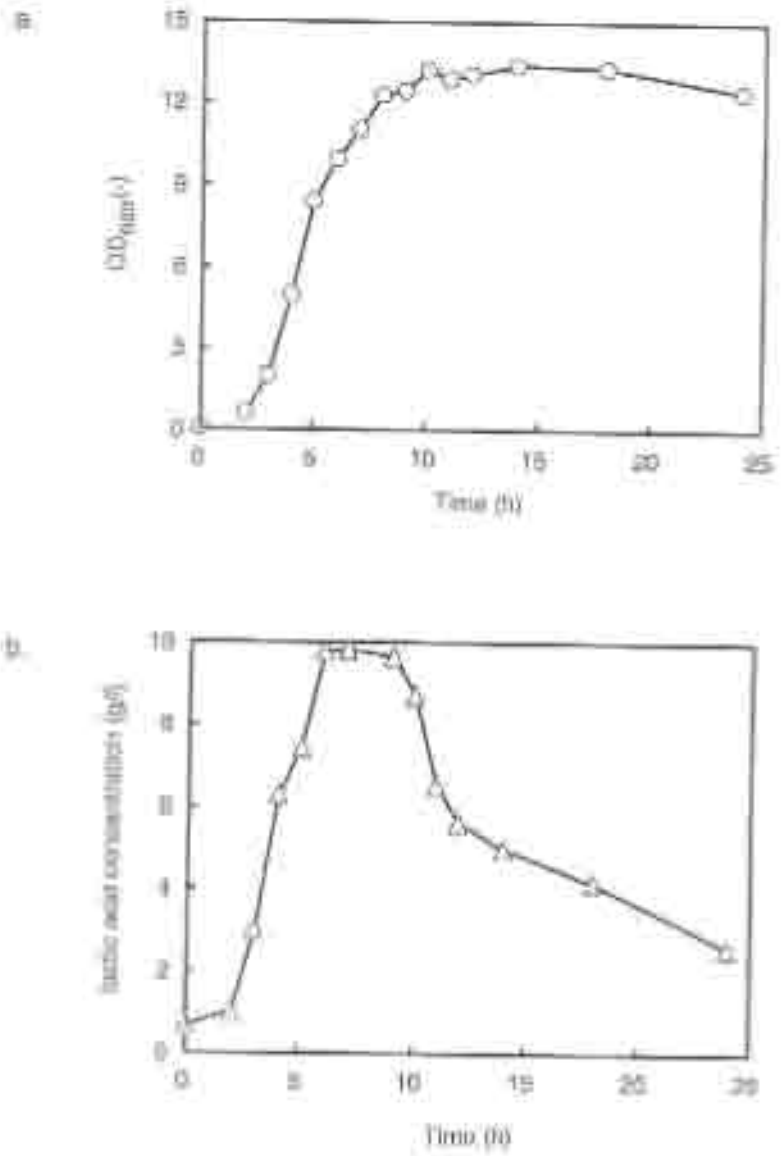


Fig. 20. Batch cultivation of *L. acidophilus* under pH control using digested fish soluble protein in modified MRS medium. a) growth, b) lactic acid formation.

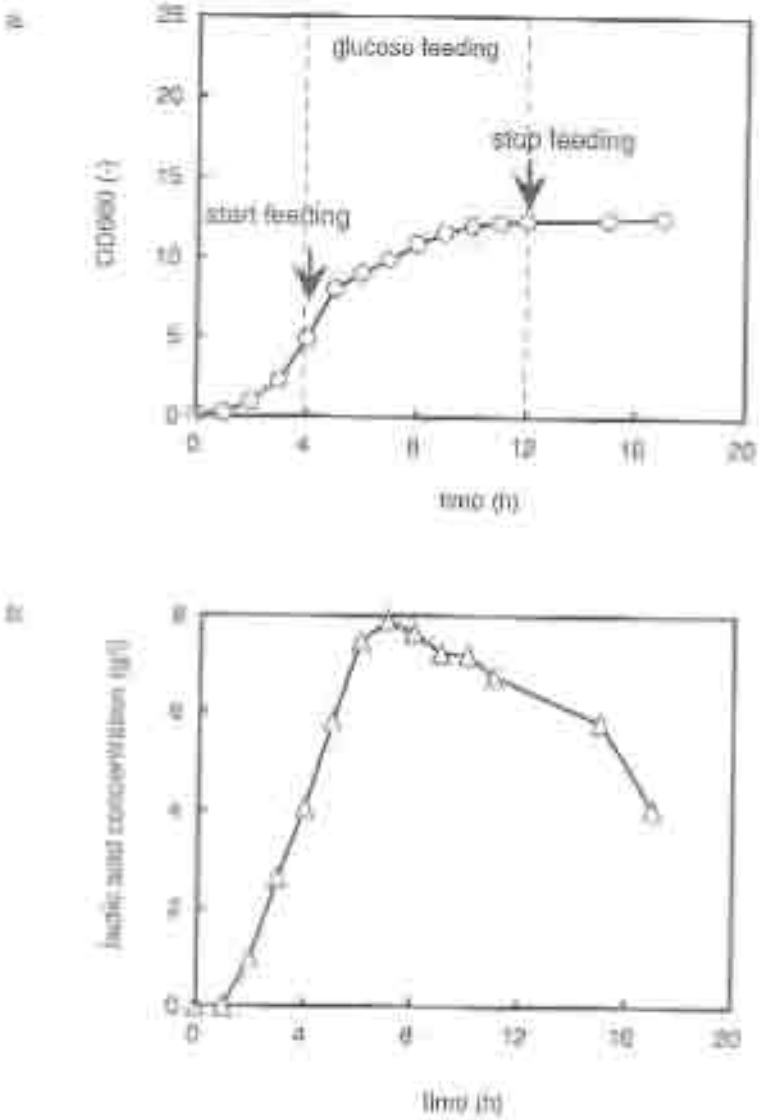
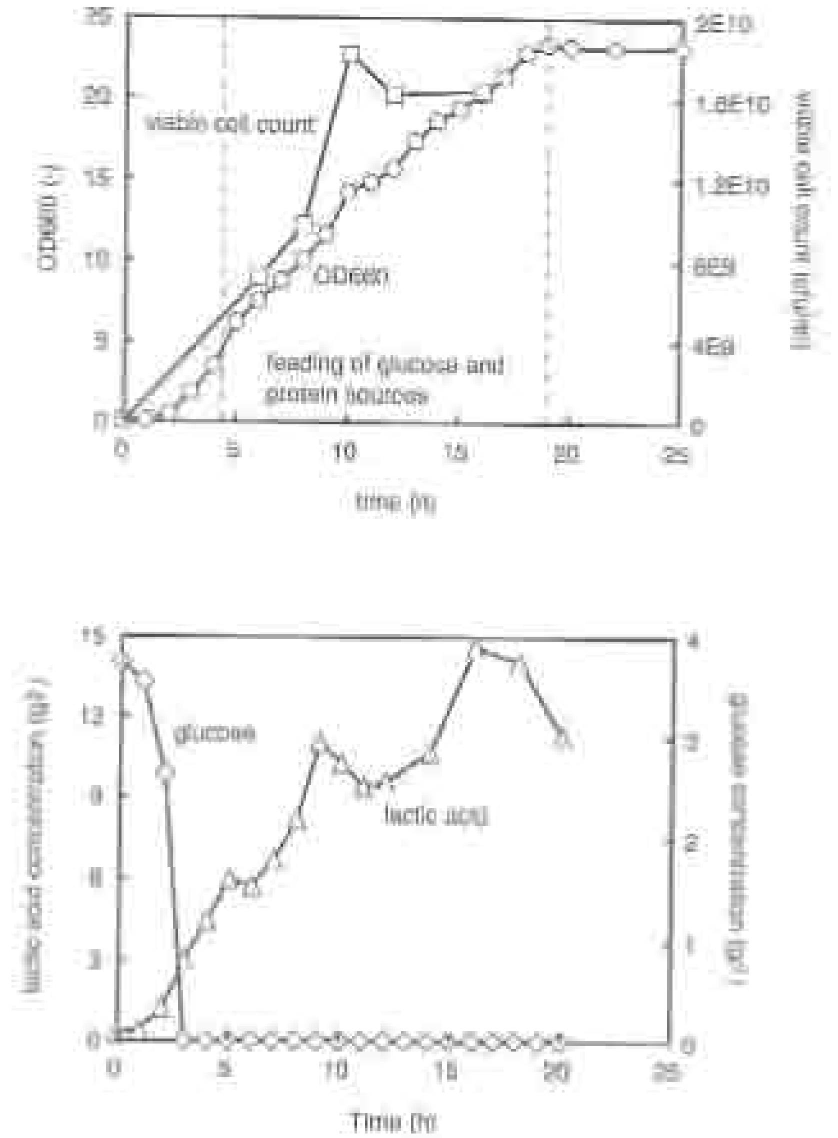


Fig. 21. Fed-batch cultivation of *L.acidophilus* by constant feed rate of glucose. a) cell density, b) lactic acid formation.

a.



b.

Fig. 22. Fed-batch cultivation by constant feeding of glucose and proteins. a) cell density and viable cell count. b) lactic acid formation and glucose concentration.

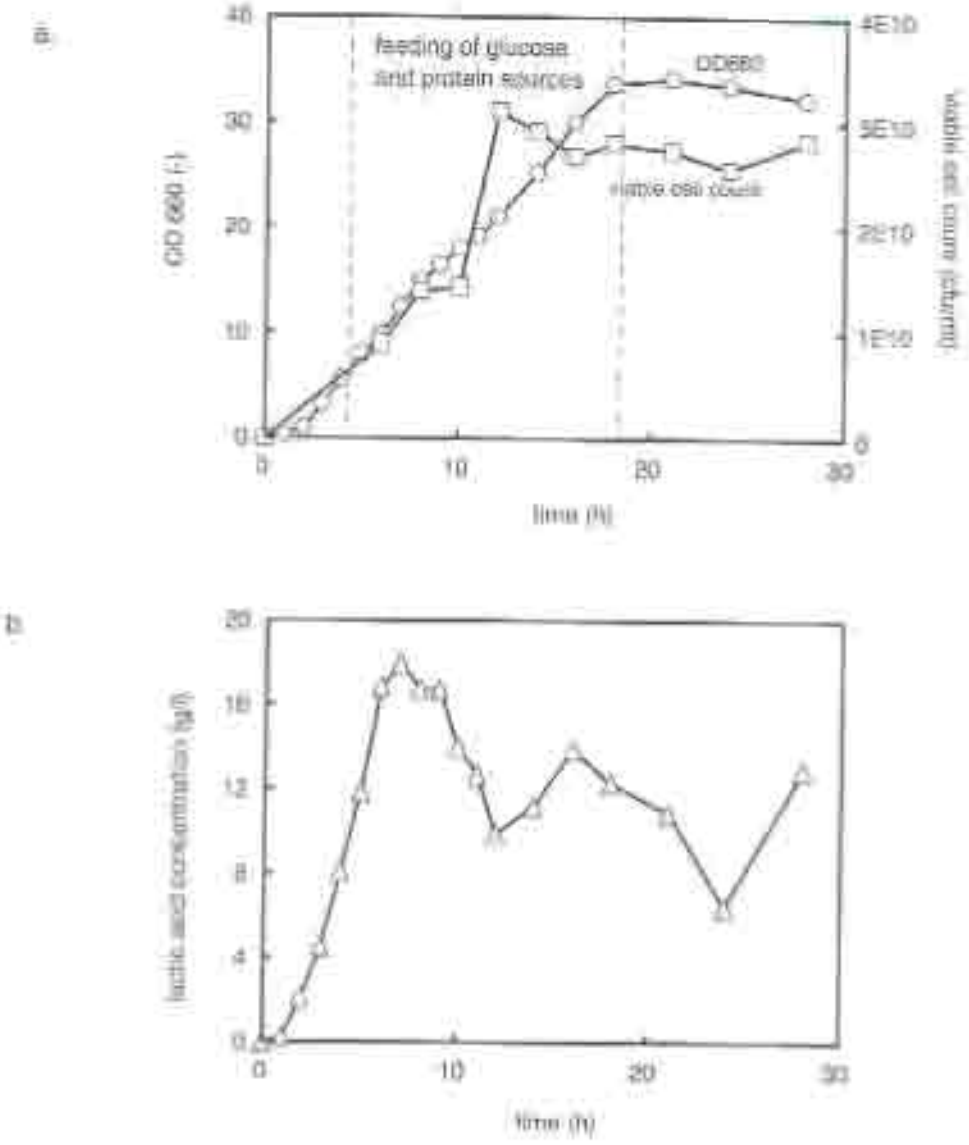


Fig. 23. Fed-batch cultivation by exponential feeding of glucose and proteins. a) cell density and viable cell count, b) lactic acid formation.

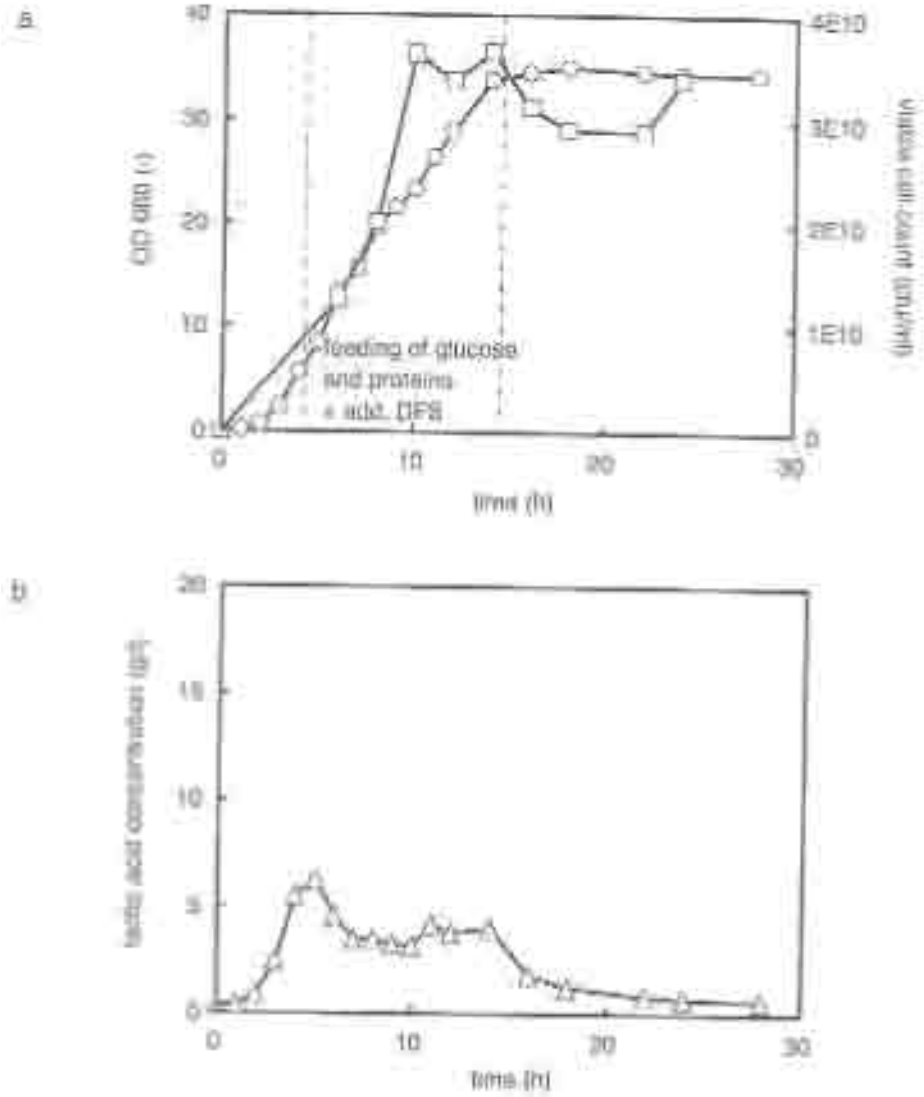


Fig. 24: Fed-batch cultivation by exponential feeding of glucose and proteins with additional DFS. a) cell density and viable cell count, b) lactic acid formation.

Note: Fermentor 4
0.1 N HCl digested DFS

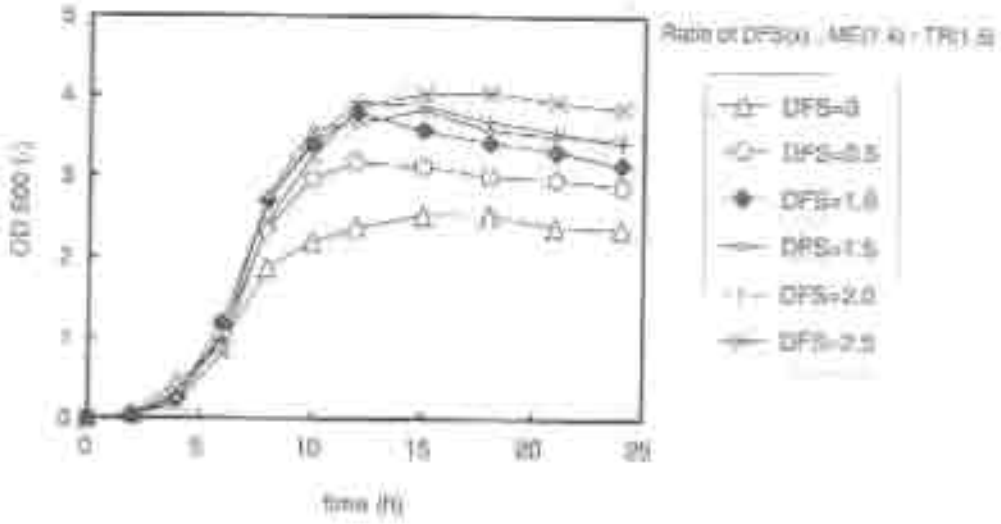
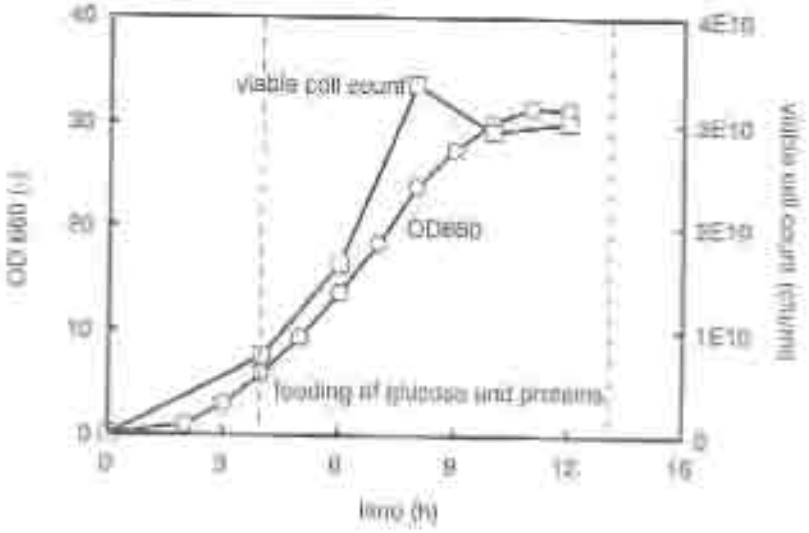


Fig. 25. Optimal ratio of DFS:ME:TR for optimal growth of *L. acidophilus*. 2% of glucose was supplemented for C-source.
Abbreviations: DFS, digested fish soluble; ME, meat extract; TR, tryptone.

Note: SN HCl digested DFS

a.



b.

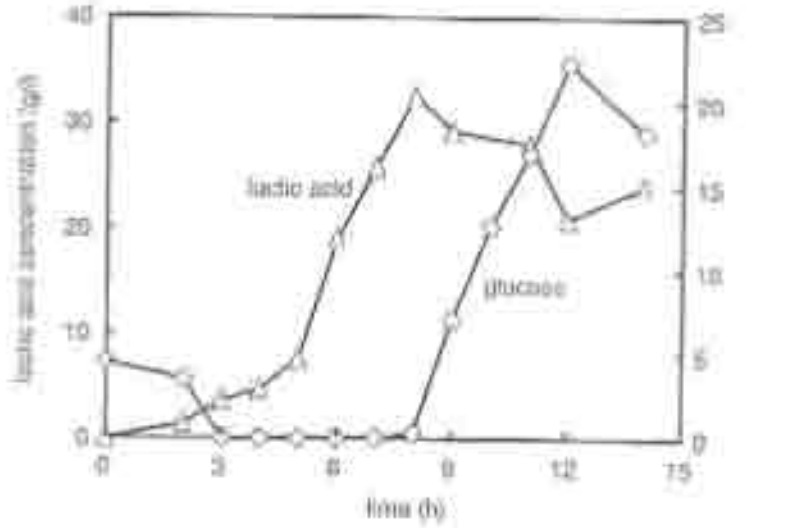
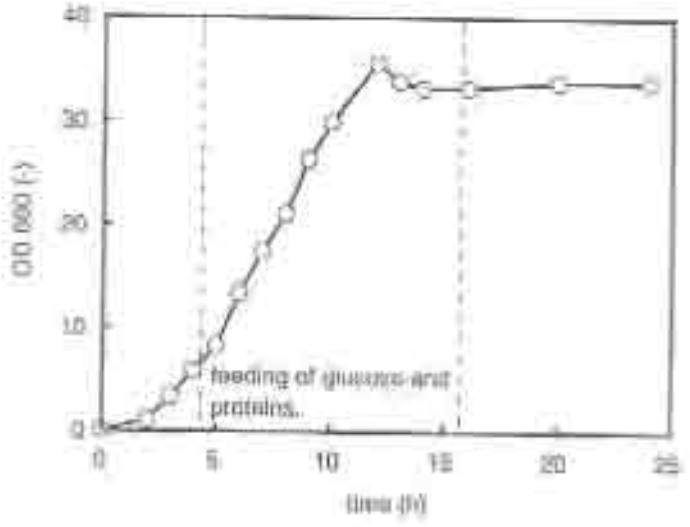


Fig. 26. Fed-batch cultivation by exponential feeding at high feed rate of glucose and proteins. a) cell density and viable cell count. b) lactic acid formation and glucose concentration.

a



b

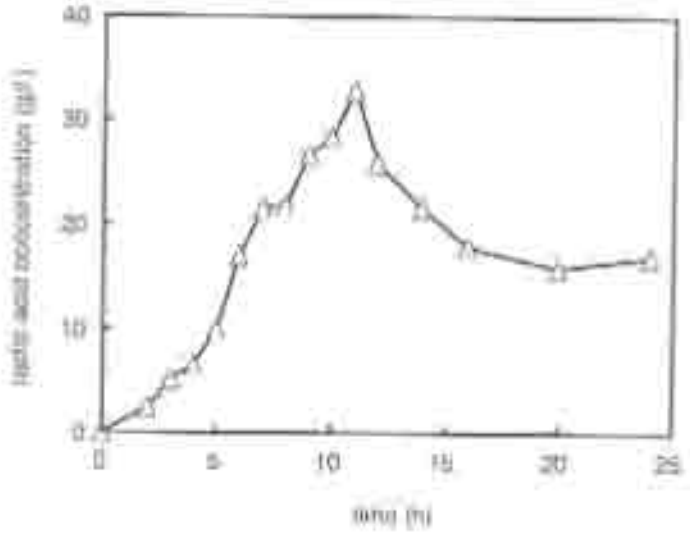
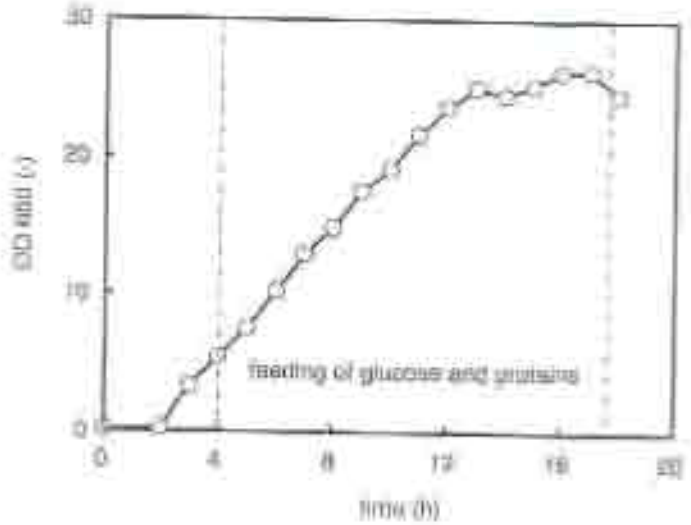


Fig. 27. Fed-batch cultivation at medium range of feeding rate. a) cell density, b) lactic acid formation.

a



b

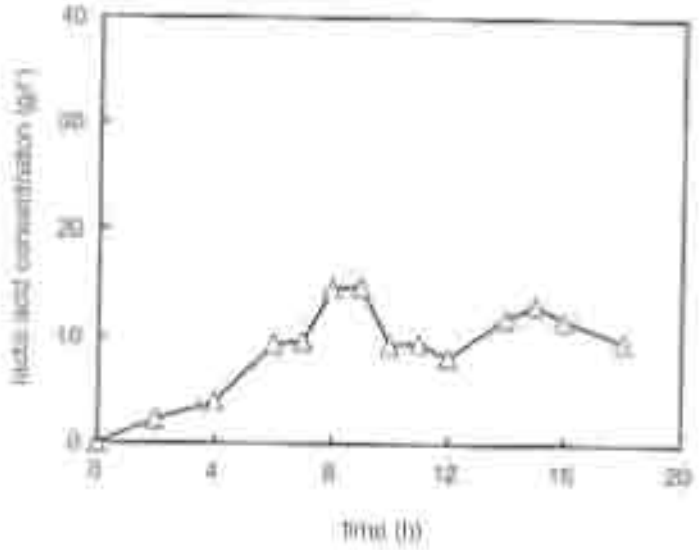


Fig. 28. Fed-batch cultivation using low range of feeding rate. a) cell density. b) lactic acid formation.

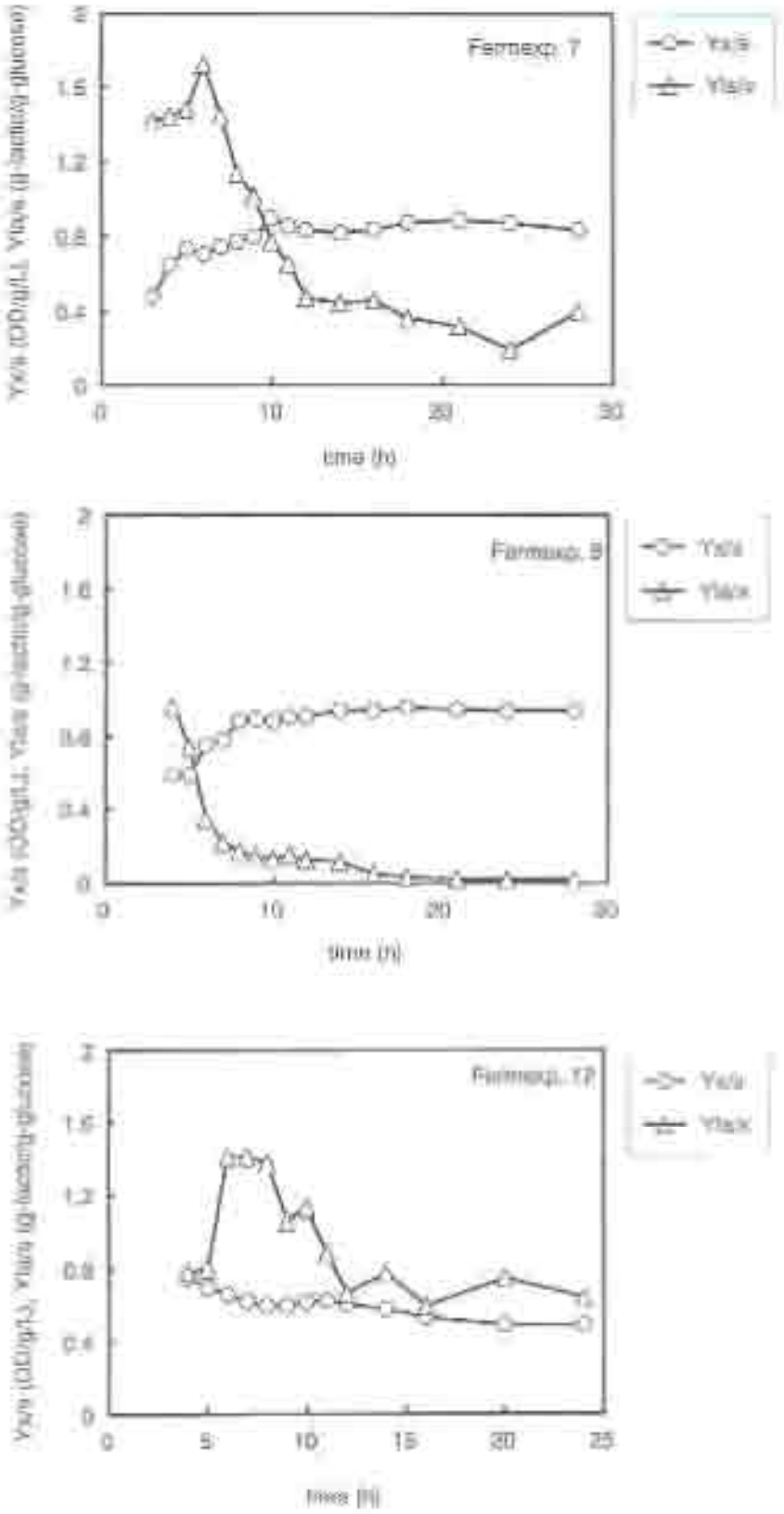
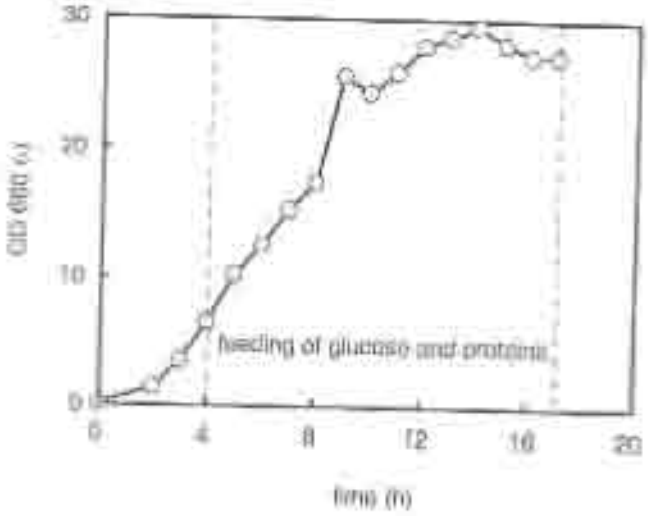


Fig. 29. Time course of growth yield (Yx/s) and lactic acid production yield (Yla/s) of various experiments.

a)



b)

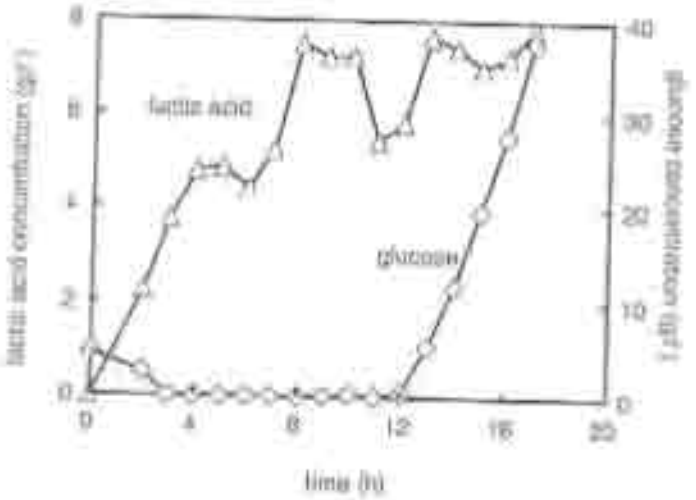
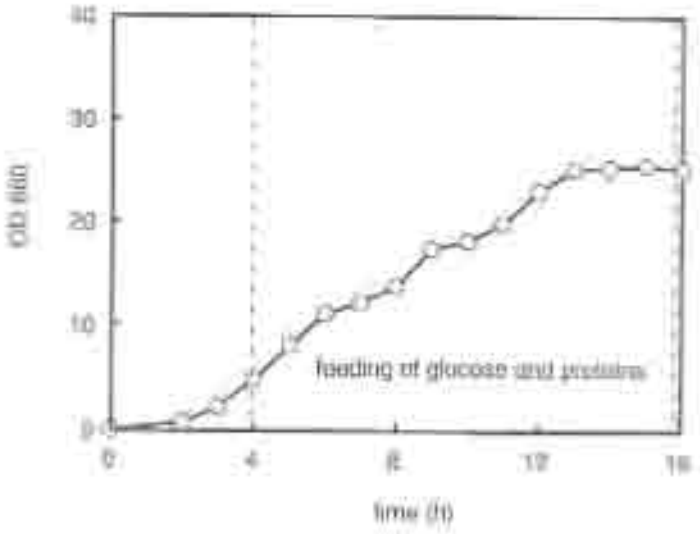


Fig. 30. Fed-batch cultivation by exponential feeding a) cell density, b)lactic acid formation and glucose concentration.

a



b

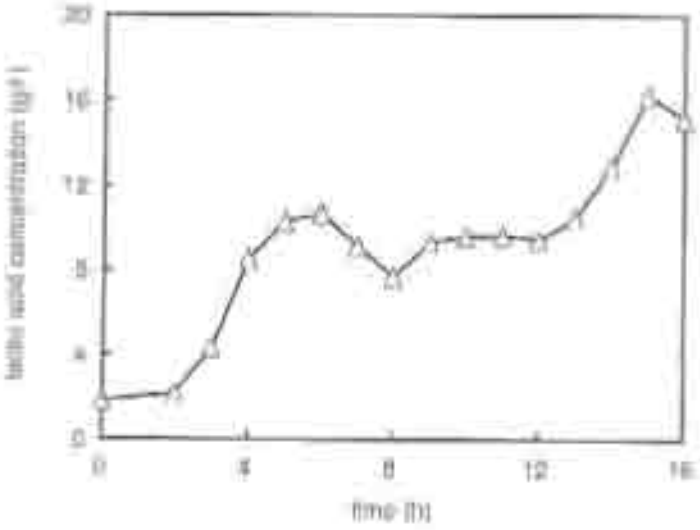


Fig. 31. Fed-batch cultivation by exponential feeding
a) cell density, b) lactic acid formation

Note: Fermentor, 1l
3N HCl, adjusted pH

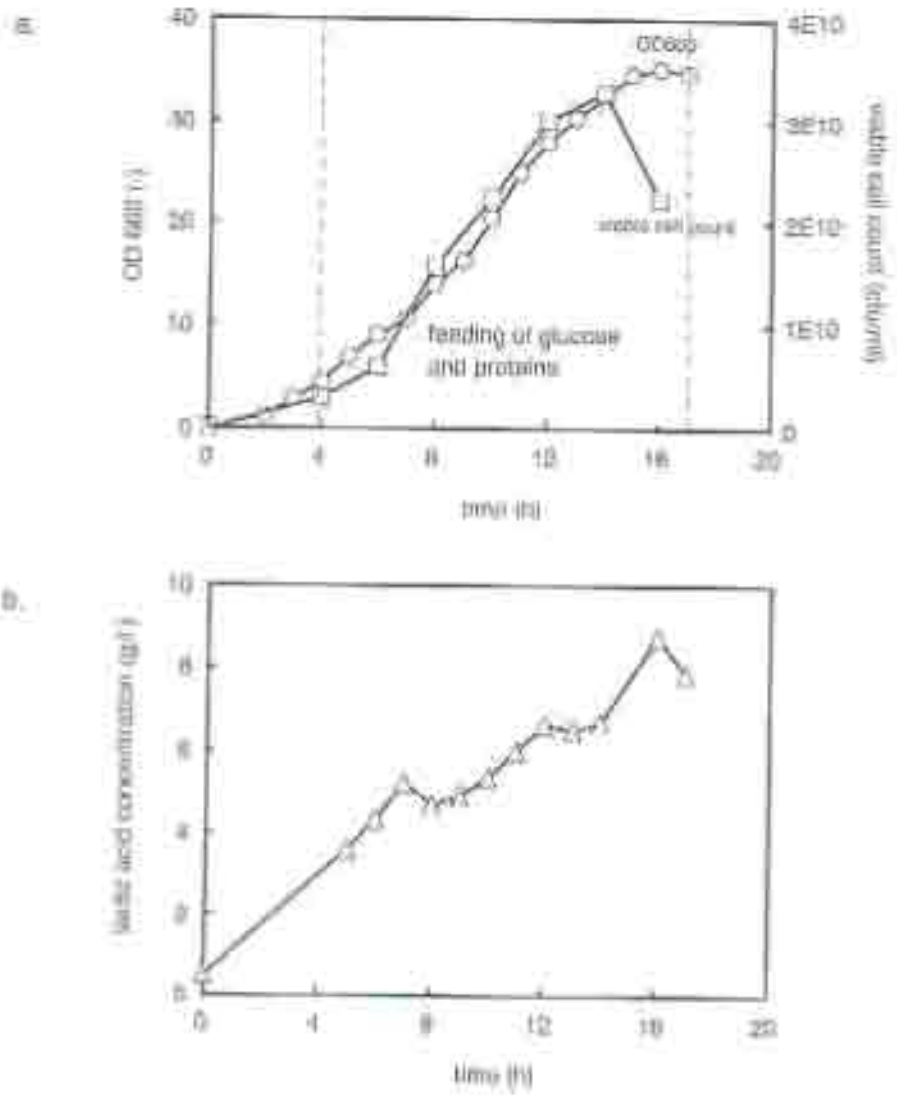


Fig. 32. Fed-batch cultivation by exponential feeding.
The DFS content in feeding protein sources was reduced by 1/2 and sterilized at 110 °C, 15 min.
a) cell density and viable cell count. b) lactic acid formation.

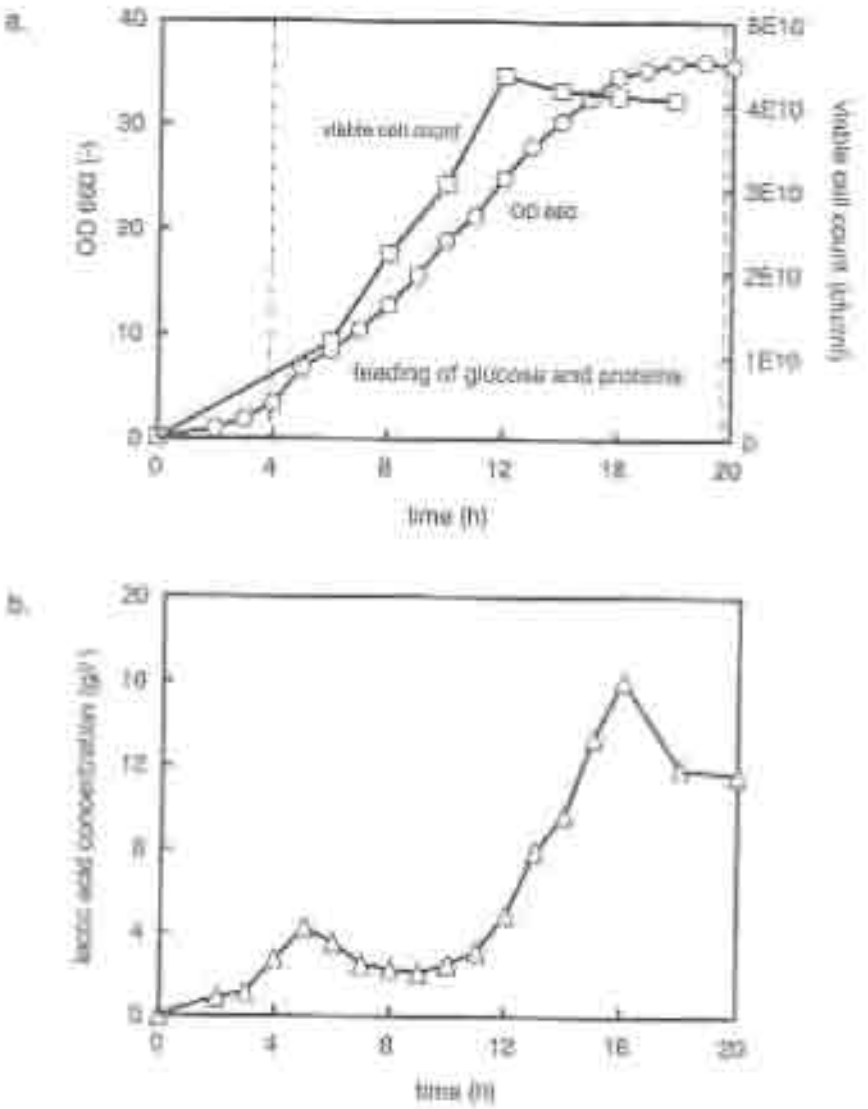


Fig. 33. Fed-batch cultivation by exponential feeding. The component of meat extract and tryptone in feeding proteins were reduced by half and separately sterilized at 121°C, 10 min.

7.ผลงานที่ได้จากการดำเนินงานตลอดโครงการวิจัย

7.1 การตีพิมพ์ผลงานทางวิชาการ

จากผลงานการศึกษาสามารถเขียนเป็นผลงานทางวิชาการเพื่อลงในวารสารวิชาการนานาชาติ ได้ 2 เรื่องตาม manuscript ในภาคผนวก คือ

1. Utilization of fish soluble protein for cell production of Lactobacillus acidophilus for probiotics use

อยู่ระหว่างการนำเสนอต่อวารสาร World Journal of Microbiology & Biotechnology เพื่อพิจารณาตีพิมพ์

2. Application of fed-batch technique for high cell density cultivation of lactobacillus acidophilus

กำลังจัดเตรียมเพื่อนำเสนอต่อวารสาร Biotechnology letter หรือ Applied microbiology and Biotechnology

7.2 การนำเสนอผลงานทางวิชาการในการประชุมวิชาการ

ได้มีการนำเสนอผลงานทางวิชาการในการประชุมวิชาการนานาชาติในรูปแบบ Poster (พร้อม Abstract ที่ตีพิมพ์ในภาคผนวก) คือ

1. " Cultivation of High Cell Density of Lactic Acid Bacteria for Probiotics" นำเสนอที่การประชุม International Conference on Asian Network on Microbial Research ระหว่างวันที่ 23 - 25 กุมภาพันธ์ พ.ศ. 2541 ณ เมือง Yogyakarta ประเทศอินโดนีเซีย

2. " Utilization of complex proteinaous sources for high cell density cultivation of *Lactobacillus acidophilus*"

นำเสนอที่การประชุม "The 5th Asia-pacific Biochemical Engineering Conference 1999 and The 11th Annual Meeting of Thai Society for Biotechnology" ระหว่างวันที่ 15-18 พฤศจิกายน พ.ศ. 2542 จังหวัดภูเก็ต

3. " Process Development for High Cell Density Cultivation of *Lactobacillus acidophillus* for Probiotics" นำเสนอที่ประชุม " International Conference on Asian Network on Microbial Researches" ระหว่างวันที่

29 พฤศจิกายน -1 ธันวาคม พ.ศ. 2542 จังหวัดเชียงใหม่

7.3 การผลิตบุคลากร

ได้มีนักศึกษาในภาควิชาเทคโนโลยีชีวภาพ คณะวิทยาศาสตร์ มหาวิทยาลัยมหิดล ทั้งระดับปริญญาตรีและโท เข้าร่วมภายใต้การดูแลในโครงการวิจัย ดังต่อไปนี้

ระดับปริญญาตรี : น.ส.ชนาใจ คลื่นเพียร สำเร็จการศึกษาปี 2540 ภายใต้หัวข้อโครงงานปัญหาพิเศษ "Lactic acid bacteria production for probiotics ; medium optimization"

ระดับปริญญาโท : น.ส.สุกัญญา แซ่เฮี้ยว จะสำเร็จการศึกษาปี 2542 ภายใต้หัวข้อวิทยานิพนธ์ "Lactic acid bacteria production for probiotics : medium and process optimization"

7.4 ความร่วมมือกับองค์กรอื่นทั้งภาครัฐและเอกชนในการเผยแพร่เทคโนโลยีทางด้านการผลิตเชื้อจุลินทรีย์สำหรับ Probiotics

ได้มีโครงการความร่วมมือกันหน่วยงานต่างๆเพื่อประยุกต์ใช้เทคโนโลยีที่พัฒนาขึ้นสำหรับการผลิตเชื้อจุลินทรีย์ในกลุ่ม lactic acid bacteria รวมถึงต่างๆที่สามารถใช้เป็น Probiotics ได้แก่

- โครงการ "การเพิ่มปริมาณชีวมวลของจุลินทรีย์ *Pediococcus pentosaceus* โดยการปรับปรุงอุดมอาหารเลี้ยงและพัฒนากระบวนการหมัก" ดำเนินการร่วมกับ ดร.พงศ์สุดา ม่องยัญญา ศูนย์พันธุวิศวกรรมและเทคโนโลยีชีวภาพแห่งชาติ

-โครงการ " งานวิจัยและพัฒนาการผลิต probiotics และ enzyme phytase เพื่อใช้เสริมในอาหารไก่ไข่" ดำเนินการภายใต้ทุนอุดหนุนการวิจัยจากมหาวิทยาลัยมหิดล โดยมีศาสตราจารย์ ดร.วิทยา มีวุฒิสม เป็นหัวหน้าโครงการ

ทางด้านการผลิตเชื้อจุลินทรีย์กรด lactic สำหรับเป็นหัวเชื้อในอุตสาหกรรม

- โครงการวิจัยและพัฒนาร่วมเพื่อศึกษาความเป็นไปได้ในการพัฒนาการผลิตหัวเชื้อจุลินทรีย์กรด lactic เพื่อใช้เป็นหัวเชื้อในการผลิตนมเปรี้ยว โดยความร่วมมือกับบริษัทในเครือเมกะ

ภาคผนวก

Utilization of fish soluble protein for cell production of *Lactobacillus acidophilus* for probiotics use

Somchai Chauvatcharin, Sukunya Oaew, Donangjai Kanungpean and Amaret Bhumiratana.

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With the aim of developing lactic acid bacteria production process, approaches of cell physiological control have been investigated to reduce the production of lactic acid, a growth inhibitor for cell production. It has been found that using protein from complex media instead of glucose in cultivation of *L. acidophilus* at aerobic conditions, resulted in fast growth and high cell density (> $1.0x10^{9}$ cfu/ml), while the lactic acid production could be reduced significantly. Fish soluble protein as cheap alternative source was recommended to replace the yeast extract for use as a complex media in the cultivation. It has been shown that the digestion of fish solubles by acid to achieve the optimal peptide size could improve the yield of *L. acidophilus*. However a growth inhibitory effect of salt, a by product produced during the digestion process occurred at salt concentration more than 1%(w/v).

Key words: [lactobacillus acidophilus, fish meal soluble, medium development, protein digestion]

Introduction

Lactic acid bacteria (LAB), where most of the strains are generally recognized as safe (GRAS), have played important roles in production of many fermented food products. LAB has also been widely used as one of the components in formulation of "probiotics", a live microbial feed supplement which beneficially affects the host animal by improving its intestinal microbial balance (1). The utilization of lactic acid bacteria in many products has led to requirements to develop an efficient and high productivity cultivation technique to produce active LABs as starter cultures or as products themselves. For the production of lactic acid bacteria, the major problem that causes low cell productivity is the growth inhibitory effect of the metabolic product, lactic acid (2-5). In order to improve the cell yield and concentration, two main approaches can be considered, one is utilizing extractive methods such as electrodialysis (6), ion-exchange adsorption (7) or solvent extraction (8) to remove the lactic acid continuously to a level that causes less effect on the cell growth. Another approach which has not often been mentioned is the physiological control of lactic acid metabolism to decrease its production. In this paper, *Lactobacillus acidophilus* strain which is commonly utilized for probiotics use (1) was studied. We investigated the approaches to reduce the lactic acid production by controlling of environmental conditions *i.e.* aeration and the development of medium which utilizes protein as an alternative complex carbon source for culturing the *L. acidophilus* at high cell density. Fish meal soluble as cheap proteineous sources was studied for their potential to be used as culture medium for industrial use. The modification of protein source was further investigated in order to improve the growth yield of *L. acidophilus*.

Materials and methods

Media and cultivation methods

Lactobacillus acidophilus was isolated from pig feces and identified according to Bergey's Manual of Systematic Bacteriology. For preculture, the strain was cultivated in the De Man, Rogosa, Sharpe (MRS) medium (Merck, Germany) at 37 °C for 12 h and then preserved in 30% glycerol and used as stock culture. When various medium formulations were investigated for their effects on bacterial growth and lactic acid production, MRS medium and its modified form in which glucose in the formulation was replaced by various carbon sources *i.e.* yeast extract, glycerol, molasses and fish meal soluble (obtained from a tuna canning factory, TC Union, Bangkok, Thailand). The fish meal soluble was either used as obtained from the factory or pre-digested with acid. The acid digested fish meal soluble was prepared as follows. The concentrated fish meal paste with total solid about 30% was mixed with 5N HCl at ratio of 1:1, the mixture was then heated up to 200 °C (the digestion time was started when the mixture was heated up) by a hot plate magnetic stirrer (Snijders Inc., the Netherlands). The digestion mixture was continuously stirred by magnetic bar to maintain homogeneity. After the required period of digestion, the digested product was neutralized to pH 7.0 using 5 N NaOH solution. The remained residual in finished products was removed by centrifugation at 6,000 rpm. The supernatant obtained was utilized as protein source for cultivation of *L. acidophilus*.

The organism was grown in shake flask at 200 rpm, 37 °C.

Analyses

Cell growth was estimated by both total plate count using MRS agar medium and spectrophotometric method measuring the OD at 660 nm (OD_{660}). ***Residual sugar*** was determined using the phenol-sulfuric method (9) and ***glucose*** was analyzed enzymatically by glucose assay kit purchased from Wako Pure Chemicals Co. Ltd., Osaka, Japan. ***Volatile acids***, acetic acid and lactic acid, were determined by gas chromatography using 80/120 Carbopack™ B-DA*/4% CARBOWAX 20M with a column length of 1.5 m. Temperature in the column chamber was controlled at 190 °C and injection port as well as detector temperature were both maintained at 210 °C. ***Total nitrogen*** (TN) was determined by Kjeldahl's method using automated total nitrogen analyzer (1026 Kjeltec®System, Tecator AB, Sweden) according to the instructions of the manufacturer. ***Amino nitrogen*** (AN) was analyzed by formol titration method (10) using glycine as a control.

Results and Discussion

Effect of carbon source on growth and lactic acid production

2% (w/v) of Glucose, molasses (prepared according to total sugar basis), glycerol and yeast extract were used as carbon source for the cultivation. As shown in Table 1, among the carbon sources those were used, yeast extract was found to be the best complex carbon source which gave a maximum viable count of 2×10^9 cfu/ml but minimum lactic acid production (0.5 g/l). For the medium using glucose and molasses, there were shown that the viable counts were rather low compared to that of yeast extract, which might be due to the inhibitory effect of produced lactic acid, and also the result of that of molasses showed slightly lower viable count compared to the

result with glucose this would be due to other effects of other inhibitors presented in molasses. The results with glycerol showed the lowest cell count which imply the difficulty of glycerol assimilation by *L. acidophilus* used in the study.

According to the results shown above, it appeared that, when the yeast extract was applied as carbon source instead of glucose, the main components of yeast extract, *i.e.* free amino acids (utilized directly) and peptides (first digested by the proteolytic system) (11-13), could enter the cell metabolism through the TCA cycle (14). Compared with the cultivation using glucose, this would cause a decrease in lactic acid production of bacteria since the main carbon flow by-passes glycolysis metabolism (where pyruvate was converted to lactate) to TCA cycle. However the utilization of yeast extract as carbon source still could not completely prevent the production of lactic acid due to two possibilities, firstly from sugars contained in yeast extract and secondly from some amino acids that can be converted to pyruvate and then to lactic acid. The analysis of total sugar in yeast extract showed a good correlation between its consumption and lactic acid production in both time course and molar balance (data not shown). This suggested that lactic acid production may occurred mainly due to sugar consumption.

Effects of aeration on growth and lactic acid production

The effects of aeration on growth and lactic acid production were investigated using glucose and yeast extract as carbon sources by comparing flask cultivation with and without shaking. As shown in Fig. 1, in case of glucose the results of growth and lactic acid production were not significantly different under different growth conditions, meanwhile, growth on yeast extract under shaking conditions, resulted in

an increase in cell growth yield, whereas the lactic acid concentration was lower when the culture was shaken. It had also found that in case of continuous shaking, lactic acid that was produced at the beginning of the cultivation was utilized again. On the other hand, in case of non shaken flasks the lactic acid produced was not re-utilized. The phenomena which have been mentioned above could be confirmed in other experiments using higher concentrations of yeast extract (data not shown).

The effect of oxygen on the growth of lactic acid bacteria has been mentioned previously (15-19). For some lactic acid bacteria oxygen is toxic, due to the accumulation of toxic oxygen derived compounds that affect the growth (15). Several strains of lactic acid bacteria (*Pediococcus, Lactobacillus, Leuconostoc, Brochothrix, Carnobacterium*) show better growth yield in aerobic conditions (16-18). It has been reported that heterofermentative *Leuconostoc mesenteroides* was able to regenerate NAD(P)$^+$ by NADH oxidase and then the ethanol branch of the heterolactic pathway was not used. Instead acetyl-phosphate was converted to acetate yielding ATP with a consequent increase in biomass (19). In the condition when the NADH was directed to NAD$^+$ by NADH oxidase, the reduction of NADH level would result in the reverse reaction of lactate to pyruvate via the coupling of NAD$^+$. This could be an explanation for the consumption of lactate during the aerobic cultivation in this study. It should be also noted that the acetate level increased during the growth under aerobic conditions (data not shown). According to the reports and results mentioned above it could be hypothesized that the *L. acidophilus* strain used in this study might have a similar activity of NADH oxidase for NADH regeneration.

Cultivation of Lactobacillus acidophilus using yeast extract at various concentration as sole carbon source

Further investigation of cultivation using higher yeast extract concentrations demonstrated that cell density as well as viability could be improved by increasing the yeast extract content and the trend was linear (Fig. 2). The result implied the growth limitation might be due to nutrient limitation. The results also implied that the cell density of lactic acid bacteria could be further increased by supplied higher amounts of yeast extract. Lactate analysis of broth culture using different concentrations of yeast extract showed that in all cases, the final lactic acid concentration was rather low (less than 0.5 g/L, data not shown) even though a high amount of yeast extract used. This might be due to the fact that the lactate those was produced by consumption of sugars in yeast extract during the beginning of cultivation period was later re-utilized to low level.

Alternative proteinous sources for Lactobacillus acidophilus cultivation

Although yeast extract proved to be a good carbon source for the high cell density cultivation of *L. acidophilus*, in view of the relatively high cost of yeast extract, it might be desirable to find alternative raw materials. In this study we have investigated another source of protein which was considered to be much cheaper than yeast extract. Fish meal soluble protein with and without pretreatment by acid digestion was used to replace total yeast extract content in the medium, and then was tested for the potential to support the growth of *L. acidophilus*. The result showed that digested fish soluble protein could provide cell yield even higher than that of yeast extract (2% w/v) at equal total nitrogen content, whereas the non-digested fish soluble poorly support the

growth (Fig. 3a). Further supplement of non digested fish soluble can improve the cell density proportionally but the effect was different for digested fish soluble at higher concentrations which caused slow growth (Fig. 3b). The phenomenon of slow growth of *L. acidophilus* at higher concentrations of digested fish soluble was considered as an inhibition effect. Since in the acid digestion process by HCl, a lot of salt (approximately 30 %w/v) was obtained during the neutralization step, the effect of salt concentration was investigated. It was found that growth inhibition of *L. acidophilus* by salt was pronounced when the salt concentration exceeded 1% w/v (Fig. 4). The growth yields obtained when yeast extract and fish soluble were utilized as complex carbon source are summarized in Table 2. It could be concluded that the digested fish soluble could support good growth of *L. acidophilus* and the extend of growth was slightly higher than with yeast extract. Although the utilization of digested fish soluble at high concentration resulted in a growth inhibition effect due to the high salt concentration, the cost for FS was low as compared with yeast extract (less than 1/10 of commercial yeast extract price on equal weight basis). The improvement of the digestion process to reduce the salt formation is currently under investigation, and removal of salt might be done by electrodialysis. Thus the utilization of digested fish soluble showed a high potential for LAB cultivation.

Modification of protein source for optimal growth of Lactobacillus acidophilus

According to the results mentioned above, the degree of protein digestion which is related to the peptide size distribution, showed an effect on the growth yield of *L. acidophilus*. Further study for the optimal degree of digestion for fish soluble protein was conducted. Fish soluble was digested by HCl to get digested fish soluble protein

with various AN/TN ratios (Fig. 5), which were then used to cultivate *L. acidophilus* for comparison of the growth yield. As shown in Fig. 6, the growth yields obtained from the culture using digested fish soluble proteins were higher than those of 2% yeast extract and much higher than non-digested fish soluble protein. The 30 minutes digested fish soluble protein which has an AN/TN ratio of 0.5 was shown to have the highest growth yield. By calculation based on average nitrogen in amino acids, and an AN/TN ratio of 0.5, it was considered to contain mainly di-peptides and free amino acids. It should be noted that the AN/TN ratio could be used for rough estimation or control of protein quality for digestion, even though optimal AN/TN ratio would be dependent on type and basic composition of protein. From the results shown in Fig. 6, fish soluble digested for 5 h has a AN/TN ratio of about 0.7 (which implies that it contains mainly free amino acids), poorly supported the growth of *L. acidophilus* compared with lower AN/TN ratio's (at shorter digestion time and considered to contain more di-, tri-, and larger peptides). The results coincide with a previous report that growth of some lactic acid bacteria depends more on oligopeptides than free amino acids as nitrogen source (20). For *Lactococcus lactis* growth on complex protein sources, the proteolytic system which includes a cell envelope-located proteinase and several peptidases located intracellularly was considered to play a crucial role (11, 12). It has been found that more than 100 different oligopeptides could be obtained according to the cleavage of peptide bonds by cell enveloped proteinase, PrtP in *Lactococcus lactis* (21). Further study revealed that only certain type of peptides could be taken up and hydrolyzed internally by peptidases to amino acids (20). In this study, the digestion of fish soluble protein to a certain degree would provide appropriate size of oligopeptides (di-, tri- peptide) which could be

taken up by the cell. It was reported by Konings *et al.*(22) that the uptake of peptides would require the same amount of ATP as that of free amino acids, thus the uptake of peptides would be preferable in term of bioenergetics and this could provide a higher growth yield. Although the protein source was shown to be a potential carbon source for high cell density cultivation of *L. acidophilus*, it should be noted that the overall consumption of protein for growth was low (overall total nitrogen consumed was less than 20%, detailed data not presented). This result was in full agreement with a previous report which claimed that in case of *Lactococcus lactis* grown on casein, only few types of peptides can be utilized (20). The reason for the low efficiency in peptide utilization might be due to several factors such as the complexity of peptides, size exclusion limits of oligopeptide transporter, competition of the peptides for entry via a single oligopeptide transport system and hydrophobicity of peptides (20).

All protein sources (fish soluble, meat extract and tryptone) used in the medium were also studied for the efficiency in growth stimulation and were compared on the basis of equal TN using modified MRS medium. It was shown that meat extract can dramatically stimulate the growth of *L. acidophilus*. The addition of free amino acids (casamino acids plus tryptophan) as well as vitamins (seven types of vitamin those has been reported to be essential for LAB (23, 24)) to the fish soluble could not substitute for meat extract to promote the growth (data not shown). It has been was mentioned elsewhere that a small peptide (with MW of 1065) from freshly prepared yeast extract was a growth stimulant for *Lactobacillus sanfrancisco* (25). According to the results, some oligopeptides in meat extract should be further studied and characterized for their essential role as growth stimulants.

Conclusion

In order to increase the growth yield of lactic acid bacteria, the inhibitory effect of lactic acid has to be reduced. In this study we have investigated the possible strategies to control the lactic acid bacterial metabolism for reducing the lactate formation. It was found that cultivation at aerobic conditions coupled with using protein as alternative complex carbon source, promotes the growth of *L. acidophilus* very well, while the lactic acid production could be reduced significantly. Further study showed that protein with optimal degree of digestion could improve the cell yield. Although when protein was used, the efficiency of protein utilization was rather low compared with glucose, a better understanding of the proteolytic system and peptide transportation mechanism in LAB would lead to improvement in the efficiency of protein utilization and cell growth yield. Also the development of protein digestion processes with less salt formation, would allow the use of higher concentrations of digested protein, and thus increased cell density. According to the results from this study, it might be possible to apply the technique for other strains of lactic acid bacteria which has similar characteristics as *L. acidophilus* for the high cell density cultivation.

Acknowledgments

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Table 1. Cultivation of *Lactobacillus acidophilus* using various carbon sources at 2%w/v.

Carbon source[1]	Total viable count (cfu/ml)±SD[2]	Lactic acid (g/l)
Glucose (MRS)	$1.24\pm0.17\times10^9$	5.8
Molasses[3]	$7.22\pm0.21\times10^8$	5.5
Glycerol	$4.82\pm0.20\times10^8$	ND[4]
Yeast extract	$2.32\pm0.14\times10^9$	0.5

[1]Modified MRS medium by substituting the glucose with mentioned carbon sources.

[2]Standard deviation of duplicated data.

[3]prepared according to a basis of equal total sugar with 2% glucose.

[4]ND is not determined.

Table 2. Comparison of the cell yield obtained by using different types of complex media[1].

Complex media	Cell density, $OD_{600\,nm}$	Ratio of $OD_{600\,nm}$/TN	AN/TN [2]
2% Yeast extract	2.03	9.38	0.64
Non digested fish soluble protein	0.50	2.33	0.26
10 h Acid digested fish soluble protein	2.52	11.72	0.70

[1]The medium used was MRS media where glucose was substituted by each complex media prepared at equal total nitrogen (TN) of 0.22 % (w/v).

[2] Ratio of amino nitrogen and total nitrogen of the complex media.

Figure captions

Fig. 1. Cultivation of *L. acidophilus* using glucose or yeast extract as media. (a) Time course of growth and (b) lactic acid production. Symbols: ○, 2% glucose; △, 2% yeast extract. Open and filled symbols denote shaken and non-shaken conditions, respectively.

Fig. 2. Effect of yeast extract concentration on growth of *L. acidophilus*. Symbols: ○, maximal OD_{660}; △, maximal viable cell count.

Fig. 3. Growth of *L. acidophilus* using complex medium of yeast extract and fish soluble protein. (a) 2% (w/v) of yeast extract in comparison with fish soluble protein and (b) 4 and 8% of fish soluble protein. Symbols: □, yeast extract; ○; △; ◇, fish soluble at 2, 4 and 8%, respectively. For fish soluble protein, open and filled symbols denote non-digested and digested, respectively. The medium of 2, 4 and 8% FS were prepared on the basis of equivalent total nitrogen to those of 2, 4 and 8%(w/v) of yeast extract.

Fig. 4. Effect of salt concentration on growth of *L. acidophilus* in MRS medium. Symbols: ○; △; ◇; □, NaCl concentration at 1, 2, 3 and 4%(w/v), respectively.

Fig. 5. Time course of AN/TN ratio in digestion of fish soluble with HCl.

Fig. 6. Growth of L. acidophilus in fish soluble digested for various time. Symbols: ●, 2%(w/v) yeast extract; ×, non-digested fish soluble; ◇; ○; □; △; ○, fish soluble digested at 10 min., 30 min., 1 h., 3 h. and 5 h., respectively. All medium was adjusted to have equal total nitrogen content.

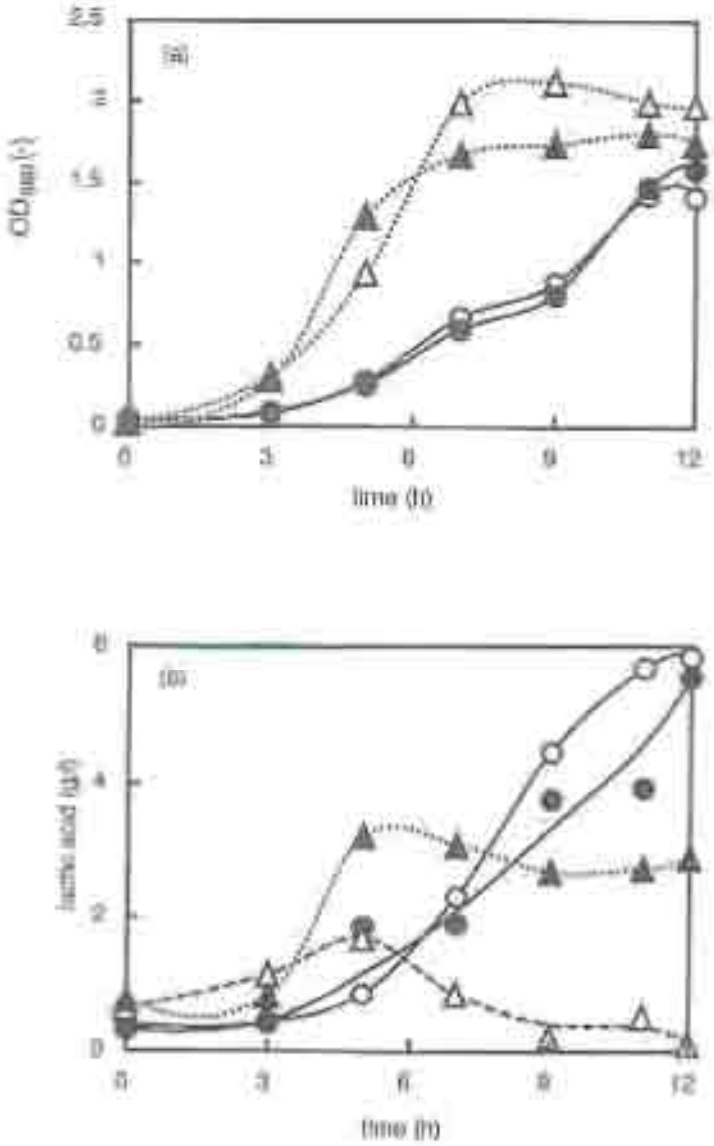


Fig. 1. Cultivation of *L. acidophilus* using glucose or yeast extract as media. (a) Time course of growth and (b) lactic acid production. Symbols: ○, 2% glucose; △, 3% yeast extract. Open and filled symbols denote shaken and non-shaken conditions, respectively.

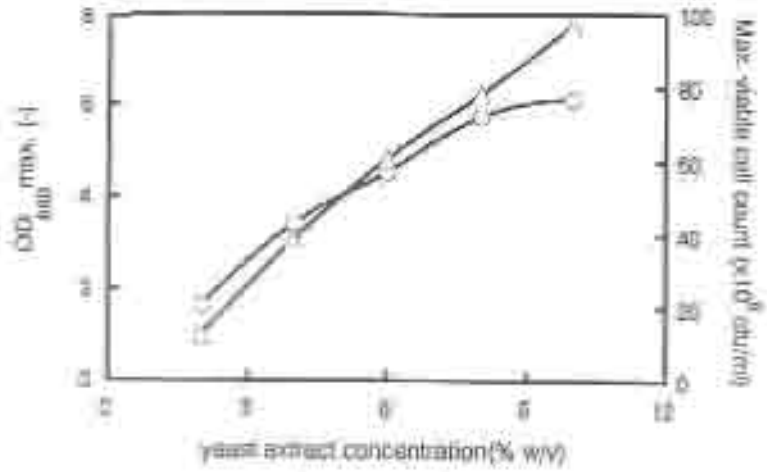
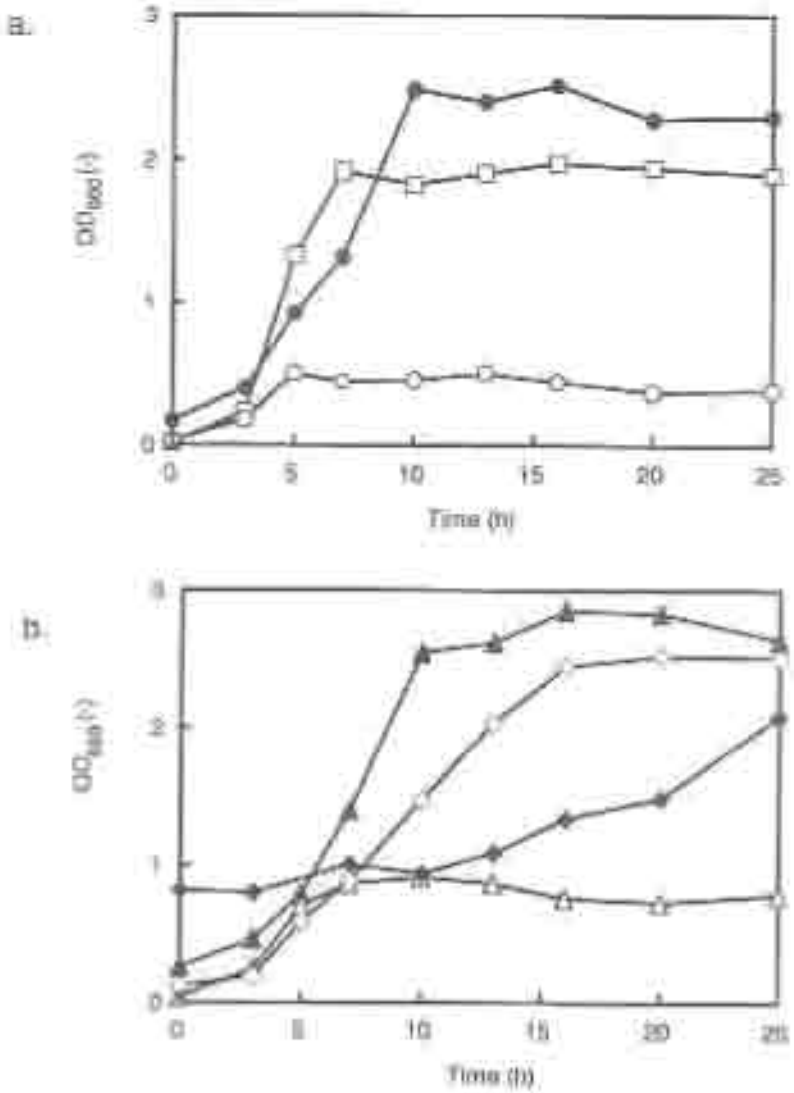


Fig. 2. Effect of yeast extract concentration on growth of *L. acidophilus*. Symbols: □ , maximal OD 660; △ , maximal visible cell count.



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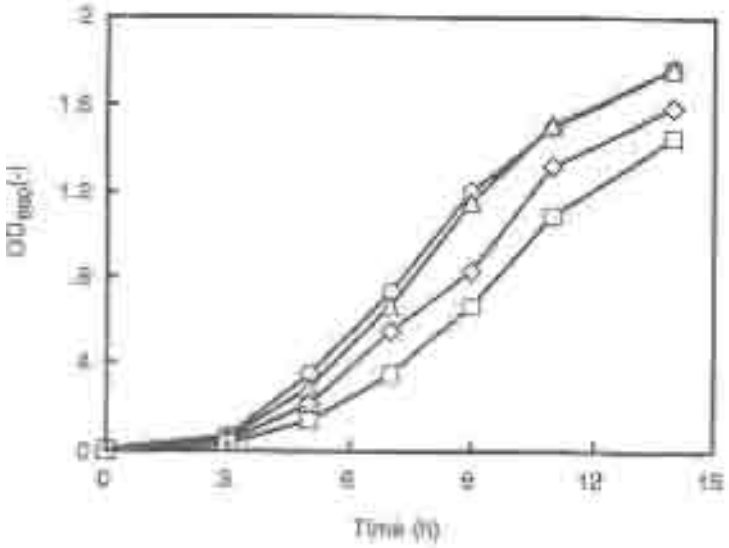

Fig. 4 Effect of salt concentration on growth of *L. acidophilus* in MRS medium. Symbols: □, △, ◇, □, NaCl concentration at 1%, 2%, 3% and 4% (w/v), respectively.

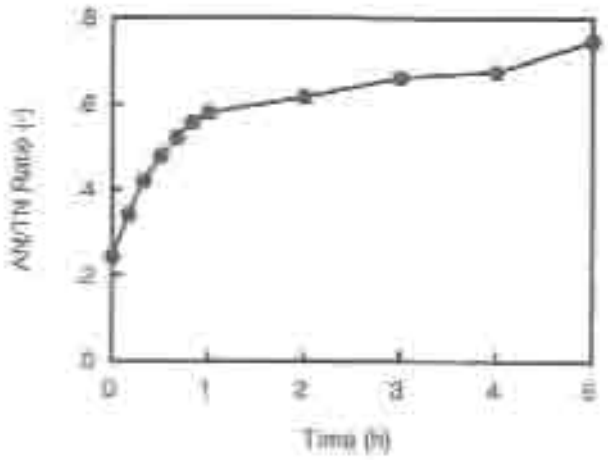

Fig. 5 Time-course of AN/TN ratio in digestion of fish soluble with HCl.

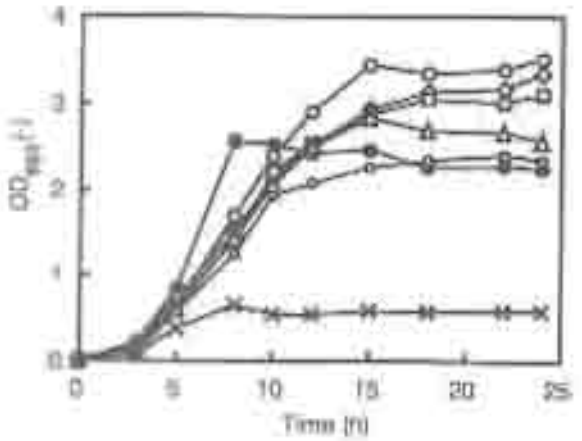

Fig. 6. Growth of *L. acidophilus* in fish soluble digested for various time. Symbols: ●, 2% (w/v) yeast extract; ×, non-digested fish soluble; ○ ; ◎ ; □ ; △ ; ＊, fish soluble digested at 10 min., 30 min., 1 h., 3h. and 5 h. respectively. All medium was adjusted to have equal total nitrogen content.

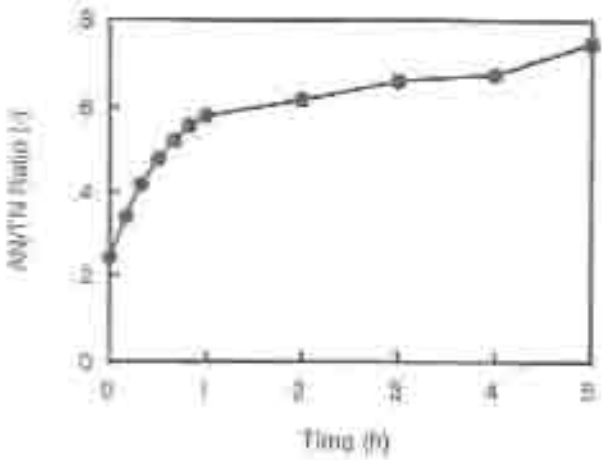

Fig. 5. Time course of AN/TN ratio in digestion of fish soluble with HCl.

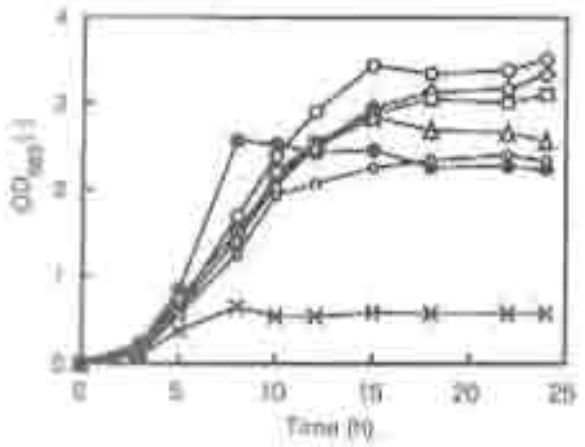

Fig. Growth of *L. acidophilus* in fish soluble digested for various time. Symbols: ●, 2% (w/v) yeast extract; ×, non-digested fish soluble; ◇, ○; □, △, ▽; fish soluble digested at 10 min., 30 min., 1 h., 3h. and 5 h. respectively. All medium was adjusted to have equal total nitrogen content.

PO-III/28

Cultivation of High Cell Density of Lactic Acid Bacteria for Probiotics

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In the production of lactic acid bacteria which is one of the major microorganisms being used for probiotics, main problem that cause the lower cell productivity is the growth inhibitory effect to of lactic acid. In order to improve the cell growth yield several approaches could be considered in order to reduce the inhibitory effect of lactic acid. In this study we investigated strategies to decrease the production of lactic acid through the use of metabolic controls in lactic acid bacteria. We investigated the effect of various of carbon sources for cultivation and aeration effect on the growth yield and lactic acid production. The result showed that by using yeast extract as sole carbon source the growth yield could be increased (with final viable count of more than 10^{11} cfu/ml) for more than 10 folds compare to normal cultivation using glucose as carbon source, while the lactic acid production was significantly decreased. The aeration accompanied with using yeast extract could improved the growth yield and also cause the produced lactic acid to be re-utilized. The results of fed-batch cultivation using yeast extract feeding coupling with aeration showed that the lactic acid concentration can be controlled at low level. The proposed strategy show the potential to be applicable with high cell density cultivation of other lactic acid bacteria.

P-IB31

Utilization of complex proteineous sources for high cell density cultivation of *Lactobacillus acidophilus*

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In lactic acid bacteria production, to improve the cell growth and obtain high cell density, the effect of produced lactic acid have to be minimized. In this study, strategy in which the metabolism control to reduce the production of lactic acid have been investigated. We have studied the effect of proteineous sources on the growth yield and lactic acid production of *Lactobacillus acidophilus*. The result showed that using protein source such as yeast extract as carbon source, high cell growth (upto $8x10^9$ cfu/ml) could be obtained proportionally to amount of protein addition while the lactic acid level was low (< 0.5 g/L under aeration condition). The improvement of cell growth was considered to be due to less inhibition effect of lactic acid compared with the cultivation using glucose as carbon source where the lactic acid concentration was more than 4.5 g/L. However, due to the high cost of yeast extract, various protein sources were investigated for their potential to use as culture medium for industrial production. It was found that fish soluble extract which was pre-digested by acid provided 1.5 times higher growth yield than that obtained from yeast extract, base on equal total nitrogen.

Process Development for High Cell Density Cultivation of *Lactobacillus acidophilus* for Probiotics

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SUMMARY

Lactic acid bacteria (LAB), where most of the strains are generally recognized as safe (GRAS), have been widely used in production of many fermented food products. It has also been utilized as one of the components in formulation of "probiotics", a live microbial food supplement which beneficially affects the host animal by improving its intestinal microbial balance. In lactic acid bacteria production, to improve the cell growth and obtain high cell density, the effect of produced lactic acid have to be minimized.

In this study, strategies in which the metabolism control to reduce the production of lactic acid have been investigated. Firstly, We have studied the effect of proteineous source on the growth yield and lactic acid production of *Lactobacillus acidophilus*. The result showed that using protein source such as yeast extract as alternative carbon source, high cell growth (upto $8x10^8$ cfu/ml) could be obtained proportionally to amount of protein addition while the lactic acid level was low (< 0.5 g/L under aeration condition). The improvement of cell growth was considered to be due to less inhibition effect of lactic acid compared with the cultivation using glucose as carbon source where the lactic acid concentration was more than 4.5 g/L. It was also found that fish soluble extract as alternative protein source which was pre-digested by acid provided 1.5 times higher growth yield than that obtained from yeast extract, base on equal total nitrogen. However the analyses of protein indicated that protein consumption was less efficient compare with that of glucose (only upto 15 % of total nitrogen). So secondly fed-batch cultivation of glucose and protein source was further investigated in order to improve the cell production yield. The results showed that higher cell density (upto $2.0x10^{10}$ cfu/mL) could be obtained by fed-batch technique while the level of lactic acid concentration could be controlled by limitation of glucose supply. The fed-batch study indicated that continuous feeding of both sugar and protein is required for high cell density cultivation of *L. acidophilus*.

The result from this study showed the potential trend to apply the technique for high cell density cultivation of other lactic acid bacteria.

INTRODUCTION

Lactic acid bacteria (LAB), where most of the strains are generally recognized as safe (GRAS), have been widely used in production of many fermented food products. It has also been utilized as one of the components in formulation of "probiotics", a live microbial food supplement which beneficially affects the host animal by improving its intestinal microbial balance (1). For lactic acid bacteria production, the major problem is the growth inhibitory effect of the metabolic product, lactic acid (2). To solve this problem, the physiological control of lactic acid bacteria metabolism to reduce its production could be considered. In this paper, *Lactobacillus acidophilus* strain which is commonly utilized for probiotics was studied. With the aim of high cell density cultivation, Firstly, the lactic acid production has been controlled by medium which utilizes protein as an alternative complex carbon source, different sources of protein have been studied for their suitability in term of efficiency and