Vitamin B_{12} Production during Tofu Fermentation by *Lactobacillus reuteri* and *Propionibacterium freudenreichii*

Dissertation

zum Erlangung des Doktorgrades der Naturwissenschaften

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The following work was conducted during the time period from April 2009 to April 2013 in the research group of Professor Dr. Bernward Bisping at the Division of Food Microbiology and Biotechnology, Department of Chemstry, University of Hamburg, Germany.

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Es wird darauf hingewiesen, dass die Ergebnisse und Aussagen dieser Arbeit, solange sie nicht publiziet wurde, vertraulich zu behandeln sind.

1. Gutachter / Reviewer: Prof. Dr. Bernward Bisping
2. Gutachter / Reviewer: Prof. Dr. Markus Fischer

Tag der Disputation / Day of the disputation: 07 June 2013

Erklärung

Ich erkäre an Eides statt, dass ich die vorliegende Dissertation selbständig verfasst habe und die angegebenen Quellen und Hilfsmittel verwendet habe. Ich habe vorher weder die vollständige Dissertation noch Teile der Dissertation an anderer Stelle eingereicht. Dies ist mein erster Promotionsversuch, um den Doktorgrad zu erlangen.

Declaration

I declare that I have worked on this dissertation independently and have used sources and equipments as specified in this work. This dissertation has not been previously submitted in part or in total to any other institution. This is my first attempt to submit a dissertation in order to obtain a doctoral degree.

Xuan Zhu

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List of Publications

Poster publications

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Zhu X, Illas N, Bisping B (2011) Determination of vitamin B_{12} in fermented soybean products by high-performance liguid chromatography (Poster), presented at The 5th Asian Vegetarian Union Congress, 8-9 November 2011, Hangzhou, China.

Zhu X, Bernhardt C, Bisping B (2013) *Acetobacter pasteurianus* DSM 3509 produces cobalamin (Poster), presented at the 2013 Frühjahrstagung der Biotechnologen DECHEMA (Gesellschaft für Chemische Technik und Biotechnologie), 4-5 March 2013, Frankfurt am Main, Germany.

Journal publication

Acetobacter pasteurianus DSM 3509 produces cobalamin. In preparation

Patent application

Vitamin B_{12} Production during Tofu Fermentation by *Lactobacillus reuteri* and *Propionibacterium freudenreichii*. In preparation

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1. Introduction

Cobalamin, which is the general name for natural occurring cobalt organometallic compounds containing substances, possesses numerous biochemically unique attributes, such as affecting DNA synthesis and regulation, fatty acid synthesis, amino acid metabolism and energy production. Vitamin B_{12} is a part of cobalamin that has biological activities for human beings. It has been reported that vitamin B_{12} can be used in the therapy of pernicious anaemia, a fatal disease of the red blood cells (Schneider and Stroinski 1987). So vitamin B_{12} has a big influence on various fields of metabolism.

1.1. History

The story of the discovery of cobalamin and its biological function began with Minot and Murphy, who in 1926 demonstrated the successful treatment of pernicious anemia by feeding of liver (Minot and Murphy 1926). In 1929, Castle observed and discovered that a protein factor existing in the stomach juice, called intrinsic factor by him, which significantly enhanced the curing effect of the liver after oral intake (William and Castle 1974). Due to ensued unsuccessful efforts to isolate and identify the anti-pernicious anaemia principle of liver, during the following twenty years more and more concentrated forms of liver replaced the ingestion of raw liver in the treatment of pernicious anemia. In 1945 the discovery and crystallization of cobalamin was achieved by two independent groups in America and England (Rickes et al. 1948; Smith 1948). However microbiologists and animal nutritionists studied on independent research fields and separately found three factors, which possibly led to the discovery of cobalamin (Castle 1975): an animal protein factor (APF) obtained from animal tissue extracts and feces, a *Lactobacillus lactis* Dorner factor (LLDF) essential for growth of this bacterial species, and a ruminant factor containing cobalt ions to cure the so-called bush sickness of sheep and cattle in Australia.

The structure of cobalamin was elucidated by X-ray crystallography (Hodgkin et al. 1956). Following the discovery and research, a considerable number of vitamin B_{12} analogues had been found. It became obvious that the primary producer of vitamin B_{12} is of microbiological origin, and not of animal origin. After Barker et al. (1958) discovered the coenzymatic function of

vitamin B_{12}, a series of biochemical attributes and enzyme systems of vitamin B_{12} were discovered and characterized.

1.2. Chemical structure (Rucker et al. 2001)

Adenosyl-cobalamin (Ado-cobalamin) is taken as a good example to elucidate the structure of cobalamin, as it is involved in a series of vital biological metabolisms. The molecular weight of Ado-cobalamin is 1580 and at least 25 enzymes are involved in the synthesis procession of cobalamin. Ado-cobalamin is made up by three parts (Fig. 1-1). They are a central ring, an adenosyl moiety, and a nucleotide loop. The central ring contains four reduced pyrrole rings (designated A-D) connected with a cobalt atom in the centre. Unlike other structurally and biosynthetically similar moieties, such as heme and chlorophyll, a direct linkage is found between the carbon of A and D porphyrin and the structure of porphyrin is decorated by methyl groups, acetamide and propionamide residues. 5′ deoxyadenosyl moiety is linked by a covalent bond to cobalt within the corrin ring and is recognized as an upper axial ligand. The cleavage of covalent bond between cobalt and deoxyadenosyl is involved in the catalysis of intramolecular rearrangement reaction (Sato et al. 1976). The other lower axial ligand of cobalt is covalently formed by dimethylbenzimidazole (Dmbi) with cobalt. 3′ phosphoribosyl-Dmbi is attached by phosphate to an aminopropanol moiety linked to a propionyl group extending from the D porphyrin of the ring.

Cobalamin includes four forms and lots of analogues different from the upper and or lower ligands. The deoxyadenosyl is replaced by a methyl group, a hydroxyl group and cyano group to form methyl-, hydroxo-, and cyano-cobalamin. This form of cyanocobalamin is not found in nature but nowadays is used as a supplement nutrient for humans and stocks. Different analogues have been isolated and identified in various Bacteria and Archaea (Brandt et al. 1979).

1.3. Chemical properties

The absorption spectrum of cyanocobalamin shows three characteristic maxima at 278 nm, 361 nm and 550 nm that are relatively independent of pH (Schneider and Stroinski 1987). Hydroxocobalamin, methylcobalmin, adenosylcobalamin and other derivatives are freely

converted to cyanocobalamin in presence of CN^- and cyanocobamin can be reserved into biological cobalamin in biological and clinic view. In alkaline solution, two cyano groups are coordinated to the cobalt atom to form dicyanocobalamin in addition of excess cyanide.

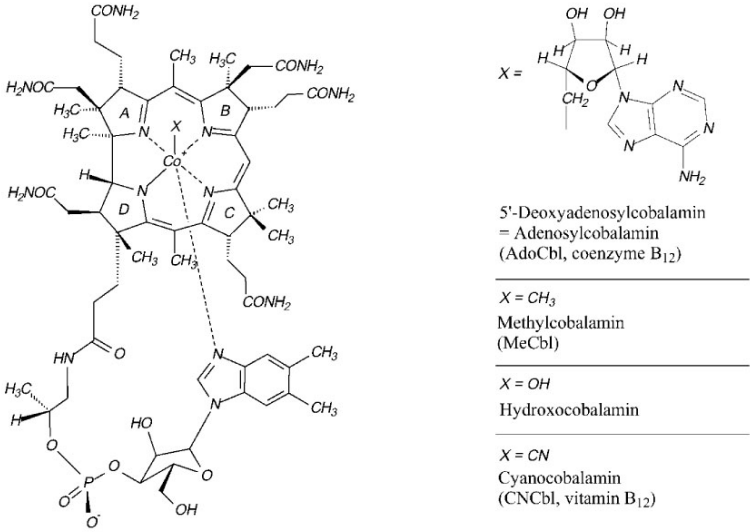


Fig.1-1 Schemtical diagram of structure of vitamin B_{12}
Cobalamin Porphyrin rings are designated with capital Letters. X stands for different upper axial ligand moieties. (Figure from Martens et al. 2002)

Photolysis of cobalamin is pH dependent and a heat-catalysed degradation (Ahmad et al. 1992; Ansari et al. 2004; Demerre and Wilson 1956). The Ado-cobalamin and methyl-cobalamin are photolabile compounds. The aerobic photodecomposition of methyl-cobalamin processes faster when exposed to oxygen, compared with irreversible decomposition of Ado-cobalamin in anaerobic conditions (Demerre and Wilson 1956; Grissom et al. 1993). The biological activities of Ado-cobalamin and methyl-cobalamin are lost and the spectrums are changed, due to the hemolytic cleavage of the C-Co bond. Nevertheless, cyanocobalamin is slowly irreversible converted to hydrocobalamin, even reversible to aquocobalamin (Ahmad et al. 1992). All forms of cobalamin can be irreversibly inactivated under the condition of prolonged irradiation. However, some enzyme requiring Ado-cobalamin and methyl-cobalamin may protect these compounds from photodecomposition (Demerre and Wilson 1956).

The stability of cobalamin is coordinated by pH and light. Cobalamin is a polyacidic base with six weak basic amide groups and has a pk_a of 3.3 which is even stronger than acetate (Ahmad et al.

1992). In the acid range, cobalamin exists as a cation, but at pH 7.0 99.9% is in neutral status (Ahmad et al. 1992). Cobalamin has a stable status ranging from pH 6.0 to pH 9.0. Over pH 9.0 the hydrolysis of amide groups may contribute to photolysis. The cyclization of the c-acetamide function, amide cyclization and amide hydrolysis may influence the stability of cobalamin solutions in basic media (Schneider and Stroinski 1987).

Compared with derivatives, cyanocobalamin has a relatively durable and stable property in air, in dry form, even at 100 °C for a few hours (Blitz et al. 1956). However, thiamine, nicotinamide or nicotinic acid, and ascorbic acid destroy cobalamin (Blitz et al. 1956), and addition of a small amount of iron can protect cobalamin (Mukherjee and Sen 1957).

1.4. Biosynthesis

The synthesis of cobalamin is a complex operation performed in living bacterial systems. There are two distinct pathways existing in the synthesis. One is the aerobic synthesis, performed by *Pseudomonas dentitrificans* as an example. *Salmonella typhimurium* is regarded as the anaerobic synthesis model bacterium (Rodionov et al. 2003). Eight main steps and intermediates will be depicted as follows (Fig. 1.2) (Rodionov et al. 2003; Roth et al. 1996; Schneider and Stroinski 1987).

1. This synthesis originates from condensation of glycine and succinyl-CoA to delta-aminolevulinic acid (ALA).

2. Two ALA molecules are condensed to form porphobilinogen.

3. Uroporphyrinogen III (Uro III) results in enzymatic condensation of four porphobilinogens moieties. Up to this step all bacteria share the same pathway. The biosynthesis of tetrapyrrole is inhibited by heme and vitamin B_{12}. When heme inhibits the formation of ALA and Uro III, the Ado-cobalamin represses the methylation of Uro III (Bykhovskii et al. 1980).

4. Cobyric acid is formed from Uro III by reductive methylation, decarboxylation, dehydrogenation, and insertion of cobalt. In this procession, two different pathways are involved. Both of them first change Uro III to precorrin 2 by different enzymes. But the GysG protein for

anaerobic pathway not only catalyzes the ring oxidation to form precorrin 2, but also appears to be involved in the catalysis of the insertion of cobalt. CobA protein, found in aerobic pathway, catalyzes only the two methylation reactions to form precorrin 2. Cobalt insertion happens later in this pathyway and is supported by a distinct protein. This unusual Co-C bond between Co and adenosyl is formed in this step.

5. Adenosylcobinamide (Ado-Cbi) is formed by the addition of L-threonine (Kurumaya and Kajiwara 1990). Threonine can generate free 1-amino-2-propanol by a simple decarboxylation reaction. 1-amino-2-propanol can be attached to cobyric acid to form adenosylcobinamide.

6. Dimethylbenzimidazole is an important part of cobalamin, which is generated from riboflavin. On the base of a different pathway, the generation of Dmbi also involves different enzymes and different conditions. For *Propionibacterium shermanii*, oxygen is required to produce Dmbi. But the pathway and genes involved in are still in question. In contrast, *Salmonella typhimurium* can produce Dmbi under anaerobic conditions. Chen et al. (1995b) have interpreted that the single CobT protein (*S. typhimurium*) catalyzes the complete synthesis of Dmbi.

7. Dmbi nucleoside is formed by transfering Ribose-PO_4 to Dmbi. The nicotinic acid mononucleotide (NaMN: an intermediate in NAD synthesis) is catalyzed to transfer Ribose-PO_4 to form DmbiMN, which has a phosphate on the 5' carbon of ribose.

8. Ado-cobalamin is completed by joining Ado-Cbi and Dmbi nucleoside. The aminopropanol group of Ado-Cbi is activated to form Ado-Cbi-GDP. The activated end of the aminopropanol side-chain attaches to Dmbi ribonucleoside at its 3' position to generate the completed Ado-cobalamin.

The biosynthesis of cobalamin is regulated by four promoters of Pcob, P1, P2 and Ppoc (Fig. 1-3) (Chen et al. 1995). The *pdu* operon adjacent to the *cob* operon encodes enzymes for propanediol degradation. The *cob* operon encodes enzymes for the synthesis of cobalamin.

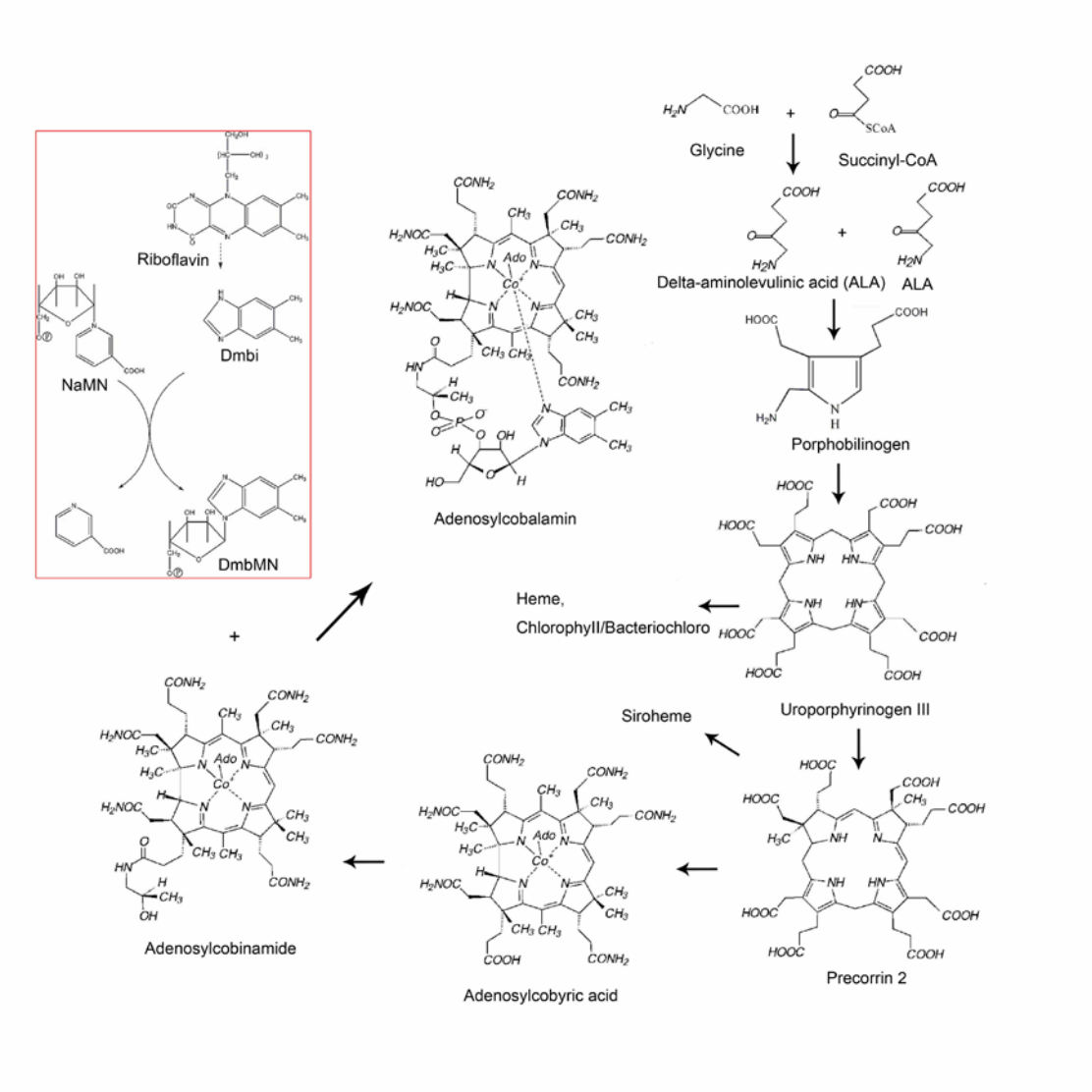


Fig. 1-2 Schematical diagram of the synthesis pathway of vitamin B_{12}. The pathway in the box designs the generation of Dmbi from riboflavin. Dmbi means dimethylbenzimidazole. NaMN stands for nicotinic acid mononucleotide and DmbMN is an abbreviation for ribofuranosyl dimethylbenzimidazole phosphate.

Two global regulatory systems (Crp/Cya and ArcA/ArcB) have controlled expressions of the cob and pdu operons (Chen et al. 1995). Both operons are additionally activated by Crp in anaerobic and aerobic conditions, but by ArcA protein only under anaerobic conditions. Four promoters (Pcob, P1, P2, and Ppdu) are positively regulated by PocR regulatory protein transcribed from gene pocR (Chen et al. 1995). As propanediol is degraded by a vitamin B_{12} dependent enzyme, propanediol has a positive effect on the production of cobalamin (Chen et al. 1995). But Ado-cobalamin also functions as an inhibitor to the Pcob (Roth et al. 1996).

1.5. Metabolic function

The characters of vitamin B_{12} catalyzed reaction may help to interpret the evolution and loss of vitamin B_{12} synthesis from different groups of bacteria. The fundamental and primary role of vitamin B_{12} in many bacteria may support fermentation of small molecules. Oxidizable compounds and electron sinks used for balancing the redox reactions are generated by catalyzing rearrangement of molecules and also ATP can be produced by substrate-linked phosphorylations during this fermentation. The vitamin B_{12} dependent degradation reactions of ethanolamine (Roof and Roth 1989), propanediol (Marcal et al. 2009; Roth et al. 1996), and glycerol (Sriramulu et al. 2008) are found in enteric bacteria. By these reactions, an aldehyde is generated under the mediation of vitamin B_{12}. This aldehyde cannot only be oxidized with the generation of ATP, but is also involved in the oxidation reaction to be a hydrogen receptor. The reactions engaged by vitamin B_{12} are the important part of anaerobic fermentation for these bacteria, which can generate reducible compounds to sustain balance of redox reactions.

The second use of vitamin B_{12} is to catalyze amino mutases (glutamic acid, lysine, leucine, or ornithine) reactions to support the fermentation of these amino acids (Schneider and Stroinski 1987). Further vitamin B_{12} dependent enzymes also involve the reactions of methionine synthesis and ribonucleotide reductase, which is a vital critical step to synthesis of DNA (Jordan et al. 1997).

The role of the complex compound vitamin B_{12} initially supports growth of bacteria under anaerobic conditions. Secondly, this compound is involved in reactions such as methionine synthesis and nucleotide reduction to maintain the physiology of organisms. With the appearance of oxygen and aerobic respiration, fermentations were not the only choice for many organisms. Many organisms chose a more efficient and quicker aerobic respiration and lose some original enzyme capabilities like vitamin B_{12} production.

Nevertheless the secondary use of vitamin B_{12} is still required by obligate aerobes and animals. In humans methionine synthetase, a vitamin B_{12} dependent methyl transferase, is recognized to be important in recycling folate and in producing methionine and it is also known to influence the

concentration of homocysteine that is a risk factor for cardiovascular disease and related with Alzheimer's disease (Stover 2004). Methylmalonyl CoA, that is also a vitamin B_{12} dependent coenzyme, plays a role in the degradation of branched chain amino acids and odd fatty acids, which are toxic for humans (Ledley 1990).

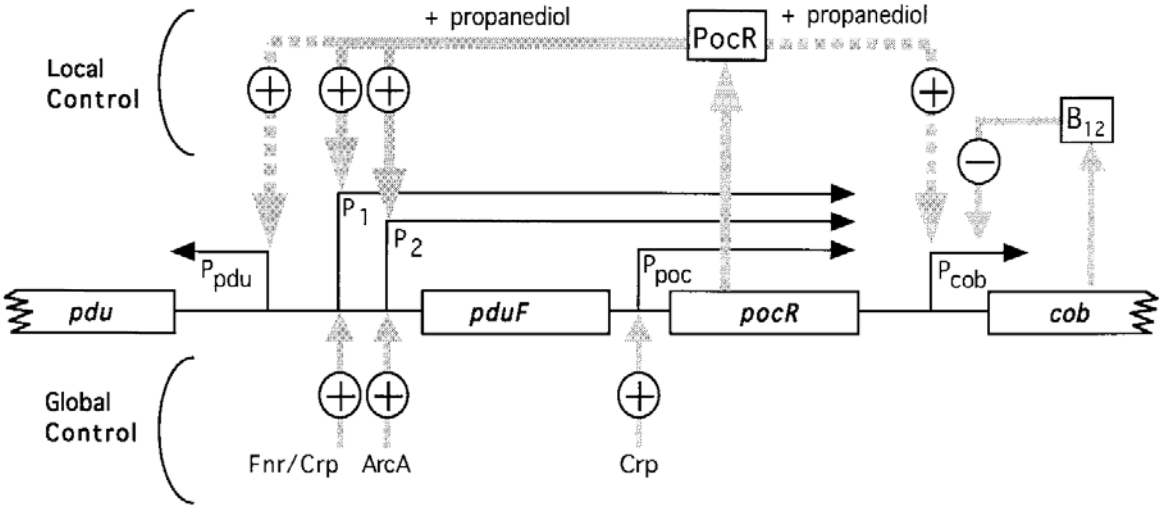


Fig. 1-3 The graph illuminates the regulation of cobalamin production. Boxes stand for operons. Black arrows indicate the transcripts. Gray arrows designate regulator influence and dash ones show an assumption that PocR may activate these promoters with the help of propanediol. (Figure from Roth et al. 1996)

Thus vitamin B_{12} is participating in a dozen of enzymatic systems mostly by two coenzymatic derivatives: methyl-cobalamin and adenosyl-cobalamin. Some important enzymes and reactions will be listed respectively on these two derivatives and diagrammed in Fig. 1-4

1.5.1. Adenosylcobalamin (Fig. 1-4a):

Propanediol dehydratase (Havemann and Bobik 2003): This enzyme catalyzes the conversion of 1,2-propanediol to propionaldehyde. Some bacteria use 1,2-propanediol as a carbon and energy source. The propionaldehyde can be further reduced to regenerate NAD to provide an electron sink for balancing the redox reaction, and its oxidation can provide a source of ATP and cell carbon.

Ethanolamine ammonia lyase (Blackwell et al. 1977; Wetmore et al. 2002): Enthanolamine is converted to acetaldehyde and by ethanolamine ammonia lyase. By this reaction, sometimes this substance can serve as a carbon, nitrogen and energy source.

Glycerol dehydratase (Roth et al. 1996; Sriramulu et al. 2008): Catalyzed by this enzyme, glycerol can be converted to hydroxypropionaldehyde, which can be further reduced to 1,3-propanediol. This reaction generates NAD to balance the reducing equivalent.

Ribonucleotide reductase (Jordan et al. 1997): This enzyme is used in many prokaryotes to generate free radicals which can convert ribonucleotides to deoxyribonucleotides to synthesize DNA.

Methylmalonyl Coenzyme A mutase (Miyano et al. 2000): This enzyme is an enzyme that catalyzes the isomerization of methylmalonyl-CoA to succinyl-CoA.

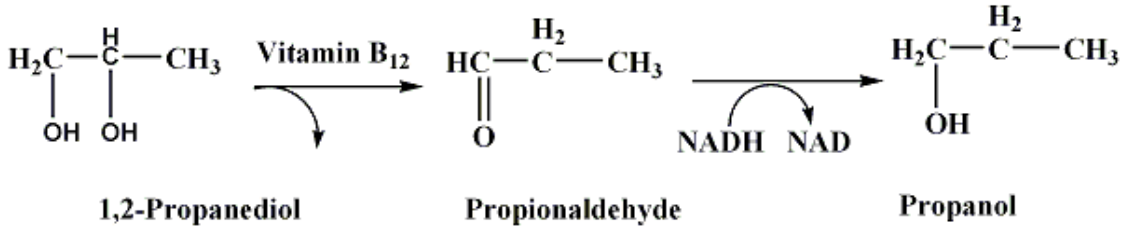
1.5.2. Methylcobalamin (Fig. 1-4b):

Methionine synthetase (Banerjee and Matthews 1990): This enzyme involves into the terminal step of methionine biosynthesis. A methyl group from methyltetrahydrofolate is transferred to homocysteine with the generation of tetrahydrofolate and methionine. In humans, megaloblastic anemia and even spina bifida are resulting from the low activity of methionine synthetase.

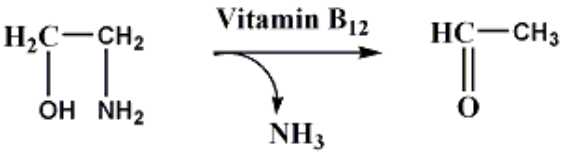
1.6. Assay method

In literature, various analytic methods have been introduced to detect vitamin B_{12} in food, such as microbiological assay (Kelleher and Broin 1991), paper- and thin layer chromatography (Szepesi and Molnar 1981), radio-isotope dilution assay (RIDA) (Lau et al. 1965), spectroscopic assay (Nepote et al. 2003), chemiluminescence (Wentworth et al. 1994), capillary electrophoresis (Schreiner et al. 2003), atomic absorption spectrometry (Whitlock et al. 1976), and high performance liquid chromatography (HPLC) (Gauch et al. 1992; Li et al. 2000; Luo et al. 2006). The methods of chemiluminescence (Wentworth et al. 1994) and atomic absorption spectrometry do not adapt to complex and fermentative food, as these methods cannot distinguish between cobalamin and cobalt bound to other substances. The methods of paper- and thin layer chromatography (Szepesi and Molnar 1981) and spectroscopic assay are not convenient to be applied for a complex matrix, especially for solid fermentation products.

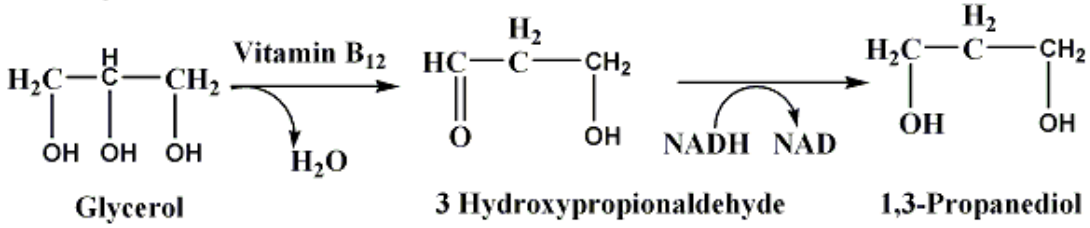
Propanediol dehydratase



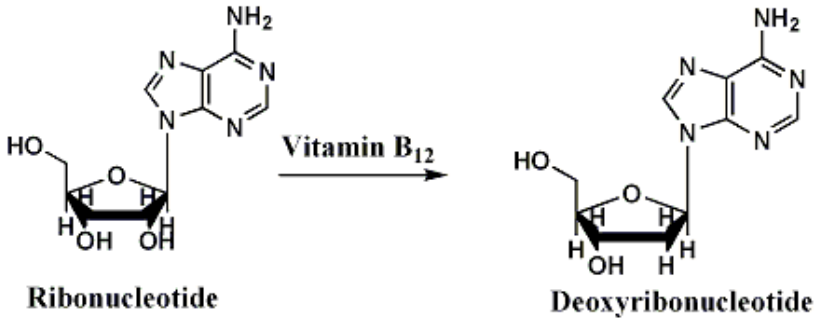
Ethanolamine ammonia lyase



Glycerol dehydratase



Ribonucleotide reductase



Methylmalonyl CoA mutase

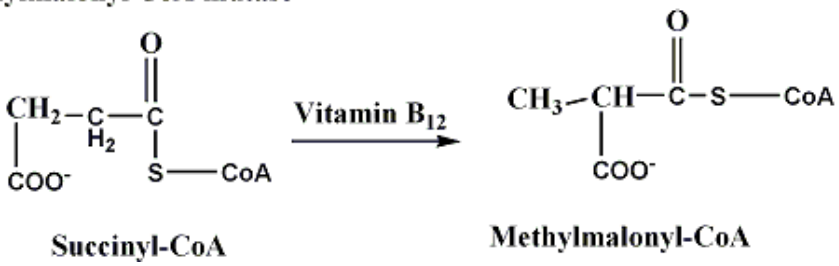


Fig. 1-4a Adenosylcobalamin dependent enzyme pathways.

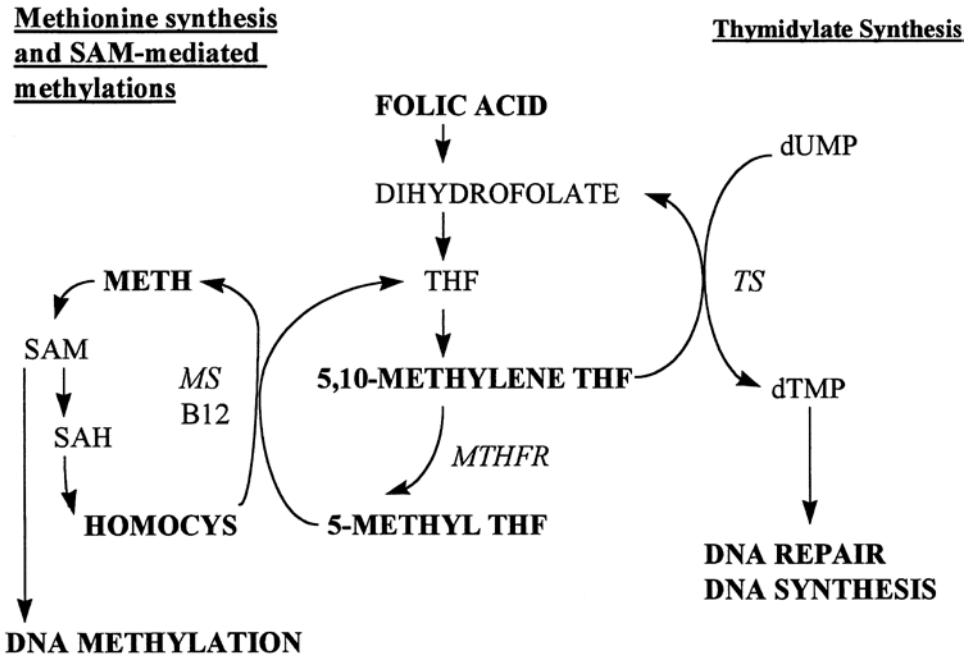


Fig. 1-4b The metabolic pathways of methionine catalyzed by the methylcobalamin dependent enzyme. (B12: Vitamin B_{12}; METH: methionine; THF:tetrahydrofolate; TS: thymidylate synthase; MS: methionine synthase; MTHFR: methylenetetrahydrofolate reductase; SAM: S-adenosylmethionine; SAH: S-adenosyl homocysteine) (Figure form Rucker et al. 2001)

In view of the requirement of radio-labeled cyanocobalamin and intrinsic factor of high purity, radio-isotope dilution assay (RIDA) is expensive although it is fast and simple. The microbiological assay, due to low cost and convenience, is very popular in the routine lab work. But there are problems such as expenditure of time, poor precision and low relative specificity. Capillary electrophoresis is a useful method to detect cobalamin, but there are many limitations in the procedure of detection. Reversed-phase HPLC method for the determination of vitamin B_{12} by UV and fluorescence has been already reported (Gauch et al. 1992; Li et al. 2000; Luo et al. 2006). However, most of these methods are only applied for vitamin tablets or milk and none of them was reported to be used to analyze low concentrations of vitamin B_{12} in solid state fermentation foods. Lou et al. (Luo et al. 2006) have succeeded in detecting vitamin B_{12} in food products by HPLC-ESI-MS.

1.7. Cobalamin deficiency

Cobalamin deficiency leads to hematologic abnormalities (Takasaki et al. 2002) and many neuropsychiatric abnormalities (Allen et al. 1998). But cobalamin deficiency is often

misdiagnosed, as it commonly presents as folate deficiency (Stover 2004). Both deficiencies of folate and cobalamin cause indistinguishable symptoms of hematologic abnormalities (Allen et al. 1993). They contain anemia, a hypercellular bone marrow with abnormal maturation, decreases white blood and platelet counts, and megaloblastic anemia (Teplitsky et al. 2003). This megaloblastic anemia stems from a metabolic derangement, a defect of DNA synthesis (Diaz Conradi et al. 2007). Cobalamin independent ribonucleotide reductase of bone marrow and other animal cells was shown (Diaz Conradi et al. 2007). A methylfolate trap hypothesis (Carmel 2000) may be used to explain the symptom. Folate is accumulated as a form of N^5-methyltetrahydrofolate under cobalamin deficiency in humans, thus blocked to transform to tetrahydrofolate, which is a precursor of N^5, N^{10}-mehylenetetrahydrofolate, the cofactor of thymidylate synthetase. Conversion of dUMP to dTMP and even DNA synthesis are impaired thereof. Many neuropsychiatric abnormalities are caused by cobalamin deficiency due to demyelination of peripheral nerves, the spinal cord, cranial nerves, and the brain (Lindenbaum et al. 1988; Lindenbaum et al. 1995; Stabler et al. 1990). Severe symptoms including decreased sensation, difficulty in walking, loss of control of bowel and bladder, optic atrophy, memory loss, dementia, depression, and psychosis result from cobalamin deficiency. Although till now the mechanisms of coabalmin deficiency leading to neuropsychiatric abnormalities are still unknown, impaired myelin synthesis, destruction of existing myelin or others have been recognized as reasons (Beck 1991; Hutto 1997). Cobalamin has been proved to be associated with the processes of fatty acid metabolism and DNA synthesis. Hence, the synthesis of myelin is depressed and myelin is incorporated into abnormal fatty acids in the cobalamine deficiency situation.

1.8. Nutritional aspects

A fatal disease, perniciuous anaemia, is a worldwide problem for both developed and developing countries. Especially, persons from European countries suffer from the cobalamin deficiency due to vegetarianism (Stabler and Allen 2004) and aging (Nelson 2001). People from India, Mexico, Central and South America, and selected areas in Africa also have a very severe dietary vitamin B_{12} deficiency problem (Stabler and Allen 2004), because of low animal product intakes. Low dietary vitamin B_{12} intake also results in hyperhomocysteinaemia and other neuropathies.

Therefore, a considerable interest is becoming drawn to synthesize and afford a high quality and cheap vitamin B_{12} to vegetarians and poor people.

1.8.1. Absorption

Absorption of vitamin B_{12} was investigated and two pathways were discovered (Abyad 2002; Baik and Russell 1999; Nelson 2001). Intrinsic factor (IF), a glycoprotein, is a normal constituent of gastric juice, which is generated by gastric parietal cells after stimulation by food and involved in the active process of absorption of vitamin B_{12}. Protein bound vitamin B_{12} is first released from food in the stomach by the action of acid and pepsin. In the stomach free vitamin B_{12} binds to haptcorrin, which is a protein to proctect acid-sensitive vitamin B_{12} through stomach and exists widely in saliva, gastric juice, bile, intestinal juice, and serum. Vitamin B_{12} is set free from haptcorrin by pancreatic enzyme in the alkaline environment of the small intestine, binding with IF to form a complex. This stable complex proceeds to ileum and is attached to specific membrane receptors of ileum. However, the capacity for absorbing vitamin is a maximum of 3µg at one meal. The other way of passive diffusion works as a complementarity, when large quantities of vitamin B_{12} are ingested. The rate of absorption by the passive process is 1% of vitamin B_{12}.

1.8.2. Sources of vitamin B_{12}

In the United States the dietary reference intake (DRI) of vitamin B_{12} is set at 2.4 µg per day (USDA 2000). The daily body loss of vitamin B_{12} is estimated to range from 2 to 5 µg per day (USDA 2000). Even though the daily recommended dosage of this vitamin was known, bioavailabilities of vitamin B_{12} from different food sources are totally varying. Animal based foods including meat, milk, egg, fish, and shellfish are considered as the main dietary sources of vitamin B_{12}, but for the plant based foods only cyanobacteria may contain vitamin B_{12} (Allen 2010; Barry et al. 2002; Venderley and Campbell 2006; Watanabe 2007). Vitamin B_{12} is synthesized only in certain bacteria (Martens et al. 2002). Vitamin B_{12} is accumulated in animal bodies by rumen bacteria (Perlman 1959) and by ingesting other animals. For humans animal

based foods are sustainable sources and some fermented plant based foods may also be sources. Some important and necessary foods are listed below (Table 1-1).

Table 1-1 Sources and bioavailability of various animal based foods. (Fischer et al. 1958; Heyssel et al. 1966; Kilshaw et al. 1982; USDA 2007)

Catalog	Vitamin B_{12} (ng/g)	Bioavailability (%)
Beef liver	833	10
Chicken	94.4	60
Turkey	330	60
Milk	3-4	65
Egg	9-14	3.7-8.9
Crustaceans	100	
Mollusk	about 1000	
Fish	30-89	20-40

The most popular vitamin B_{12} source of animal products is meat. According to United States Department of Agriculture database (USDA 2007), the cooked beef liver contains the highest amount of vitamin B_{12} among different meats, 83.3 µg per 100 gram. Other reliable sources are chicken at 9.44 µg per 100 gram and turkey at 33 µg per 100 gram (USDA 2007). Researchers (Ortigues-Marty et al. 2006) reported that 27-33% of this vitamin was destroyed when these meats were cooked, but the loss of water by evaporation can increase the content of vitamin B_{12} from 15.5% to 37%. Heyssel et al. (1996) reported that with increasing feeding amount of vitamin B_{12} (less than 3 µg per meal), bioavailability of vitamin B_{12} rises from averaged 56% to 89%. An average bioavailability of this vitamin for liver (normally at 80µg per 100 gram) is lower than 10%. The absorption rate of chicken meat is averaged at 60% (Doscherholmen et al. 1978).

Milk is a basic and important food for western people. Because of high daily intake of milk, milk and other dairy products contribute vitamin B_{12} to the general population, even including only 0.3 - 0.4 µg per 100 gram (USDA 2007). 65% of milk is absorbed (Daniel et al. 1953). The losses of vitamin B_{12} are significant from 30% to 50% through the different heating treatments (Kilshaw et al. 1982).

Relative to other animal food products the bioavailability of eggs is poor between 3.7% to 8.9% by different treatments (Doscherholmen et al. 1975; Levine and Doscherholmen 1983). Most of

this vitamin is accumulated in the egg yolk (Fischer et al. 1958), the content of vitamin B_{12} is about 0.9-1.4 µg per 100 gram (Fischer et al. 1958).

Crustacean and molluscs are popular and consumed widely. These animals contain large quantities of vitamin B_{12} due to vitamin B_{12} synthesizing microorganisms (Bourre and Paquotte 2008). The highest vitamin B_{12} content in crustaceans is almost 10 µg per 100 gram, but the content in molluscs is up to about 100 µg per 100 gram (USDA 2007). All of these animals are recognized as rich sources of vitamin B_{12}.

Fish has a high vitamin B_{12} content and contributes to the vitamin B_{12} supplementation in Asia. The content of this vitamin ranges from 3.0 to 8.9 µg per 100 gram of fish (USDA 2007). The losses of this vitamin from fish by different treatment range from 5% to 15% (Banerjee and Chatterjea 1963). Fish meat bioavailability of vitamin B_{12} is ranging from 20% to 40% (Doscherholmen et al. 1981).

Fermented foods including plant based food is a vital important and necessary source for people, especially for vegetarians. Perlman (1959) reported that *Aerobacter*, *Agrobacterium*, *Alcaligenes*, *Azotobacter*, *Bacillus*, *Clostridium*, *Corynebacterium*, *Flavobacterium*, *Micromonospora*, *Mycobacterium*, *Norcardia*, *Propionibacterium*, *Protaminobacter*, *Proteus*, *Pseudomonas*, *Rhizobium*, *Salmonella*, *Serratia*, *Streptomyces*, *Streptococcus*, and *Xanthomonas* are vitamin B_{12} producing genera. More attention has been paid to strains of *Propionibacterium shermanii* and *Pseudomonas denitrificans* because of their natural high vitamin B_{12} productivity and rapid growth. Theoretically, *Propionibacterium shermanii* and *Propionibacterium freudenreichii* are suitable for industry, because they have obtained the GRAS status from the United States Food and Drug Administration.

Pseudomonas denitrificans is successfully applied in commercial production of vitamin B_{12} by Aventis company (Fabregas et al. 2000). *Propionibacterium freudenreichii* and *Propionibacterium shermanii* that are used to make cheeses for thousands of years are also utilized in vitamin B_{12} production for human beings under strict anaerobic conditions (Mantere-Alhonen 1995). Hugenschmidt et al. (2011) reported that *Lactobacillus plantarum*

SM39 and *Propionibacterium freudenreichii* used in a cofermentation of whey produced up to 8 µg per mL of cobalamin. However, 5,6-dimethylbenzimidazole (Dmbi), an important precursor, can only be formed in the presence of oxygen by *Propionibacterium freudenreichii* and *Propionibacterium shermanii* (Hoellriegl et al. 1982). Because of this, after several days of fermentation under anaerobic conditions for *Propionibacterium freudenreichii* or *Propionibacterium shermanii*, the fermentation should be switched to aerobic conditions. In spite of this fact, Santos et al. (2008) found that the gene of cobT of *Lactobacillus reuteri* is 59% similar with *Salmonella typhimurium*, which means that *L. reuteri* has the ability to form Dmbi without oxygen.

Fermented and salted fish and fish sauce are preferred food by people in East Asia. The traditional Thailand fish sauce procedure (Saisithi et al. 1966) is beginning with Fish mixed with salt in the ratio of 3:1 on a concrete floor. The mixture is transferred to fermentation tanks, which are sealed for at least 6 months, until the fish is liquefied. The mush is filtered, transferred to earthenware containers and ripened under sun for 1 to 3 months. During this process, *Bacillus*, *Lactobacillus*, *Streptococcus*, *Micrococcus*, and *Staphylococcus* were found in fish sauces (Saisithi et al. 1966; Tanasupawat et al. 1998). These bacteria may be involved in the synthesis of vitamin B_{12}. These sauces appear to constitute a major source of vitamin B_{12} in Thailand, due to the considerable amounts of vitamin B_{12} from 0.8 to 2.4 µg per 100 gram (Hadioetomo 1983). But this source may not be suitable for humans as it contains a high amount of salt. The important part cobalt (16.3 ng per L) originates from sea water to synthesize this vitamin.

Tea is treated as a daily drink. By an investigation from Japan, vitamin B_{12} was detected in green tea, red tea and black tea leaves (Kittaka-Katsura et al. 2004). They found out that green tea contains 0.1-0.5 µg vitamin B_{12} per 100 gram, red tea contains about 0.7 µg per 100 gram and black tea can contain up to 1.2 µg per 100 gram (Kittaka-Katsura et al. 2004). For green tea, there are no special fermentation processes. Various bacteria and fungi are involved in the fermentation of tea, in different types of red and black teas (Greenwalt et al. 2000). Tea is rich in cobalt (0.18 µg per gram) (Han et al. 2005), affording enough precursor for vitamin B_{12}.

Table 1-2 The sources and bioavailability of fermentated foods

Catalog	Vitamin B_{12} (ng / g)	Fermentation microorganism
Fermented fish	8.0-24	*Lactobacillus*, Streptococcus*, Micrococcus, and Staphylococcus*
Green tea	0-5.0	Various bacteria* and fungi
Red tea	7	Various bacteria* and fungi
Black tea	12	Various bacteria* and fungi
Stinky tofu	1000	*Bacillus* sp.* and Lactic acid bacteria*
Tempeh	1	*Lactobacillus* spp.*, Citrobacter freundii, Klebsiella pneumonia*, Pseudomonas fluorescens, Streptococcus** and *Bacillus** and *Rhizopus* spp.
Natto	15	*Bacillus natto**
Soy sauce	1	fungi
Miso	1	fungi
Fermented whey	8000 ng / mL	*Lactobacillus plantarum* SM39* and *Propionibacterium freudenreichii**

a. The symbol * stands for microorganism to produce vitamin B_{12}

Soybean products including soy paste, soy sauce, tempeh, sufu, soy nuggets, stinky tofu, natto, and soy yogurts are regarded as a good and cheap source of vitamin B_{12} for people all around the world. Among them, stinky tofu contains the highest amount of vitamin B_{12} (100 µg per 100 gram) (Li et al. 2004), which is much higher than liver of animals. The production of stinky tofu is starting with tofu (Friberg and Hui 2005). The tofu is inoculated with *Bacillus* sp. and lactic acid bacteria and soaked into prepared brine, which includes cabbage, bamboo shoots, shrimp, and salt. This mixture is stored at 4 °C over night. Cobalt derives from bamboo shoots (Wu et al. 2005) and shrimp (Sinanoglou et al. 2007). Meanwhile shrimp is suspected to contribute a part of vitamin B_{12} to this product.

Tempeh may contain vitamin B_{12} during soaking step contamined by bacteria from the family of *Enterobacteriaceae* (Keuth and Bisping 1993; Keuth and Bisping 1994). Regarding the different spots and methods used by tempeh makers, the content of vitamin B_{12} may be varying from 0.1 to 14.8 µg per 100 gram (Denter and Bisping 1994; Liem et al. 1977). The traditional way to make tempeh is described below (Friberg and Hui 2005). Soybeans are cleaned and soaked overnight. The next morning the beans are dehulled, split and cooked in open kettles. After the beans are

cooled and dried, strains of *Rhizopus* are inoculated and the mixture is incubated at 30 °C for 48 hours. Then inoculated beans are wrapped into banana leaves. Keuth and Bisping (1993) have conducted a research of bacteria and fungi which may synthesize vitamin B_{12} in tempeh. They made a conclusion that *Rhizopus* has no capability to produce vitamin B_{12}. The overnight soaking of beans is the main cause for vitamin B_{12} production. Some bacteria existing naturally on the beans such as *Lactobacillus* (Feng et al. 2005), *Citrobacter freundii*, *Klebsiella pneumoniae*, *Pseudomonas fluorescens*, *Streptococcus* (Denter and Bisping 1994) and *Bacillus* (Nout et al. 1987) may generate this vitamin. Soybeans can accumulate cobalt from earth, which can offer enough precursors.

Natto is a typical Japanese traditional food, which includes 1.5µg per 100 gram (Hadioetomo 1983). Natto is produced from steamed soybean with *Bacillus natto* starter culture (Friberg and Hui 2005). The smell and taste of natto is not fit for all people due to the high content of ammonium.

Other fermented soybean products such as soy sauce (0.1 µg per 100 gram) and miso (0.1 µg per 100 gram) include low contents of vitamin B_{12}. Most of these products are fermented by fungi (Friberg and Hui 2005). The vitamin B_{12} may be produced by some contaminating bacteria. Miso uses rice, in which the cobalt content is low, as the medium for fermentation (Friberg and Hui 2005).

Fermented milk products are also a source of vitamin B_{12}. The concentrations of vitamin B_{12} in fermented milk drop dramatically down during 14 days, compared with fresh milk (Watanabe 2007). But with the help of fermentation, about 20 - 60 % of vitamin B_{12} present in milk is recovered in cottage cheese, hard cheese, and blue cheese (Arkbågea et al. 2003). The removing of whey is the main reason of vitamin B_{12} reduction. Hugenschmidt et al. (2011) reported that a co-fermentation of whey by *Lactobacillus plantarum* SM39 and *Propionibacterium freudenreichii* has achieved to obtain 8 µg per mL of cobalamin.

1.9. *Lactobacillus* spp.

The lactic acid bacteria (LAB) are comprised by a cluster of similar physiological and metabolic bacteria. They are characterized as gram positive cocci or rods with low G C content. They are non-spore forming and no respiring. The genera of LAB are *Lactobacillus, Leuconostoc, Pediococcus, Lactococcus, Streptococcus, Aerococcus, Carnobacterium, Enterococcus, Oenococcus, Tetragenococcus, Vagococcus, Weissella*, and the spore forming *Sporolactobacillus* (Meng et al. 2009). According to the end products of glucose metabolism, LAB can be divided into homofermentative and heterofermentative. Homofermetative LAB produce lactic acid as the major or sole end product. Those LAB producing equal mol amounts of lactate, carbon dioxide, and ethanol or acetic acid are recognized as heterofermentative LAB. LAB have the ability of inhibiting spoilage and pathogenic microorganisms by acidification and bacteriocins. LAB also give an organoleptic and textural profile to a fermented food.

The genera of *Lactobacillus* are involved in many parts of food procession in industry. Normally, the morphology of *Lactobacillus* varies from long to slender, sometimes bent rods to short, often coryneform, coccobcilli, and chain formation (Kandler and Weiss 1982). Growth temperature ranges from 2 to 53 °C; optimum generally is at about 30-40 °C. *Lactobacillus* spreads widely from milk products, grain products, meat, wine, beer, sour dough, water, sewage, silage, and even intestinal tract and vagina of homothermic animals including humans (Kandler and Weiss 1982). These kinds of bacteria are acid tolerant and can grow from pH 5.5 to 6.2. Some can survive below pH 5.0.

One of the species in L*actobacillus*, named *Lactobacillus reuteri* is able to colonize the gastrointestinal tract of humans and other animals (Casas and Dobrogosz 2000), and was isolated originally from human feces. It was determined to be a probiotic and possesses properties of lowering the blood cholesterol level in mice (Taranto et al. 2000) and anti-inflammatory activity in human cell lines (Ma et al. 2004). A unique ability of *L. reuteri* is to convert glycerol to reuterin, catalyzed by glycerol dehydratase, a vitamin B_{12} dependent enzyme (Taranto et al. 2003). This broad spectrum antimicrobial compound is a mixture of monomeric, hydrated monomeric,

and cyclic dimeric forms of 3-hydroxypropionaldehyde (3-HPA) (Taranto et al. 2003). Taranto (2000) demonstrated that *Lactobacillus reuteri* which belongs to lactic acid bacteria and possesses a GRAS (generally recognized as safe) status can synthesize cobalamin.

The ability of utilization of organic nitrogen of *Lactobacillus* is so weak that nitrogen will be a growth limit for *Lactobacillus*. *Lactobacillus* has a full proteolytic system, including proteinases and peptidases, to hydrolyze proteins into small peptides and peptides and amino acids (Rollan and Font de Valdez 2001). These small substances can be transported through cell membranes. The system plays a vital role not only in propagation of cells and acidification but also in developing of precursors for flavor (Rollan et al. 2005).

L. reuteri can use arabinose, fructose, galactose, glucose, gluconate, lactose, maltose, sucrose, ribose, melibiose, raffinose as carbon sources (Kandler and Weiss 1982). *L. reuteri*, belonging to heterofermentative LAB, has phosphoketolase. Instead of EMP (Embden-Meyerhof-Parnas) pathway for glucose degradation, hexose monophosphate or pentose pathway is used by these microorganisms (Jay et al. 2005). The reaction equation is as follows:

$$C_6H_{12}O_6 + ADP + Pi \longrightarrow CH_3CH_2OHCOOH + CH_3CH_2OH + CO_2 + ATP$$

In the pathway, NAD^+ is used as hydrogen receptors to form $NADH_2$. Only with help from other hydrogen receptors like acetaldehyde, the balance of NAD^+ and $NADH_2$ can be kept in balance to continue the pathway, but large concentrations of ethanol will inhibit the growth of cells. A conversion from glycerol to 1,3-propanediol (1,3 PD) was found by the coenzyme B_{12} dependent glycerol dehydratase and NAD^+ dependent oxidoreductase (Fig. 1.5) (Taranto et al. 2003). Glycerol is conversed into reuterin via coenzyme, and then NAD^+ dependent oxidoreductase renders reuterin to change into 1,3-propanediol, simultaneously with generation of NAD^+. During carbohydrate co-fermentation, glycerol is used as an alternative hydrogen acceptor. Depending on this economic metabolism, yield of ATP, growth and the accumulation of biomass are developed (Luthi-Peng et al. 2002b). This phenomena of auxiliary pathway was also found in other bacterial species such as *Klebsiella pneumoniae* (Huang et al. 2002). Some researchers also supposed that 3-HPA played a role of quorum sensing (Bauer et al. 2010b). *Lactobacillus reuteri* appears to

uniquely produce and store more 3-HPA than required, but for others 3-HPA functions as a transient metabolite that is immediately reduced to 1,3-propanediol. Glycerol is shown as an inducer of synthesis of cobalamin. However, lactic acid bacteria have a very limited activity to hydrolyze triglycerides of fat during ripening cheeses (Dupuis et al. 1993).

The accumulated 3-HPA can reversibly be dehydrated to acrolein (Fig. 1-5), hydrated to HPA hydrate and also dimerized to HPA cyclic dimer. Acrolein is a pulmonary toxicant and an irritant of mucous membranes (Esterbauer et al. 1991) and develops bitterness in wine (Noble 1994). At 20 °C, approximately 92% of acrolein is hydrated to 3-HPA, which is increasing with the rise of pH and decreasing of temperature (Bowmer and Higgins 1977). Acrolein decays faster under field conditions, due to hydration, volatilization, adsorption or uptake by organisms and sediments (Bauer et al. 2010a).

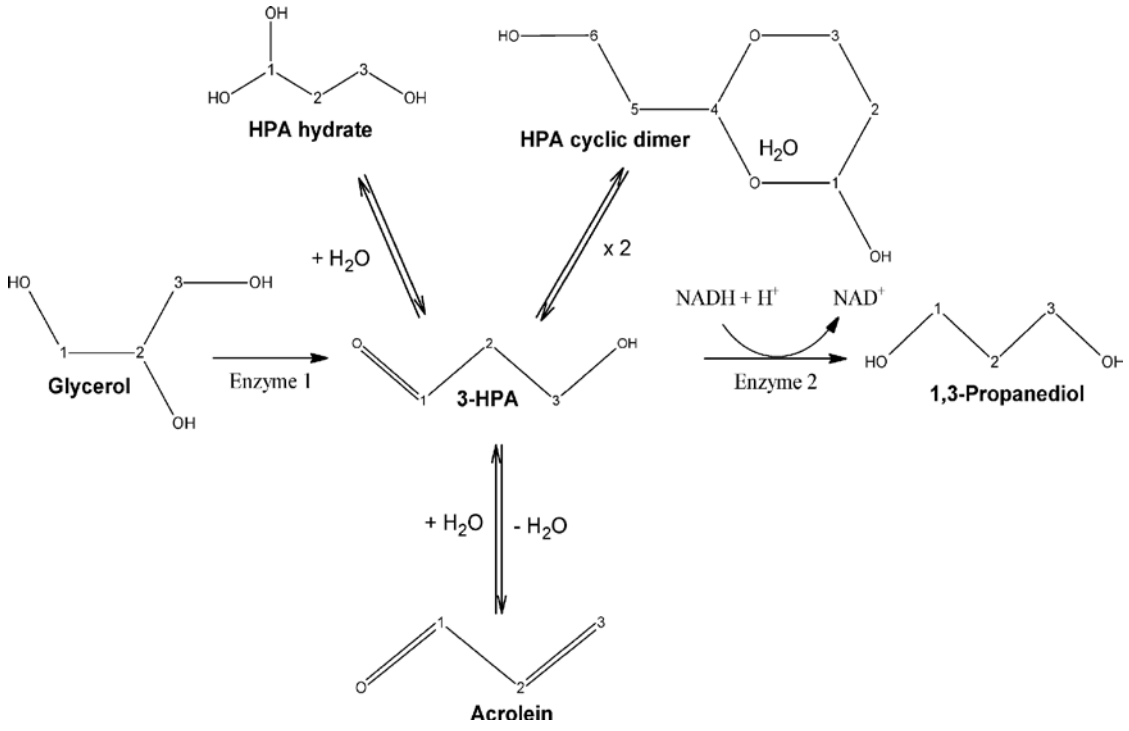


Fig. 1-5 the reactions from glycerol to HPA and the reversible reaction between HPA and derivative Enzyme1 indicates a vitamin B_{12} dependent dehydratase. Enzyme 2 designs an NAD dependent oxidoreductase enzyme. (Bauer et al. 2010a)

1.10. *Propionibactium* spp.

Propionibacterium (PBA) is a genus of bacteria producing large amounts of propionic acid besides acetate and CO_2 during fermentation (Cummins and Johnson 1984). The genus *Propionibacterium* is described as Gram positive, catalase positive, nonmotile, and non-spore forming rod and prefers predominately microaerophilic to anaerobic conditions (Cummins and Johnson 1984). The optimum growth temperature for propionibacteria ranges from 30 °C to 37 °C (Cummins and Johnson 1984). The optimum pH for propionibacteria is from 6.8 to 7.2 and pH minimum is 5.0 or 5.1 (Weber 1996). As high GC content bacteria, the G + C content of total genomic DNA of the genus *Propionibacterium* is from 53–67 % (NCBI), which can be used to distinguish from other propionic acid producing but low GC content bacteria, e.g. *Clostridium* (Cummins and Johnson 1984). Nowadays, some reseachers (Stackebrandt et al. 1997) suggested to classify them into the class of *Actinobacteria* in taxonomic terms. Propionibacteria can be isolated and counted from sodium lactate agar, in which 1.0 to 2.5 mm dull brown colonies with a lighter margin appear after 7 to 9 days of anaerobic fermentation at 30 °C (Tharmaraj and Shah 2003).

The genus *Propionibacterium* includes two principal groups of classical or dairy, and cutaneous propionibacteria distinguished on the basis of their habitats (Vorobjeva 2000).

Cutaneous propionibacteria are predominant anaerobic microorganisms found in normal human skin (Evans et al. 1950). These bacteria also can be isolated from intestinal tract (Vorobjeva 2000), facial acne (Evans et al. 1950), and even from wounds (Benediktsdottir and Kolstad 1984), bone marrow (Cummins and Johnson 1984) and tissue abscesses (Cummins and Johnson 1984). Five species of cutaneous propionibacteria (*Propionibacterium acnes*, *Propionibacterium avidum*, *Propionibacterium granulosum*, *Propionibacterium propionicum* and *Propionibacterium lymphophilum*) were involved in pathology of diseases (Richad and Keith 2004). *P. acnes* is a common contaminant of clinic specimens and seems clearly to cause lesions, although it is recognized to have a low level virulence (Guenthard et al. 1994). Till now, production of vitamin B_{12} by cutaneous propionibactera has not been described.

The group of classical propionibacteria was first isolated from Emmental cheese by Freudenreich and Jensen in 1906 (Cummins and Johnson 1984). In 1909 Orla-Jensen originally described *Propionibacterium* (Cummins and Johnson 1984). Four species of classical propionibacteria were isolated from cheese and other dairy products, and also some exist in soil, silage, fermenting olives, and also in intestines of rats (Cummins and Johnson 1984; Mantere-Alhonen 1995). Those are *Propionibacterium freudenreichii* with subspecies *freudenreichii*, *Propionibacterium globosum, Propionibacterium shermanii*, *Propionibacterium thoenii, Propionibacterium acidipropionici,* and *Propionibacterium jensenii* (Britz and Riedel 1991). The propionibacteria play a critical role in several industrial processes. These kinds of bacteria are widely used and applied in cheese making (Piveteau 1999), propionic acid production (Himmi et al. 2000), ensilage inoculums (Lind et al. 2005), cobalamin production (Quesada-Chanto et al. 1994) and probiotics production (Mantere-Alhonen 1995). Especially, *P. freudenreichii* is used to ripen Swiss type cheese and produce propionic acid, acetate, and CO_2, which influence the quality of cheese, synthesis of flavor compounds, and eyes of cheese (Piveteau 1999). *P. freudenreichii* ssp. *freudenreichii* and *P. freudenreichii* ssp. *shermanii* are distiguished by two tests. *P. freudenreichii* ssp. *freudenreichii* has an ability of nitrate reduction, but *P. freudenreichii* ssp. *shermanii* not. However, only *P. freudenreichii* ssp. *shermanii* can ferment lactose.

P. freudenreichii can utilize different carbon sources such as glucose, xylose, sucrose, lactate, and lactose (not for *P. freudenreichii* ssp. *freudenreichii*) as energy sources (Cummins and Johnson 1984). For PBA, hexoses are utilized via Embden-Meyerhof-Parnas (EMP) pathway and also pentose phosphate pathway exists, but most of glucose is utilized through the EMP pathway alone under anaerobic conditions (Piveteau 1999). The unique feature of PBA is that lactate is initially oxidized to pyruvate via a NAD^+ dependent lactate dehydrogenase (Piveteau 1999). Even though the same mol of sugars can generate much more ATP and trigger higher growth rates and cell yields than lactate, lactate is prefered by PBA to be taken up in a mixture of substrates (Lee et al. 1974). *Propionibacterium* sp. has a very strong activity of hydrolyzing triglycerides of fat, whose activity is 100 fold more lively than that of lactic acid bacteria (Dupuis et al. 1993).

Although normally lactate is recognized as a fast but not efficient energy substance in the cheese fermentation, Crow (1986) described that aspartate was metabolized to succinate and ammonia during lactate fermentation. Apartate is converted to fumarate and ammonia by the enzyme aspartase, and then subsequent reduction is from fumarate to succinate (Crow 1986). Serin, Alanin, and Glycin can also be degraded by *P. freudenreichii* (Weber 1996). As others (Langsrud et al. 1995) noted, *Propionibacterium* only contains 2 weak proteinases: one cell wall associated and one intracellular or membrane bound, but *Propionibacterium* includes a wide variety of peptidases such as amino peptidases, proline aminopeptidase, arginine aminopeptidase, leucin aminopeptidase, alanine aminopeptidase, glycine aminopeptidase, histidin aminopeptidase, phenylalanine aminopeptidase, and tyrosin aminopeptidase (Weber 1996). Large amounts of free proline will be produced when media contain pepetides (Cummins and Johnson 1984). Various volatile flavor compounds in cheese can be formed through the catabolism of amino acids by propionibacteria, such as branched-chain acids, which are important flavor compounds in cheese (Thierry and Maillard 2002). Different compounds of nitrogen and carbon sources do not only affect the taste of cheese, but also influence the growth of microorganisms.

Propionate is the main compound produced by reduction of pyruvate by PBA. Several vitamins are needed to join into this fermentation process. Biotin is a cofactor of pyruvate carboxytransphosphorylase; Thiamin functions as a component of dehydrogenases in oxidative phosphorylation of α-ketoacids; pantothenate is a constituent of CoA; riboflavin is a constituent of FAD and FMN; and cobalamin (Vorobjeva 2000). PBA can synthesize the last two kinds of vitamins.

Adenosylcobalamin (coenzyme B_{12}) is involved in the conversion from succinyl-CoA into methylmalonyl-CoA (Kellermeyer et al. 1964) (Fig. 1-6). Cobalamin is covalently bound to succinyl-CoA to generate free radicals (Marsh and Harding 1993; Woelfe et al. 1986). There is a hypothetical mode of action of this enzyme (Woelfe et al. 1986). The cleavage of Co-C covalent bond of coenzyme leads to the change of the charges of cobalt from +3 to +2 and also a 5'-deoxyadenosyl free radical. After a hydrogen atom of succinyl-CoA is taken by the radical, consequently the new radical is generated from succinyl-CoA. A rearrangement of the acyl-CoA

group to the position formerly occupied by the hydrogen atom in this new radical happens. After a hydrogen atom binds with product-like radical again, methylmalonyl-CoA and 5'-deoxyadenosyl radical are generated. The rebinding of Co-C bond renders the charge of cobalt back to +3 and the enzyme is ready for the new catalytic cycle.

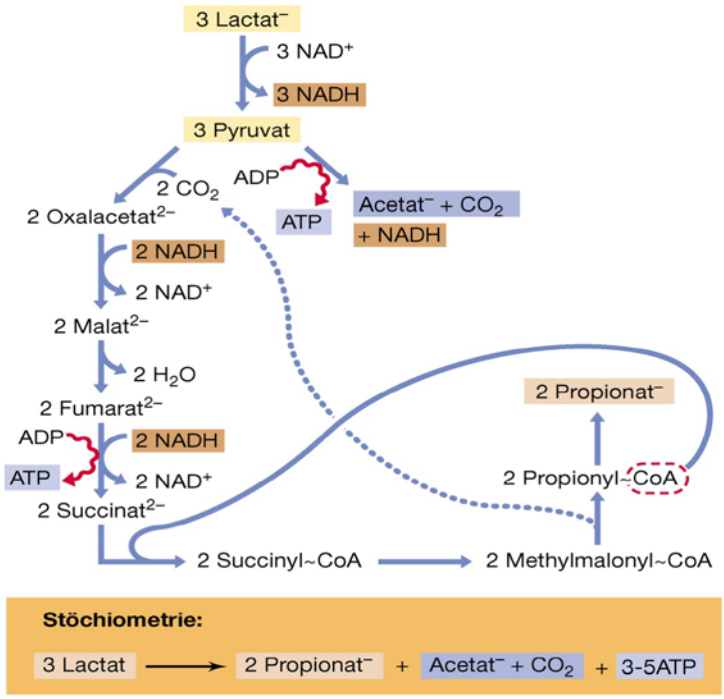


Fig. 1-6 The metabolic pathway of propionate fermentation (Madigan and Martinko 2009).

The production of propionate is obviously affected by oxygen. Some researchers (Miyano et al. 2000; Ye et al. 1999) found that under aerobic conditions, growth of PBA was much slower and the formation of propionate, acetate and succinate was inhibited and pyruvate accumulated. In this condition, the propionate was completely decomposed. However, under anaerobic conditions a large amount of propionic acid is produced and inhibits the growth of PBA (Foschino et al. 1988). Lactate fermentation is also influenced by the presence of nitrate. Nitrate is reduced to nitrite until nitrate is exhausted from the medium. Nitrite can be further converted into N_2 or N_2O. The production of propionic acid decreases and pyruvate accumulates, accompanying with high concentration of nitrate (Van Gent-Ruijters et al. 1975).

1.11. Soybean

Soybean, *Glycine max* (L.), belongs to the family *Leguminosae* and grows annually. Soybean seeds are yellow with spherical or long oval forms, but some are green, dark brown, or purplish black. There is evidence that soybean is originating from northern part of China almost 5000 years ago (Gai et al. 2002). During the development of soybean cultivation, Chinese gradually created various types of soybean products, such as tofu, soymilk, soy sprouts, soy paste, and soy sauce. With the development of methods of soybean cultivation, soybean was spread to Korea, Japan, and even to Europe and America. Currently, global soybean production is almost 180 million metric tons (Liu 2009). Soybean is usually regarded as an efficient and important nutrient source. High quality and quantity protein and oil compounds are made up out of soybeans. A high proportion of unsaturated fatty acids such as oleic, linoleic, and linolenic acids (two essential fatty acid) and all essential amino acids, which matched those required by humans, were found in soybeans (Liu 1997). However, the presence of lectin and inhibitors of digestion enzyme in consumption of raw soybean results in adverse nutritional and other effects (Friedman et al. 1991). With the purposes of enhancing the quality of soybean proteins, a heating treatment to inactivate the biologically active compounds of soybean was introduced.

1.11.1 Tofu

Tofu is a curd. It is prepared by coagulating traditional soymilk with a coagulant, resembling a soft cheese or a firm yogurt. It also can be defined as a water extracted, and acid or salt coagulant soy protein gel with water, lipids and other constituents. Liu An (Fig. 1-7) is recognized as inventor of tofu in Han Dynasty (122 BC).

Because of inexpensive, nutritious and versatile properties, tofu is still and always a popular product of soybeans in China, Japan and other countries. Because the healthy food of plant origin and vegetarian menu are popular worldwide in recent years, the sales market of tofu in the US was dramatically increasing from 380 millian dollars in 1980 to 2.6 billion dollars in 2003 (Liu 2009). Traditionally in eastern Asia, tofu, which is treated as substitution of meat, serves to cook together with other soups or vegetables. It also can be further processed into various secondary products

such as deep-fried tofu, grilled tofu, frozen tofu, dried-frozen tofu, and fermented tofu. New commercial products based on tofu that were recently developed in western countries result in invention of baked, flavored and smoked varieties. These kinds of further procession or new treatment can not only retain the beany taste but also impart the different types of flavoring to suit peoples' different tastes.

Fig. 1-7 Inventor of tofu: Liu An

Tofu is one of the best nutritious and natural soy products. Except for addition of coagulation, tofu can be made from whole soybeans. On a wet basis, a classical tofu with a moisture of 85% includes 7.8% protein, 4.2% lipids and 2 mg per gram of calcium; on a dry basis it contains 50% protein, 27% lipids and the remains are carbohydrates and minerals (Wang et al. 1983). Besides the character of enrichment of protein, tofu is also known as having a low content of unsaturated fat and no trans fatty acid and cholesterol (Ashton et al. 2000). All of the fat content in tofu is in the natural state. In the remaining compounds, isoflavones are one of the remaining nutraceutical constituents after procession. On a dry matter basis, the total isoflavones content ranges from 2.03 to 3.88 mg per gram, even though a big part of isoflavones were lost into whey and okara and some are changed in chemical form by modification during procession (Coward et al. 1993). Scientists (1994) have suggested that consumption of tofu may contribute to the relatively low rates of breast, colon, and prostate cancers in countries such as China and Japan (Messina et al. 1994).

Nowadays, there are varieties of tofu produced at home or at commercial plants in different regions. The basic principles and procedures are still the same as what Chinese invented 2000

years ago. But some modification, including variation of coagulation, different temperatures and equipment, are applied in order to suit the diverse requirements of tofu products. There are seven steps to produce tofu from soybeans.

1. Soaking: Dry whole soybeans are cleaned and soaked in water overnight. The ratio of volume of water and bean is normally 2 or 3 times to one.
2. Draining and rinsing: The soaked beans are drained and rinsed with fresh water 2 or 3 times.
3. Grinding: The overnight soaked beans are ground in a mill and simultaneously fresh water is added up to the volume of water 6 to 10 times as much as the bean volume. The slurry is deposited in a clean and big container.
4. Filtering: The bean slurry is filtered through a screen, cloth, or pressing sack. The residue, called okara, is separated from the slurry. In order to yield maximum volume of soymilk, okara is normally washed once or twice with cold or hot water, stirred and re-pressed. The total bulk volume of raw soymilk is almost 6 to 10 times of the original.
5. Cooking: The raw milk is heated up to boiling with frequently stirring to avoid burning of the milk at the bottom of the cooking vessel and maintained in this situation for 5 to 10 min. A treatment of boiling the slurry before filtering is popular in Japan.
6. Coagulating: The powdered coagulant, such as calcium sulfate, glucono-delta-lactone (GDL) or magnesium chloride, is dissolved in hot water. The heated milk and dissolved coagulant are transferred and mixed into another container. The mixture is kept to stand for about 20 to 30 min for coagulation to complete.
7. Molding: The formed soy curd is broken by stirring, and then transferred into a shallow forming box lined with cloths at each edge. By pressing out whey, tofu becomes firm and hard. Some tofu such as silken tofu and lactone tofu is made without the pressing steps. The cooled tofu cake is served or immersed in cold water for short storage or sale at local markets.

Regarding the procedure mentioned, tofu making technology in some aspects has similarities with cheese making. Both of them involve protein coagulation and whey removal. The three

differences are also obvious. Tofu is made from plant milk but cheese from cows milk. The coagulant for cheese is rennet but for tofu is a salt. As a nutriceutical and natural food, tofu does not contain cobalamin.

1.11.2. Fermented soybean products

The fermentation treatment introduced in soybean procession results in the production of large amounts of amino acid, vitamins and long shelf life to suit the demand of seasoning and nutrition. There are seven traditional fermented soyfoods, including soy paste, soy sauce, tempeh, sufu, soy nuggets, natto and soy yogurts. In the fermented products of natto and soy yogurt only bacteria are involved. Especially, preparation of natto requires *Bacillus natto* (Wang and Fung 1996). For other products, fungi such as *Aspergillus* sp. and *Rhizopus* sp. are used for fermentation. Preparation of tempeh and natto takes only a few days, while the rest types in general demand several months. The soy products, including soy paste, soy sauce, sufu and soy nuggets, are normally recognized as seasonings in cooking. The high salt content, added during the second fermentation stage, as well as the side products like alcohols and acetate can inhibit spoilage of these products. As no salt foods, tempeh, soy yogurt and natto can contribute protein and oil as well as their special flavor.

Compared with our project, sufu and stinky tofu (fermented tofu) are the traditional soy foods invented in China around 1500 years ago. There are two stage fermentations from tofu to sufu and stinky tofu. After tofu cubes are completed, fungi (*Rhizopus chinensis* var. *chungyuen*, *Mucor hiemalis* etc.) are inoculated on to tofu and fermented until their mycelia cover the surface of tofu. Subsequently, they are soaked in brine (or partially covered with salt) and immersed in wine, miso, or soy sauce. The Chinese soybean cheese, sufu, can offer salty taste with a smooth texture and stinky flavour. After the fermentation of tofu, protein nitrogen decreases significantly, and the amino nitrogen and ammonia nitrogen increase (Friberg and Hui 2005). Finally, sufu contains 60-70% moisture and 12-17% protein, 63-68% protein nitrogen, 10-12% amino nitrogen, and 7-10% ammonia nitrogen (Friberg and Hui 2005). On the dry matter basis, sufu contains 0.42-0.78 mg per 100 gram vitamin B_{12} (Li et al. 2004). The difference between stinky tofu and

sufu is that different microorganisms are used. The tofu curds mixed with the stinky brine contain *Bacillus* sp., *Streptococcus* sp., *Enterococcus* sp. and *Lactobacillus* sp. (Lu et al. 2007). As a result of mixture of bacteria, the pH value of tofu in the stinky brine first drops from 6.5 to 4.6 due to the production of lactic acid and growth of bacteria (Lu et al. 2007). Subsequently, the pH increases gradually to 7.5 as the protein is hydrolyzed and further degraded to form ammonia (Lu et al. 2007). Because of this alkali situation, the alkali tolerant bacteria grow instead of the lactic acid bacteria. The stinky tofu is considered as a fermented and alkaline food. On the dry matter basis, stinky tofu contains 9.8 - 18.8 mg per 100 gram vitamin B_{12} and up to 3400 mg per liter of supernatant (Li et al. 2004). However, strange taste and odour from ammonia of sufu and stinky tofu can hardly be accepted by western people.

2. Aim of the work

The present work is aimed to produce vitamin B_{12} in tofu by fermentation with microorganisms. The study consists of the following stages:

2.1. Single fermentation

Microorganisms from our culture collection and isolates are screened and selected to determine which strains not only grow well on tofu but also produce vitamin B_{12} in tofu. The best strain should be used to do further optimization of carbon sources, nitrogen sources, Dmbi and cobalt by single factor, FFD, and CCD designs.

2.2. Cofermentation

L. reuteri and *P. freudenreichii* have to be used to do a cofermentaion to improve production of vitamin B_{12} in tofu. A series of supplementations and different environmental conditions should be investigated to optimize and to ensure the maximal production of vitamin B_{12}.

2.3. Scaling up

A 1 kg batch fermentation and a fed batch experiment should be used to improve vitamin B_{12} production in a bigger scale. Natural materials such like seaweed, spinach and banana should be selected to substitute pure chemical substances to reduce cost and avoid harm from cobalt chloride.

Simultaneously, a novel and safe method should be developed to produce high vitamin B_{12} contents by microorganisms. This product should be used to offer enough nutrition to vegetarian people, so that they do not have to take vitamin B_{12} in form of pills.

3. Materials and Methods

All values of tofu are given on wet weight basis, unless stated otherwise. Other necessary information is listed in Appendix.

3.1. Microorganisms and media cultures

All microorganisms were taken from the culture collection of division of Food Microbiology and Biotechnology, Institute of Food Chemistry, University of Hamburger isolated from natural samples. The stocks of cells were maintained in glycerol 80% (v/v) at -70°C. The bacteria were propagated in de Man, Rogosa, and Sharpe (MRS) broth (Carl Roth, Karlsruhe, Germany) in standing cultures over night for 37°C.

Table 3-1 Names and sources of microorganisms used in our work

Name	Source
Lactobacillus delbrueckii spp. *lactis* DSM 20355	Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ)
Lactobacillus sp. LMH T.10	Isolated out of tempe sambal
Lactobacillus rhamnosus EK4	Emmental Cheese
Lactobacillus casei spp. *casei*	DSMZ
Lactobacillus rhamosus DSM 20021	DSMZ
Streptococcus sp. LMH T.11	Tempe Perringan
Lactobacillus sp. LMH T.4	Isolated out of cooked bean
Propionibacterium freudenreichii spp. *shermanii* DSM 20270	DSMZ
Propionibacterium. freudenreichii spp. *freudenreichii* DSM20271	DSMZ
Lactobacillus sp. LMH T.12	Islolated out of tempe from North Jakarta
Lactobacillus reuteri DSM 20016	DSMZ

Lactobacilli Broth AOAC (Difco, Kansas, US)

Lactobacilli Agar AOAC was used for maintaining stock cultures for microbiological assays of vitamins and amino acids, and also used for preparing inocula for microbiological assays of vitamins and amino acids.

38 g powder was suspended in 1 L of double distilled water (DDW) and mixed thoroughly. The mixture was heated with frequent agitation and boiled for 2-3 min to completely dissolve the powder and autoclaved at 121 °C for 15 min.

Vitamin B_{12} assay broth (Merck, Darmstadt, Germany)

Vitamin B_{12} Assay Medium was used for determining vitamin B_{12} concentration by the microbiological assay technique.

83 g of dehydrated vitamin B_{12} (*Lactobacillus*) Assay Broth together with 2 mL Tween® 80 was dissolved in 1 L DDW by briefly boiling. The pH was controlled at 6.8 at 25 °C. The solution was sterilized for 10 min at 115 °C.

Table 3-2 Composition of Lacotbacilli Broth AOAC

Substance	Content (g)
Peptonized milk	15
Yeast extract	5
Dextrose	10
Tomato juice base	5
Dipotassium phosphate	2
Polysorbate	1
Bidistilled water	1000 mL
pH	6.6 – 7.0

MRS broth (Carl Roth, Karlsruhe, Germany)

MRS is an abbreviation for de Man, Rogosa and Sharpe, which are names of its inventors: This medium was designed to favour the luxuriant growth of lactobacilli for lab study.

52 g powder was dissolved in 1 L, adjusted to pH between 6.2 - 6.5 and autoclaved at 121 °C for 12 min.

Modified MRS broth agar (Carl Roth, Karlsruhe, Germany)

This agar was adjusted to pH 5.0, by which the growth of P*ropionibacterium* spp. is inhibited. This media can be used to count *L. reuteri* in tofu. 62 grams of powder was used. Then 12 gram per L of agar was added in the formulation. Others are the same as MRS broth.

Sodium lactate agar (NaLa agar) (Tharmaraj and Shah 2003)

Propionibacteria can be distinguished from *L. reuteri* and calculated by formed colonies that were dull brown with lighter margin of 1.0 to 2.5 mm in diameter by this medium.

The medium was prepared, adjusted to pH 7.0 and autoclaved at 121°C for 15 min.

Table 3-3 Ingredients of vitamin B_{12} assay broth

Substance	Content
D(+)Glucose, anhydrous	40 g
Casein hydrolysate "Vitamin-free"	15 g
L-Asparagine	200 mg
L-Cysteinium chloride	200 mg
L-Cysteine	400 mg
L-Tryptophane	200 mg
Adenine	20 mg
Guanosin	40 mg
Uracil	20 mg
Xanthine	20 mg
4-Aminobenzoic acid	2 mg
L(+)Ascorbic acid	4 g
D(+)Biotin	0.01 mg
Calcium D(+)pantothenate	1 mg
Folic acid	0.2 mg
Nicotin acid	2 mg
Pyridoxol hydrochloride	4 mg
Pyridoxamine hydrochloride	0.8 mg
Riboflavin	1 mg
Thiaminium dichloride	1 mg
Di-potassium hydrogen phosphate	1 g
Iron(II) sulfate	20 mg
Potassium dihydrogen phosphate	1 g
Magnesium sulfate	400 mg
Manganese(II) sulfate	20 mg
Sodium acetate, anhydrous	20 g
Sodium chloride	20 mg
Bidistilled water	1000 mL
pH	6.6 - 6.8

3.2. Buffers

Sodium acetate buffer (pH 4.5)

18 grams of Sodium acetate and 9.8 grams of acetate were mixed and filled to 1000 mL.

Sodium acetate buffer (pH 6.0)

54.6 grams of Sodium acetate was added into 20 mL of 1 mol per L acetate. Then the solution was filled up to 500 mL.

Table 3-4 Ingredients of MRS broth

Substance	Content (g)
Peptone	10
Yeast extract	4
Beef extract	8
Glucose	20
Dipotassium phosphate	2
Sodium acetate	5
Ammonium citrate	2
Magnesium sulphate ($MgSO_4$)	0.2
Manganese sulphate	0.05
Tween 80	1
Bidistilled water	1000 mL
pH	6.2 – 6.5

Table 3-5 Ingredients of NaLa agar

Substance	Content (g)
Pancreatic digest of casein	10
Yeast extract	10
Sodium pyruvate	2
Glycine	2
Dipotassium hydrogen phosphate	0.25
Tween 80	0.5
agar	12
Sodium chloride	0.5
Sodium lactate	10
Bidistilled water	1000
pH	6.9 – 7.1

3.3. Methods

3.3.1. HPLC

Vitamin B_{12} was extracted from 10 gram of product with 50 mL sodium acetate buffer (pH 6.0) in the presence of sodium cyanide (1%) (Merck, Darmstadt, Germany) and heated in a water bath (Type 1004 water bath, GFL Gesellschaft für Labortechnik, Burgwedel, Germany) for 40 min at 90 °C. After that, the solution was adjusted to pH 7.0 and mixed with 10 mL hexane (Extra pure N-hexane, Merck, Darmstadt, Germany) and then centrifuged for 15 min at 4010 g (Varifuge 3.0, Heraeus centrifuge, Heraeus Instruments, Hanau, Germany). The aqueous solution was collected

and passed through a solid phase extraction column (SPE) (CEC181M6 United Chemical Technologies, Bristol, PA, USA), which had been washed with 3 mL methanol (Merck, Darmstadt, Germany) and 3 mL double distilled water (DDW) (from Reversed osmosis Mill-Q water (18Ω) (Millipore, Billerica, MA, USA)), with the aid of a pump (AL 15, Knf Neuberger, Hamburg, Germany) to control the speed of drops at 1 drop per second. After 3 times washing by DDW, 3 mL methanol was utilized as the eluate. After the solvent was evaporated to dryness, the residue was dissolved by 1 mL DDW. The solvent was filtered through a membrane filter (0.2 µm) (Macherey-Nagel, Düren, Germany) and the filtrate was analyzed by HPLC using a RP-18 column (250*4mm I.D., 5µm, Merck, Darmstadt, Germany).

All of the chromatographic separations were carried out at room temperature. A flow of 0.5 mL per min, methanol with 0.1% formic acid (A) (Merck, Darmstadt, Germany) and DDW with 0.1% formic acid (B), which were degassed by an ultrasonic water bath (Sonorex TK 52, ultrasonic waterbath, Bandelin electronics, Berlin, Germany), were used as mobile phases and the gradient elution was programmed as follows; 0-2 min 20% A; 2-3 min 20-25% A; 3-11 min 25-35% A; 11-19 min 35-20% A; 20-22 min 100-100% A; 22-26 min 100-20% A; 26-36 min 20% A. The injection volume was 100 µL and the column eluate was monitored by DAD at 361 nm.

The HPLC system utilized in this study consisted of a Merck Hitachi L-7100 pump (Merck Hitachi, Darmstadt, Germany), a Merck Hitachi D-7000 interface (Darmstadt, Germany), a Merck Hitachi L-7250 sample injector (Darmstadt, Germany) and a Merck Hitachi L-7455 Diode Array Detector (Darmstadt, Germany). The software, D-7000 HPLC-System-Management HSM, Version 4.1 (Hitachi, Tokyo, Japan) was used to manage the HPLC system, and to evaluate and quantify the results. The column used was a reversed phase RP-18 column (250*4mm I.D., 5µm, Merck, Darmstadt, Germany).

The standard solutions were prepared by adding cyanocobalamin (Merck, Darmstadt, Germany) into the tofu matrix and its concentrations were adjusted from 200 ng per mL to 10,000 ng per mL.

Samples were dealt with four different methods as follows;

Grinding method

10 gram of samples were ground with liquid nitrogen and mixed with 50 mL of buffer pH 6.0.

Ultrasonic method

Ultrasonication (UPS200, ultrasonic processor, Hielscher ultrasound technology, Teltow, Germany) was used with 10 gram of tofu mixed with 50 mL of buffer pH 6.0 in the presence of sodium cyanide. The parameters were set as follows: cycle 0.5 and amplitude 55%.

Microwave method

10 gram of samples were put into microwave oven (R-15AM, Sharp, Osaka, Japan) and mixed with 50 mL of buffer pH 6.0 in the presence of sodium cyanide at 200 watt.

Boiling method

10 gram of samples were boiled in a water bath (Type 1004 water bath, GFL Gesellschaft für Labortechnik, Burgwedel, Germany) and mixed with 50 mL of buffer pH 6.0 in the presence of sodium cyanide at 100 °C.

3.3.2. Microbiological assay

Extraction

2 grams of samples were ground in liquid nitrogen and mixed with 10 mL of sodium acetate buffer (pH 4.5), 0.4 mL of sodium cyanide (0.5%) and 40 mL of double distilled water. Samples were homogenized for 15 min and heated for 10 min at 121°C. After cooling 0.6 mL of metaphosphoric acid solution (10%) (Fluka, Buchs, Switzerland) are added in ice water bath for 30 min. The solution was filled up to a volume of 100 mL. The solution was filtered through a 0.2 µm filter. The fluid was divided into two 10 mL portions. One portion was adjusted to pH 6.0 and the volume was adjusted with double distilled water to 20 mL. The other portion was adjusted to pH 11-12 and heated to 121°C for 30 min, and then adjusted with double distilled water to 20mL.

Preparation of test microorganisms

Lactobacillus delbrueckii spp. *lactis* DSM 20355 was used as a test microorganism, which was inoculated into a Lactobacilli Broth AOAC broth at 37 °C for 24 hours. Then the culture was

centrifuged at 4,000 g for 5 min (Biofugo pico centrifuge, Heraeus Instruments, Hanau, Germany) and rinsed three times with physiological saline and adjusted to a microbial count of 10^8 bacteria per mL. A mixture was made by 3 mL of culture and 100 mL of vitamin B_{12} assay broth.

Calibration and samples

A storage solution of vitamin B_{12} (20 mg/L) was prepared by dissolving 10 mg cyanocobalmin in 130 mL ethanol (Merck, Darmstadt, Germany) and filling up to 500 mL.

A concentration series of 0, 10, 20, 30, 40, 50 pg of cyanocobalamin per mL was made by adding 200 µL of mixtures and 100 µL of corresponding reference solutions, which was incubated at 37°C for 48 hours. As with reference solutions, 100 µL of sample solutions and 200 µL of mixtures were filled into holes of a 96-well microtitre plate.

Evalutation

The optical density (OD) value of 96-well microtitre plates was measured at 620 nm against blank by a microplate spectrophotometer (SLT Labinstruments, Salzburg, Austria). A calibration curve was made by applying OD values as the Y axis and concentrations as the X axis on the linear ordinate.

3.3.3. Methods for different parameters and fermentation

General fermentations

Tofu was cut into 2*2 cm blocks. 100 gram of tofu were weighted, transferred into 500 mL flasks and fermented.

pH

Solid tofu was separated from samples, diluted by a ratio of 1 to 10 and measured by a pH meter (pH 21, Hanna, Rhode Island, USA). Fermented liquid was transferred from samples and also measured by a pH meter.

Microorganism counts

Solid tofu was separated from samples and diluted by 0.9 % sodium chloride (Carl Roth, Karlsruhe, Germany) from 1 to 10. 0.1 mL of this solution was spread onto solid agars such as pH 5.0 MRS and NaLa agar. 0.1 mL of fermented liquid was transferred and spread onto solid agars as above. The dishes with pH 5.0 MRS were incubated at 37°C for 72 hours and the dishes with NaLa agar were incubated at 30 °C for 7 days. *Lactobacillus reuteri* DSM20016 was counted through MRS agar by forming white shiny smooth colonies. *P. freudenreichii* spp. *freudenreichii* DSM 20271 was indentified and counted through NaLa agar by the morphology of 1.0-2.5 mm, dull brown, lighter margin colonies. A substraction method, as a control, could also be used to determine the counts of propionibacteria by reducing the number of *L. reuteri* from the total count in NaLa agar (Tharmaraj and Shah 2003).

Microorganism optical density values

1 gram of samples with 9 mL of 0.9 % sodium chloride buffer were stomached (Stomacher 400, Seward Medical, London, UK) and diluted. The concentrations of cells were determined by measuring the optical density at 600 nm (Spectrophotometer U-2000, Hitachi, Tokyo, Japan).

Metabolites

The important metabolites were detected through HPLC (Merck Hitachi, Darmstadt, Germany) with an organic acid column (850 BP-OA H^+, 300*7.8mm, Benson Polymeric, Sparks, USA) as a solid phase. All of the chromatographic separations were carried out at 60 °C. A flow of 0.6 mL per min with 26 mM sulfuric acid was used as mobile phase. The injection volume was 10 µL and the column eluate was monitored by Lachrom RI Dectector. 1mL of fermentation liquid was centrifuged for 10 min at at 17,000 g for 5 min (Biofuge pico centrifuge, Heraeus Instruments, Hanau, Germany) 10 µL of supernatant was used and injected into HPLC.

The HPLC system utilized in this study consisted of a Merck Hitachi L-6200 pump (Merck Hitachi, Darmstadt, Germany), an ERC-3512 degasser (Erma, Tokyo, Japan), an AS-2000A sample injector (Darmstadt, Germany) and a Merck Hitachi Lachrom RI detector L-7490

(Darmstadt, Germany). The software, D-7000 HPLC-System-Management HSM, Version 4.1 (Hitachi, Tokyo, Japan) was used to manage the HPLC system, and to evaluate and quantify the results. The column used was an 850 BP-OA H+ Organic Acid Column (300*7.8mm, Benson polymeric, Sparks, USA).

Amino acid analysis by ninhydrin colorimetric method

Ninhydrin is originally yellow. After reacting with amino acid, ninhydrin is forming a final complex, deep purple, which is detected by this method.

Alpha-amino acid + 2 ninhydrin → CO_2 + aldehyde + final complex + $3H_2O$

Ninhydrin reacts with any compound with a free alpha-amino group, which exists in all amino acids, peptides, or proteins. However, the decarboxylation reaction only proceeds for free amino acids, it does not happen for peptides and proteins. Thus, theoretically only free alpha-amino groups can lead to the development of color.

Reagents

Ninhydrin reagent solution was prepared by dissolving 0.5 g of ninhydrin into 10 mL enthanol and filling up to 100 mL with sodium acetate buffer (pH 6.0).

20 mg of glycine (Carl Roth, Karlsruhe, Germany) was dissolved into 100 mL DDW to form 200 µg/mL of storage solution.

Procedures

A concentration series of 0, 10, 20, 30, 40, 50, µg of glycine per mL was made. 1 mL of ninhydrin reagent solution was added into 4 mL of reference solutions and incubated at 100 °C for 15 min.

5 mL of fermentation liquid was centrifuged for 10 min at 4010 g (Centrifuge 5840R, Eppendorf, Hamburg, Germany). 4 mL of supernatant was added into 1 mL of ninhydrin reagent solution and incubated at 100 °C for 15 min.

After cooling to room temperature in a cold water bath, the solutions were determined by spectrophotometer at 570 nm.

3.4. Isolating and Screening of vitamin B_{12} producing microorganisms

Sufu and stinky tofu (Wangzhihe, Beijing, China) was separated from samples and diluted by 0.9 % sodium chloride (Carl Roth, Karlsruhe, Germany) from 1 to 10. 0.1 mL of this solution was spread onto solid agars such as Plate count agar (Carl Roth, Karlsruhe, Germany). Single colonies were picked up and maintained in glycerol 80% (v/v) at -70°C.

Microorganisms from the culture collection of division of Food Microbiology and Biotechnology, Institute of Food Chemistry, University of Hamburg and isolations were growing in tubes with vitamin B_{12} Assay Broth (Merck, Darmstadt, Germany), which contained all the necessary nutrients except vitamin B_{12}. Growth was determined by measuring the optical density at 600 nm in a spectrophotometer (U-2000, Hitachi, Tokio, Japan). A 100 µL sample was transferred from a tube which indicated growth of bacteria into another tube with vitamin B_{12} (*Lactobacillus*) Assay Broth and this passage was repeated eight times. The strain that survived all the process showed an ability to produce vitamin B_{12}.

The selected microorganisms were inoculated in 10 mL of MRS broth at 37°C for 24 hours. The medium was centrifuged, washed by 0.9% sodium chloride three times and adjusted to 10^8 bacteria per mL. 1 mL of this medium was inoculated into 100 grams tofu under anaerobic conditions for 3 days.

Vitamin B_{12} was analyzed by microbiological assay.

3.5. Single fermentation

All of the optimization experiments were used the same conditions described in the next single fermentation paragraphs. *Lactobacillus reuteri* was inoculated into 10 mL of MRS broth at 37°C for 24 hours. The inoculation medium was centrifuged at 4,000 g for 5 min (Biofuge pico, Heraeus Instruments, Hanau, Germany), washed by 0.9% sodium chloride three times and adjusted to 10^8 bacteria per mL.

3.5.1. Screening of nitrogen sources

To selecte the most fitting nitrogen source, a series of anaerobic fermentations with 1 mL of this medium together with 100 grams of tofu and 100 mL of water with 10 grams of corresponding nitrogen sources such as peptone, yeast extract, and casein were processed at 37 °C for 4 days. The vitamin B_{12} was analyzed by a microbiological assay.

3.5.2. Optimization of moisture

Experiments of moisture optimization were designed and conducted as described below. 1 mL of inoculation medium was inoculated into 100 grams of tofu supplied with a series of corresponding water of 10 mL, 50 mL, 100 mL, and 300 mL and was fermented anaerobically at 37°C for 4 days. The vitamin B_{12} was analyzed by a microbiological assay.

3.5.3. Optimization of fermentation days

In order to find out the optimal time to stop fermentation, an anaerobic fermentation including 1 mL of this medium, 100 grams of tofu and 100 mL water was performenced at 37°C for 4 days. Vitamin B_{12} was analyzed by microbiological assay and a growth curve was made by measuring optical density values.

3.5.4. Screening of carbon sources

With an aim of checking the most efficient carbon source, a series of anaerobic fermentations with 1 mL of this medium together with 100 grams of tofu and 100 mL of water supplied with 10 grams of corresponding carbon sources such as glucose, fructose, sucrose, glycerol, corn flour, rice flour, maltose, and soluble starch were perfomanced at 37°C for 3 days. Vitamin B_{12} was analyzed by a microbiological assay.

3.5.5. Effects of glucose, glycerol, and fructose

3.5.5.1. Effects of glucose, glycerol, and fructose, in vitamin B_{12} test broth

100 μL of medium was transferred into 200 mL of modified vitamin B_{12} test broths and was fermented anaerobically at 37 °C for 3 days. Combinations of glucose, glycerol, and fructose were shown in Table 3-6, in order to confirm if they affect the production of vitamin B_{12}. Vitamin B_{12} was measured on the 3rd day by HPLC.

Table 3-6 Designs of combinations of glucose, glycerol, and fructose in vitamin B_{12} test broths

g/L	Glucose	Glycerol	Fructose
1	40	0	0
2	40	0	0.05
3	40	0	0.1
4	40	0.04	0.01
5	40	0.04	0.05
6	40	0.04	0.1

3.5.5.2 Effects of glucose, glycerol, and fructose in tofu

Though positive effects were checked in pure media, they had also to be checked in tofu. 1 mL of inoculation medium was transferred into 100 grams of tofu with 100 mL water and fermented at 37 °C for 3 days. Combinations of glucose, glycerol, and fructose are shown in Table 3-7. Vitamin B_{12} was measured on the 3rd day by HPLC. The metabolites were also analyzed by HPLC. A growth curve was made by measuring OD value. Fermented liquid was transferred from samples and also measured by a pH meter.

Table 3-7 Designs of combinations of glucose, glycerol, and fructose in tofu.

g/kg	Glucose	Glycerol	Fructose
1	20	0	0
2	20	2	0.5
3	20	2	2
4	20	5	0.5
5	20	5	2

3.5.6. Optimizations of fermentation by *Lactobacillus reuteri*

Data analysis SAS (statistical analysis system) was applied to perform the regression analysis of experiment data. The fit quality of equation was checked by the coefficient value of R^2, and its significance was checked by the value of F test. The significant coefficient was tested by a T-test. The level of significance was given as ***$p<0.01$, **$p<0.05$, and *$p<0.1$.

3.5.6.1 Fractional factorial design (FFD)

To identify the significant important ingredients in the synthesis of vitamin B_{12}, factorial designs were calculated and conducted to expose information about most important features and interactions between two factors or among these factors. Compared with one factor experiment, this design can identify the most important factor in relatively few experiments, without loss of the information from main effects. A first-order model fitted to the data obtained from FFD experiments was sufficient for approaching from a remote region to the optimum vicinity.

In view of fractional factorial design principle, 2^{5-1} design ('2' indicates each factor has two levels; '5' indicates five factors; '1' indicates half fraction to be run) with four center points was chosen to reveal the information of five factors, but only 20 times experiments were requested. A basic medium in a glass flask was prepared out of 100 gram of tofu, and 100 mL of DDW. All the designs (Table 3-8) were fermented under anaerobic conditions at 37 °C for 3 days. In order to investigate the effective factors, a first-order model was fitted to the data obtained from FFD experiment by SAS. Vitamin B_{12} was measured on the 3rd day by HPLC. Fermented liquid was transferred from samples and also measured by a pH meter.

3.5.6.2 Steepest design

After the most important factors were found, the concentration of the factors should be moved forward to the optimum vicinity. The FFD results represented an incline. Along the path of steepest ascent, a maximum increase of responses could be obtained by varying factors in a relative range determined by counter lines. A basic medium in a glass flask was prepared out of 100 gram of tofu and 100 mL of DDW. For the supplementations, $CoCl_2$ was fixed at 2.5 mg/kg and DMBI at 3.5 mg/kg, fructose at 1.5 g/kg. The changing direction of both varieties and

experimental design are shown in Table 3-9. The concentration of glucose was decreasing at interval by 1 g/kg and the concentration of glycerol was increasing serially by 0.25 g/kg.

3.5.6.3. Central composite designs (CCD) design

The response surface method (RSM) is represented based on the results of FFD. However, the center points are not fixed in the maximum region by comparing the value of center points with other values through T test. As a result of that, the steepest ascent method was applied in order to investigate the initial experiment region, along the path of the steepest ascent till no increase in the response was observed. In order to describe the nature of response surface of an optimum region through the steepest ascent experiment, the central composition design with 5 coded levels was performed. Glucose (X_1), glycerol (X_2) were chosen as independent variables.

Table 3-8 Experimental design of FFD

Run	X_1	X_2	X_3	X_4	X_5
1	1	1	-1	-1	1
2	-1	1	-1	-1	-1
3	0	0	0	0	0
4	0	0	0	0	0
5	1	-1	1	1	-1
6	-1	1	1	-1	1
7	1	-1	-1	1	1
8	1	-1	-1	-1	-1
9	-1	-1	1	-1	-1
10	-1	1	1	1	-1
11	-1	-1	-1	-1	1
12	0	0	0	0	0
13	1	1	-1	1	-1
14	-1	1	-1	1	1
15	1	-1	1	-1	1
16	1	1	1	1	1
17	-1	-1	-1	1	-1
18	1	1	1	-1	1
19	1	1	1	-1	-1
20	0	0	0	0	0

X_1 = (X1 -20)/1.5, X_2 = (X2 -0.5)/0.25, X_3 = (X3 -2.5)/1.5, X_4 = X4 -1.5, X_5 = (X5-1.75)/1.75. X1, X2, X3, X4, and X5 stand for natural variables of glucose (g/kg), glycerol (g/kg), fructose (g/kg), $CoCl_2$ (mg/kg), and Dmbi (mg/kg).

Table 3-9 Experimental design of the ascent

Run	X_1	X_2
1	12. 5	2
2	13. 5	1.75
3	14. 5	1.5
4	15. 5	1.25
5	16. 5	1
6	17. 5	0.75
7	18. 5	0.5

X_1 and X_2 stand for the natural variables of glucose and glycerol (g/kg).

For a 2^2 CCD design with 4 axial points (α=1.414) with five replications in the center point of two factors, a set of 13 runs experiment (Table 3-10) was carried out. A second-order polynomial equation was fitted to the data by the multiplied regression procedure. For two factors the equation was:

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_{11} X_1 * X_1 + \beta_{21} X_2 * X_1 + \beta_{22} X_2 * X \qquad \text{(Eq. 3-1)}$$

Y is the predicted response; β_0 is the intercept coefficient; β_1 , β_2 are the linear coefficients; β_{12} are the interaction coefficients; β_{11} , β_{22} are the quadratic coefficients (Eq. 3-1)

Table 3-10 Experimental design and results of a central composite design

Run	X_1	X_2
1	-1	-1
2	1	-1
3	-1	1
4	1	1
5	-1.414	0
6	1.414	0
7	0	-1.414
8	0	1.414
9	0	0
10	0	0
11	0	0
12	0	0
13	0	0

X_1 = (X1 -16.5)/3.5, X_2 = (X2 -1)/0.5. X1 and X2 stand for the natural variables of glucose (g/kg) and glycerol (g/kg).

3.6. Cofermentation

For all optimization experiments the same conditions described in next co-fermentation paragraphs were used. *Lactobacillus reuteri* was inoculated in 10 mL of MRS broth at 37 °C for 24 hours. *Propionibacterium* sp**.** was inoculated in 10 mL of MRS broth at 30 °C for 48 hours. These inoculation media were centrifuged at 4,000 g for 5 min (Biofuge pico, Heraeus Instruments, Hanau, Germany), washed by 0.9% sodium chloride three times and adjusted to 10^8 bacteria per mL.

3.6.1. Cofermentation of *Lactobacillus reuteri* and *Propionibacterium* spp. in vitamin B_{12} test broths.

Due to unsatisfying results of the single fermentation, a co-fermentation was performed. Anaerobic fermentations based on 200 mL of modified vitamin B_{12} test broth were processed with various combinations of inoculation of 100 µL of *L. reuteri* and 100 µL of *Propionibacterium* sp. and temperatures at 37 °C for 4 days. Combinations are shown in Table 3-11. Vitamin B_{12} was measured on the 3rd day by HPLC. Fermented liquid was transferred from samples and also measured by a pH meter. The growth curve was made by measuring OD value.

Table 3-11 Designs of co-fermentation at different temperatures in vitamin B_{12} assay broth

Run	*Lactobacillus reuteri*	*Propionibacterium freudenreichii* spp.	Temperature (°C)
1	--------	*P. freundenreichii* spp. *shermanii*	30
2	*L. reuteri*	*P. freudenreichii* spp. *freudenreichii*	37
3	*L. reuteri*	--------	37
4	--------	*P. freudenreichii* spp. *freudenreichii*	30
5	*L. reuteri*	*P. freudenreichii* spp. *freudenreichii*	30
6	*L. reuteri*	*P. freundenreichii* spp. *shermanii*	37
7	*L. reuteri*	*P. freundenreichii* spp. *shermanii*	30

3.6.2. Co-fermentation in tofu by *Lactobacillus reuteri* and *Propionibacterium freudenreichii* spp. *freudenreichii*

As experiments in pure media had led to satisfying results, co-fermentation was conducted in tofu. A basic medium in a glass flask was prepared out of 100 gram of tofu, 100 mL of DDW and 10 g of glucose. There were three parallel designs. A basic medium with 1 mL of *Lactobacillus reuteri* inoculation medium was fermented at 37 °C for 8 days. A basic medium with 1 mL of *Propionibacterium freudenreichii* inoculation medium was fermented at 37 °C for 8 days. A basic medium with 1 mL of *Lactobacillus reuteri* inoculation medium and 1 mL of *Propionibacterium freudenreichii* inoculation medium was fermented at 30 °C for 8 days. Vitamin B_{12} was measured by a microbiological assay. Fermentation liquid was transferred from samples and also measured by a pH meter. The growth curves were made by measuring OD value. The metabolites were analyzed by HPLC.

3.6.3. Co-fermentation on tofu supplemented with glycerol and glucose at different temperatures.

Regarding to knowledge of both of carbohydrates and temperatures, complex and complicated combination designs and experiments were performed in order to interpret effects of factors. A basic medium in a glass flask was prepared out of 100 of gram tofu, 100 mL of DDW, and 1 mL of *Lactobacillus reuteri* inoculation medium and 1 mL of *Propionibacterium freudenreichii* inoculation medium. Anaerobic fermentations based on the basic medium were processed with various combinations (Table 3-12) of glucose, glycerol, and temperatures at 30 °C and 37 °C for 8 days. Vitamin B_{12} was measured by HPLC. Fermentation liquid was transferred from samples and also measured by a pH meter. Growth curves were made by OD values. Metabolites were analyzed by HPLC.

3.6.4. Effects of supplements

Because of the complex structure and the complicated synthesis procedure of vitamin B_{12}, abundances of precursors and factors was involved into vitamin B_{12} formation. The inoculation medium was cultured and treated as described in the paragraph above. A basic medium in a glass

flask was prepared out of 100 gram of tofu, 100 mL of DDW, 0.1 gram glucose, 1 mL of *Lactobacillus reuteri* inoculation medium and 1 mL of *Propionibacterium freudenreichii* inoculation medium.

Table 3-12 Designs of different concentrations of glucose, glycerol, and temperatures

Runs	Glucose (g/kg)	Glycerol (g/kg)	Temperature (°C)
1	0.5	0	37
2	5	0	37
3	0	0.5	37
4	0.5	0	30
5	5	0	30
6	0	0.5	30

Important precursors and factors (Table 3-13) which have an effect on the production of vitamin B_{12} were supplemented into a basic medium and fermented at 30 °C for 7 days. Vitamin B_{12} was measured on the 7th day by HPLC

Table 3-13 Design of different supplementations in different concentrations

Runs	Substances	Concentration (g/kg)
1	Lactose	1
2		10
3	Succinyl	0.1
4		1
5	1,2-Propanediol	0.1
6		1
7	$CoCl_2$	0.5
8	L-Threonine	1
9	L-Methione	1
10	Glutamate	1
11	Fructose	0.1
12		1
13	Riboflavin	5 mg /kg
14		50 mg/kg
15	Glycine	0.1
16		1
17	Betaine	0.1
18		1
19	Dmbi	5 mg /kg
20		50 mg/kg

3.6.5. Optimization of fructose and glycine

A basic medium in a glass flask was prepared out of 100 gram of tofu, 100 mL of DDW, 0.1 gram glucose, 1 mL of *Lactobacillus reuteri* inoculation medium and 1 mL of *Propionibacterium freudenreichii* inoculation medium.

Concentrations of fructose added were 0.05, 0.1, and 0.5 g/kg. Concentrations of glycine added were 1, 5, 10, and 15 g/kg. Vitamin B_{12} was measured on the 7th day by HPLC. The metabolites were analyzed by HPLC. Growth curves were made by the spread plate method.

3.6.6. Optimization of $CoCl_2$, vitamin B_2, and betaine

After selection experiments, an emphasis attention was paid to these three precursors. The inoculation medium was cultured and treated as described in the paragraph above. A basic medium in a glass flask was prepared out of 100 gram of tofu, 100 mL of DDW, 0.1 gram of glucose, 1 mL of *Lactobacillus reuteri* inoculation medium and 1 mL of *Propionibacterium freudenreichii* inoculation medium.

3.6.6.1 Effects of riboflavin, Dmbi, and fermentation days

Furthermore, riboflavin, and Dmbi played a vital important role in the synthesis of vitamin B_{12}. 12 µg of riboflavin and 20 µg of Dmbi were added into the basic medium. All of the fermentations were processed at 30 °C for 7 days by two steps, first anaerobic, then aerobic. The fermentations with riboflavin or Dmbi were designed as 5 sets including 2 days of anaerobic fermentation, 3 days of anaerobic fermentation, 4 days of anaerobic fermentation, 5 days of anaerobic fermentation and 6 days of anaerobic fermentation. Vitamin B_{12} was measured on the 7^{th} day by HPLC.

3.6.6.2. Effects of $CoCl_2$

Further on, cobalt is also a limiting factor for vitamin B_{12} production. This fermentation medium was made out of the basic medium, 40 µg of riboflavin and a series out of $CoCl_2$ of 0.2 mg, 2mg and 20 mg. Vitamin B_{12} was measured on the 7^{th} day by HPLC.

3.6.6.3. Full factorial design of riboflavin, betaine, and $CoCl_2$

Furthermore, 2^3 designs with two center points were chosen to reveal information of 3 factors. 10 times experiments were requested. A basic medium in a glass flask was prepared out of 100 gram of tofu, 100 mL of DDW, 0.1 gram glucose, 1 mL of *Lactobacillus reuteri* inoculation medium and 1 mL of *Propionibacterium freudenreichii* inoculation medium. All designs (Table 3-14) were fermented at 30 °C under anaerobic conditions for 5 days and under aerobic conditions for 2 days. In order to investigate effective factors, a first-order model was fitted to the data obtained from FFD experiment by SAS. Vitamin B_{12} was measured on the 7th day by HPLC.

Table 3-14 Experiment designs of Full Factorial design

Run	X_1	X_2	X_3
1	-1	-1	-1
2	-1	-1	1
3	-1	1	-1
4	-1	1	1
5	1	-1	-1
6	1	-1	1
7	1	1	-1
8	1	1	1
9	0	0	0
10	0	0	0

X_1 = (X1 -200)/100, X_2 = (X2 -5000)/2500, X_3 = (X3 -500)/250, X1, X2, and X3 stand for natural variables of riboflavin (µg/kg), $CoCl_2$ (µg/kg), and betaine (mg/kg).

3.6.6.4. Further optimization of riboflavin

Moreover, regarding to the importance of riboflavin, a further single factor experiment was performed. The fermentation medium was made out of the basic medium, 0.2 mg of $CoCl_2$, and 0.1 g of betaine with a serial corresponding riboflavin supplement of 0.8, 1, 1.2, 1.6, and 2 µg. The fermentation was carried out at 30°C for 5 days under anaerobic conditions and for 2 days under aerobic conditions. Vitamin B_{12} was measured on the 7^{th} day by HPLC.

The fermentation media was made up by the basic medium 0.2 mg $CoCl_2$, and 0.1 g betaine. The fermentation was carried out at 30°C in 5 days anaerobic and 2 days aerobic conditions. 1.6 µg of

riboflavin were added into the fermentation flasks on the 0, 2nd, 3rd, and 4th day. The vitamin B_{12} was measured on the 7th day by HPLC.

3.6.7. Effects of pH, temperature, oxygen, and inoculation titer of microorganisms

The fermentation conditions should also be thought about since they influence the physiology and morphology of microorganisms. A basic medium in a glass flask was prepared out of 100 gram tofu, 100 mL of DDW, 0.2 gram of glucose, 16 µg of riboflavin, 0.2 mg $CoCl_2$, 0.5 g of betaine, 1 mL of *Lactobacillus reuteri* inoculation medium and 1 mL of *Propionibacterium freudenreichii* inoculation medium.

3.6.7.1. Initial pH

The initial pH was set at 6.0, 6.5, 7.0, 7.5, and 8.0 before autoclavation. The fermentation was carried out at 30°C for 5 days under anaerobic conditions and for 2 days under aerobic conditions. Vitamin B_{12} was measured by HPLC. Fermentation liquid was transferred from samples and also measured by a pH meter. Growth curves were made by the spread plate method. Metabolites were analyzed by HPLC.

3.6.7.2. Temperatures

Every bacterium has its own optimal growth temperature. Hence a manifold temperature design was performed. The fermentation medium was adjusted to pH 7.0 before autoclavation. Fermentations were carried out at 28, 30, 35, 37 °C for 5 days under anaerobic conditions and for 2 days under aerobic conditions. Vitamin B_{12} was measured by HPLC. Fermentation liquid was transferred from samples and also measured by a pH meter. Growth curves were made by the spread plate method. Metabolites were analyzed by HPLC.

3.6.7.3. Effects of oxygen

CobG is a oxygen dependent enzyme involved in cobalamin synthesis of *Propionibacterium* spp.. Also, *Propionibacterium* spp. are found to produce Dmb only when exposed to oxygen. Therefore an oxygen supplementation experiment was conducted. The fermentation medium was adjusted to

pH 7.0 before autoclavation. Fermentations were conducted at 30 °C. The first group was fermented for 5 days under anaerobic conditions and for 2 days on a rotary shaker (200 rpm) (Type 3015 shaker, GFL Gesellschaft für Labortechnik, Burgwedel, Germany) aerobically. The second group was fermented for 6 days anaerobically and 1 day on a rotary shaker (200 rpm) (Type 3015 shaker, GFL Gesellschaft für Labortechnik, Burgwedel, Germany) aerobically. The third group was fermented for 7 days anaerobically. Vitamin B_{12} was measured by HPLC. Fermentation liquid was transferred from samples and also measured by a pH meter. Growth curves were made by the spread plate method. Metabolites were analyzed by HPLC.

3.6.7.4. Inoculation titers of microorganisms

Different initial cell numbers of bacteria can lead to totally different results. The fermentation medium was adjusted to pH 7.0 before autoclavation. All fermentations were performed at 30°C for 5 days anaerobically and for 2 days aerobically. The first group was inoculated with 1 mL of *Lactobacillus reuteri* inoculation medium and 1 mL of *Propionibacterium freudenreichii* inoculation medium. The second group was inoculated with 0.5 mL of *Lactobacillus reuteri* inoculation medium and 0.5 mL of *Propionibacterium freudenreichii* inoculation medium. The third group was inoculated with 0.1 mL of *Lactobacillus reuteri* inoculation medium and 0.1 mL of *Propionibacterium freudenreichii* inoculation medium. Vitamin B_{12} was measured by HPLC. Fermentation liquid was transferred from samples and also measured by a pH meter. Growth curves were made by the spread plate method. Metabolites were analyzed by HPLC.

Additionally, other treatments of tofu with *L. reuteri* were further introduced. The fermentation medium was adjusted to pH 7.0 before autoclavation. The fermentation was conducted at 30°C for 5 days anaerobically and for 2 days aerobically. All fermentations were inoculated with 0.5 mL of *Propionibacterium freudenreichii* inoculation medium and 0.01 mL, 0.1 mL and 1 mL of *Lactobacillus reuteri* inoculation medium were added into fermentation media. For the first group, all of the inoculation volumes of *L. reuteri* were fermented as normal. For the second group, 0.01mL, 0.1 mL and 1 mL of *Lactobacillus reuteri* inoculation medium were fermented for 1 day and then 0.5 mL of *Propionibacterium freudenreichii* inoculation medium was inoculated and pH

was adjusted to 6.5 to start fermentation for 7 days. For the third group, three levels of *L. reuteri* inoculation medium were fermented for 2 days and then 0.5 mL of *Propionibacterium freudenreichii* inoculation medium was inoculated and pH was adjusted to 6.5 to start fermentation for 7 days. For the fourth group, three levels of *L. reuteri* inoculation medium were fermented for 3 days and then 0.5 mL of *Propionibacterium freudenreichii* inoculation medium was inoculated pH was adjusted to 6.5 to start fermentation for 7 days. Vitamin B_{12} was measured by HPLC. Fermentation liquid was transferred from samples and pH was also measured by a pH meter. Growth curves were made by the spread plate method. Metabolites were analyzed by HPLC. Amino acids were detected by the ninhydrin method.

3.7. Scaling up and fed batch

3.7.1. Scaling up to 1 kg tofu with 1 g, 5 g, and 7 g of glucose

After small size experiments, a scale up experiment was done. The basic medium in a glass flask was prepared out of 1 kg of tofu, 1 L of DDW, 80 µg of riboflavin, 2 mg of $CoCl_2$, 0.5 g of betaine, 5 mL of *Lactobacillus reuteri* inoculation medium and 5 mL of *Propionibacterium freudenreichii* inoculation medium.

The fermentations with supplementation of 1 g, 5 g and 7 g of glucose were performanced at 30 °C for 5 days anaerobically and for 2 days aerobically. Vitamin B_{12} was measured by HPLC. Fermentation liquid was transferred from samples and pH was measured by a pH meter. Growth curves were made by the spread plate method. Metabolites were analyzed by HPLC.

3.7.2. Fed batch

With the target to improve the product, fed batch fermentations would be a good choice. The inoculation medium was cultured and treated as described in the paragraph above. A basic medium in a glass flask was prepared out of 1 kg of tofu, 1 L of DDW, 5 g of glucose, 160 µg of riboflavin, 2 mg of $CoCl_2$, 1 g of betaine, 5 mL of *Lactobacillus reuteri* inoculation medium and 5 mL of *Propionibacterium freudenreichii* inoculation medium.

A fed batch design was made. For the first fed batch fermentation, 4 gram of glucose were added after 4 days of fermentation. For the second fed batch fermentation 4 gram of glucose were added after 5 days of fermentation. For the third fed batch fermentation 4 gram of glucose were added after 6 days of fermentation. For the fourth fed batch fermentation 1 gram of glucose was added every day. For the fourth fed batch fermentation 2 gram of glucose were added every 2 days. Vitamin B_{12} was measured by HPLC. Fermentation liquid was transferred from samples and also measured by a pH meter. Growth curves were made by the spread plate method. Metabolites were analyzed by HPLC.

3.8 Fermentations with natural substrates

In order to lower cost and to fit for the requirements of bio food and vegetarian menus, many fresh and nutritional materials were put to the experiments. The inoculation medium was cultured and treated as described in the paragraph above. A basic medium in a glass flask was prepared out of 1 kg tofu, 1 L of DDW, 5 mL of *Lactobacillus reuteri* inoculation medium and 5 mL of *Propionibacterium freudenreichii* inoculation medium.

Natural materials such as banana, spinach, and seaweed were used to replace the pure chemical substances. Besides supplementation at the beginning, 10 grams of banana were added every day up to the 4th day. An orthogonal experimental design was made (Table 3-15), which can explain the effect of main factors by fewer experiments without main information loss. The results were calculated and analyzed by intuitive analysis method, which was fast and easy to solve the complex problems because of complicated compounds. Vitamin B_{12} was measured by HPLC. Fermented liquid was transferred from samples and pH was also measured by a pH meter.

Table 3-15 An $L_4(2^3)$ orthogonal experiment design of fermentations with natural substances

	Banana (X_1)	Spinach(X_2)	Seeweed(X_3)
1	1	1	1
2	1	-1	-1
3	-1	1	-1
4	-1	-1	1

X_1 = (X1 -200)/100, X_2 = (X2 -400)/200, X_3 = (X3 -400)/200, X1, X2, and X3 stand for natural variables of banana (g/kg), spinach (g/kg), and seaweed (g/kg).

3.9. Preculture and culture optimization

During tofu producing, lots of waste liquid is produced, which contains carbohydrates, nitrogen and even other growth factors. A design of preculture based on waste liquid was made. *Lactobacillus reuteri* was inoculated in 10 mL of MRS broth at 37 °C for 24 hours. *Propionibacterium freudenreichi*i was inoculated in 10 mL of MRS broth at 30 °C for 48 hours. These inoculation media were centrifuged, washed by 0.9% sodium chloride three times and adjusted to 10^8 bacteria per mL. 100 µL of *L. reuteri* and *P. freudenreichii* was inoculated into 10 mL of modified waste liquid at 37°C for 24 hours and at 30°C for 48 hours. An FFA design (Table 3-16) was made to find out key factors to improve the growth of microorganisms.

Table 3-16 Experiment design of FFD for preculture

Run	X_1	X_2	X_3	X_4
1	-1	-1	1	1
2	-1	1	-1	-1
3	1	-1	-1	1
4	-1	1	-1	1
5	-1	-1	-1	-1
6	1	1	-1	-1
7	1	-1	1	-1
8	1	1	1	1
9	0	0	0	0
10	0	0	0	0
11	0	0	0	0
12	0	0	0	0

X_1 = (X1 -0.6)/0.1, X_2 = (X2 -0.7)/0.1, X_3 = (X3 -0.2)/0.2, X_4 = (X4 -0.5)/0.5. X1, X2, X3, X4, and X5 (g/L) stand for natural variables of meat extract, peptone, yeast extract, maize extract.

3.9.1. Further single factor optimzations

For *L. reuteri*, maize extract plays a critical role in enrichment of microorganisms. Corresponding concentrations of maize extract at 0.5, 1, 5, 10, 15, 20, 25, and 30 g per L were done. Fermentations with 0.4 g/L of meat extract were performanced at 37 °C for 24 hours.

For *P. freudenreichii*, meat extract plays a critical role in increments of microorganisms. Corresponding concentrations of maize extract were made at 0.5, 1, 5, 10, 15, 20, and 25 g per L.

Fermentation with 0.5 g/L of maize extract were performed at 37 °C for 24 hours. The values were determined by a spectrophotometer (SLT Labinstruments, Salzburg, Austria) at 600 nm.

3.9.2. Heme preculture

The pathway to produce vitmain B_{12} can also synthesize heme, which can inhibit the pathway at high concentrations. A hypothesis of reversed evolution was proposed. 100 µL of *Lactobacillus reuteri* and *Propionibacterium freudenreichii* were inoculated in 10 mL of modified vitamin B_{12} assay broth with heme (10 mg per L) at 37 °C for 24 hours and at 30 °C for 48 hours and transferred into the same medium. Then passages were repeated up to 20 times. In comparison to this, the inoculation into B_{12} assay broths for 20 generations was used as control.

100 µL of *Lactobacillus reuteri* and *Propionibacterium freudenreichii* cultured in modified heme medium after 20 generations were inoculated into 100 mL of both normal and modified media at 37°C for 24 days and at 30 °C for 48 hours. The control microorganisms were treated in the same way. Vitamin B_{12} concentration was determined by HPLC.

3.10. Model

A Lotkae Volterra model of competition, historically proposed in ecology as a mechanistic model, was introduced into our work to interpret the interacting impacts between both microorganisms in different conditions. An assumption was made that both microorganisms were grown naturally without any inhibition from themselves.

$$\frac{dL}{dT} = \mu_{\text{maxL}} L\left(\frac{Q_1}{1+Q_1}\right)\left(1-\frac{L-aP}{L_{\text{max}}}\right) \qquad (3\text{-}2)$$

$$\frac{dP}{dT} = \mu_{\text{maxP}} P\left(\frac{Q_2}{1+Q_2}\right)\left(1-\frac{P-bL}{P_{\text{max}}}\right) \qquad (3\text{-}3)$$

L and P stand for population densities of *Lactobacillus reuteri* and *Propionibacterium freudenreichii* at time t. Q1 and Q2 respectively represent the physiological state of both microorganisms. μ_{maxL} and μ_{maxP} separately show the maximum growth of both species and L_{max}

and P_{max}. The coefficients of a and b means the interspecific competition paramenters of *Propionibacterium freudenreichii* on *Lactobacillus reuteri* and vice versa.

This work was done with the help of Dr. Chao Xiong from Wuhan Universtiy to use least squares method with Matlab to estimate coefficients a and b.

According to the assumption we have made, $Q_i/(1+Q_i)$ was set as 1. The integration of equation was made from t_{i-1} to t_i. These kinds of differential equations ((3-6), (3-7)) can normally not be dissolved. Hence, least squares method was introduced to estimate the coefficients a and b by Matlab (Version 5.3.0.10183, Mathworks Inc). The transpose of A is A^T.

$$\ln L_{1i} - \ln L_{1i-1} = \mu_{\max L}(t_i - t_{i-1}) - \frac{\mu_{\max L}}{L_{\max}} A_{1i} - \frac{\mu_{\max L} a}{L_{\max}} A_{2i} \quad ---(3-4)$$

$$\ln P_{1i} - \ln P_{1i-1} = \mu_{\max P}(t_i - t_{i-1}) - \frac{\mu_{\max L}}{P_{\max}} A_{1i} - \frac{\mu_{\max P} b}{P_{\max}} A_{2i} \quad ---(3-5)$$

$$A_{ki} = \int_{t_{i-1}}^{t_i} L(t)dt$$

$$i = 1,2,3...m$$

$$AX = B_L \quad ----(3-6)$$

$$AX = B_P \quad ----(3-7)$$

$$A = \begin{bmatrix} t_1 - t_0 & -A_{11} & -A_{21} \\ : & : & : \\ t_m - t_{m-1} & -A_{1m} & -A_{2m} \end{bmatrix}$$

$$X = \begin{bmatrix} \mu_{\max L} \\ \frac{\mu_{\max L}}{L_{\max}} \\ \frac{\mu_{\max L} a}{L_{\max}} \end{bmatrix}$$

$$Y = \begin{bmatrix} \mu_{\max P} \\ \frac{\mu_{\max P}}{P_{\max}} \\ \frac{\mu_{\max L} b}{P_{\max}} \end{bmatrix}$$

$$B_L = \begin{bmatrix} \ln\frac{L_1}{L_0} & \ln\frac{L_2}{L_1} & ... & \ln\frac{L_m}{L_{m-1}} \end{bmatrix} ----(3-8)$$

$$B_P = \begin{bmatrix} \ln\frac{P_1}{P_0} & \ln\frac{P_2}{P_1} & ... & \ln\frac{P_m}{P_{m-1}} \end{bmatrix} ----(3-9)$$

$$X = (A^T A)^{-1} A^T B_L \quad ----(3-10)$$

$$Y = (A^T A)^{-1} A^T B_P \quad ----(12)$$

4. Results

An average value plus standard deviation (X ± SD) was used to express results of measurements and calculations. The standard deviation was used to plot as error bars in graphs. All values were based on a wet weight, unless stated otherwise. Values with two or three asterisk superscripts were significantly different (***$p<0.01$, **$p<0.05$, and *$p<0.1$) through statistic analysis of variance (ANOVA).

4.1. HPLC

4.1.1. Stability of cobalamin

The stability of cyanocobalamin plays an important role in the extraction and recovery, as all samples were boiled in a water bath for 40 min at 90 °C. 2,000 ng of cyanocobalamin were dissolved in a buffer (pH 6.0) and put in a water bath for 20, 40, 60, and 80 min at 100 °C. The recoveries of all these treatments were not significantly different by statistical analysis of ANOVA. The boiling treatment from 0 to 80 min does not obviously destroy cobalamin in the pH 6.0 buffer, although the recovery after 40 min was reduced a little bit (Fig. 4-1). The treatment can be used to release cobalamin from bound proteins.

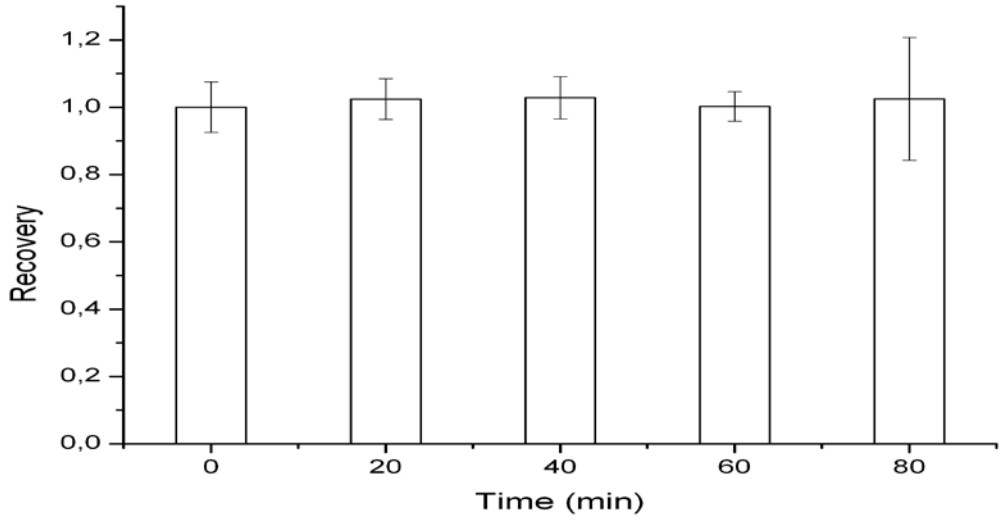


Fig. 4-1 Effect of heating time on the recovery of cobalamin in a buffer (pH 6.0)

4.1.2. Effects of pH on SPE procedures

SPE can not only purify the samples, but also concentrate vitamin B_{12} by polar effects. Nevertheless, polarity of molecules is altered by changing of pH. 2,000 ng of cyanocobalamin was added into 10 gram of sample. The samples were handled as described before passing SPE. Then the solution was adjusted to pH 4.0, pH 5.0, pH 6.0, pH 7.0, and pH 8.0 and passed through SPE to calculate the recovery. The recovery dramatically increased up to 81.4% from pH 4.0 to pH 7.0, but decreased again at pH 8.0 (Fig. 4-2). As a result of ANOVA, the recovery at pH 7.0 is significantly different with others. A conclusion can be drawn that at pH 7.0 most of the cyanocobalamin can be detected.

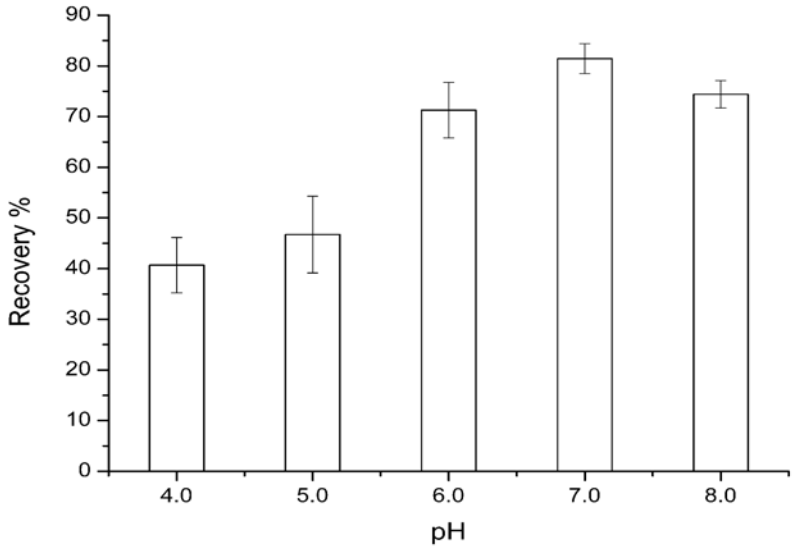


Fig. 4-2 Effect of pH on the recovery of cobalamin in matrix

4.1.3. Calibration and recovery

In accordance with the spectrograph (Fig. 4-3), the peaks at 361 nm and 521 nm were intensive response peaks, but remarkable interference by matrix at 521 nm was found. A clear peak at 361 nm appeared at 12.7 min (Fig. 4-4). Consequently, the peak at 361 nm was chosen and the calibration was made from 500 to 10,000 ng by matrix standard solutions, which were prepared

by adding cyanocobalamin into the matrix. The straight line was defined by the following equation:

$$Y = 90.0X - 3439.5 \quad \text{(Equ. 4-1)}$$

(absorbance values as Y and vitamin B_{12} concentration as X), $r^2 = 0.9991$ and the limit of detection defined as the signal to noise ratio of 3 was 200 ng. Concerning our samples, the detection limit was 5 ng per gram when vitamin B_{12} was extracted from 100 gram of samples.

The recovery experiment was performed by adding standards at different concentrations into soybean products (Table 4-1) and extraction was done as described above. For every concentration, it was repeated 5 times. Recoveries however were only ca. 75%. In brief, this method can be used to detect vitamin B_{12} in tofu but only in large quantities.

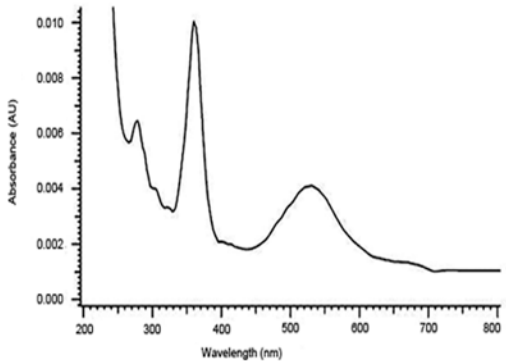


Fig. 4-3 Spectrograph of vitamin B_{12} in the eluent of methanol-water (30:70) with 0.1% formic acid

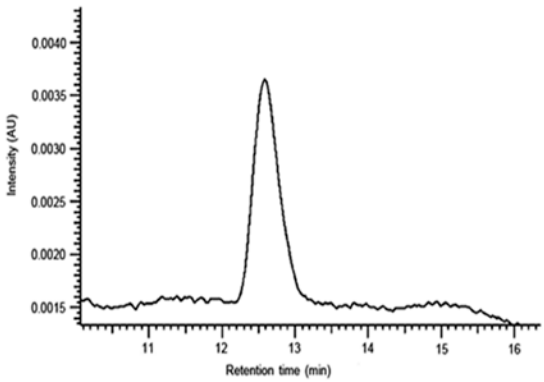


Fig. 4-4 Chromatograph of vitamin B_{12} at 1 µg/mL in the matrix
Conditions: column, RP-18 column: eluent, methanol (A) – water (B) with 0.1% formic acid; gradient (0-2 min 20% A; 2-3 min 20-25% A; 3-11 min 25-35% A; 11-19 min 35-20% A; 20-22 min 100-100% A; 22-26 min 100-20% A; 26-36 min 20% A); flow-rate, 0.50 mL min-1; detection, DAD at 361 nm; injection volume, 100 µL.

4.1.4. Sample handling

Four methods were studied to disrupt cells of *Propionibacterium freudenreichii* ssp. *freundenreichii* DSM 20271. Thereupon, a convenient method with high recovery to analyze vitamin B_{12} in fermented food was set up. Most of vitamin B_{12} is bound to proteins and located inside of cells. Therefore, the method that releases vitamin B_{12} from cells plays a very essential role in detection. The following experiments were designed based on that. Water bath heating and ultrasonic disruption were used for 10, 20, 30, 40, and 50 min (n=3). Meanwhile, the microwave oven was used separately for 2, 4, 6, and 8 min (n=3).

By comparing the results (Fig. 4-5 and Fig. 4-6), microwave treatment led to a good release of cobalamin after 6 min. But the cobalamin concentration released by this treatment was only two thirds of that released by boiling treatment. In addition, concentrations of cobalamin released by ultrasonic treatment increased from less than 40 to up 104.6 µg/g and stayed overall stable for the next 30 min. Furthermore, the results by boiling treatment started at 64.7 µg/g and decreased manifestly. Later, it increased rapidly up to 121.7µg/g at 40 min. Thus, the best result of 121.7 ng/g can be obtained after 40 min of boiling at 90 °C. Maximum 44.0 ng per g was obtained by a grinding method. Compared with the boiling treatment, only half of the time was needed by ultrasonic treatment to obtain a maximum concentration. Generally, the ultrasonic and boiling disruption work will be a good choice for the lab.

Table 4-1 Recovery of vitamin B_{12} in fermented tofu (n=5) by HPLC

Concentration (ng/mL)	Found concentration (ng/mL)	Recovery	Relative standard deviation
2000	1727.46	71%	0.14
1000	756.60	75%	0.03
500	345.62	69%	0.21

Table 4-2 Recovery of vitamin B_{12} added to tofu without fermentation (n=3) by microbiological assay

Standard concentration (ng/L)	Concentration determinated (ng/L)	Recovery	R. S. D
40	34.23	85.60%	16.37
10	7.82	78.29%	15.11

4.2. Microbiological assay

An advantage of microbiological assay is a low detection limit, which can detect tiny changes of vitamin B_{12} concentrations. A first order model, y = 0.0021x + 0.2303, was fitted (Fig. 4-7). The calibration was made from 10 ng/L to 50 ng/L. The detection limit was 0.02 ng per gram when vitamin B_{12} was extracted from 100 gram of samples. The rest standard deviation and standard deviation of formula are respectively 0.017 and 0.59. The coefficient of variations is only 2.9 %. The coefficient of determination (R^2) is 0.9812, which means this model can predict 98.12 % of results. The recoveries of matrix with adding vitamin B_{12} have similarity with the results from HPLC (Table 4-2). Conversely, the relative standard deviation was much more fluctuant than HPLC. Subsequently, microbiological assay was used in detection of vitamin B_{12} for low concentrations, but the repeatability and stability of this method was lower than HPLC. HPLC method was chosen as a routine method to detect vitamin B_{12} in fermented tofu.

4.3. Single fermentation

Coupled with the ability of vitamin B_{12} production, microorganisms should be recognized as safe to be used in food. Therefore, lactic acid bacteria and propionibateria from our culture collection were screened for vitamin B_{12} production. 7 strains of *Lactobacillus* spp. and 1 strain of *Streptococcus* spp. from our lab have the ability to survive in substrate broths without supplementation of vitamin B_{12}. These were *Lactobacillus reuteri* DSM 20016, *Lactobacillus* sp. LMH T.10, *Lactobacillus rhamnosus* EK4, *Lactobacillus casei* ssp. c*asei*, *Lactobacillus rhamosus* DSM 20021, *Streptococcus* sp. LMH T.11, *Lactobacillus* sp. LMH T.4, and *Lactobacillus* sp. LMH T.12, *Priopiniobacterium freudenreichii* ssp. *shermanii* DSM 20270 and *P. freudenreichii* spp. *freudenreichii* DSM 20271. Even though these strains had the property to form cobalamin, we can not confirm whether they can adapt to tofu to grow and synthesize vitamin B_{12}. After a 5-day fermentation, the strain *Lactobacillus reuteri* DSM 20016 was significantly different from other microorganisms and produced more vitamin B_{12} in tofu (Fig. 4-8). We also found, that *L. reuteri* produced 3 ng/g of analogues. Unfortunately, vitamin B_{12} producted by propionibateria

that is preferred by food industries cannot be detected in tofu. *L. reuteri* was used to carry out further experiments.

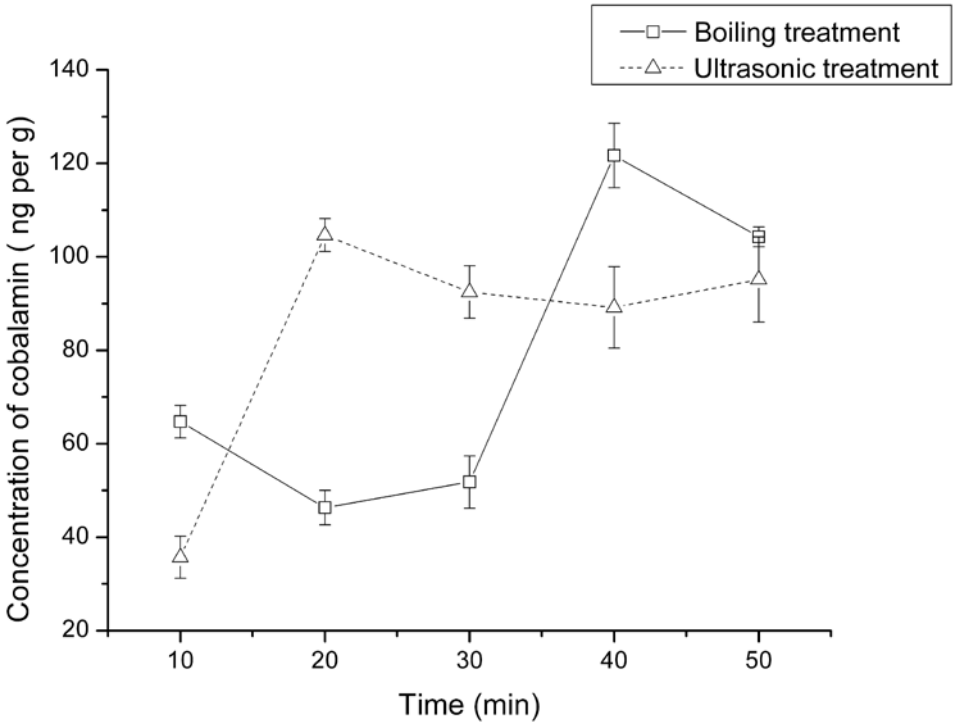


Fig. 4-5 Results of extraction of vitamin B_{12} by ultrasonic and boiling treatment.
Triangles with dashed lines indicate the results of extraction of cobalamin by ultrasonic treatment; square with full lines show the results of extraction of cobalamin by boiling

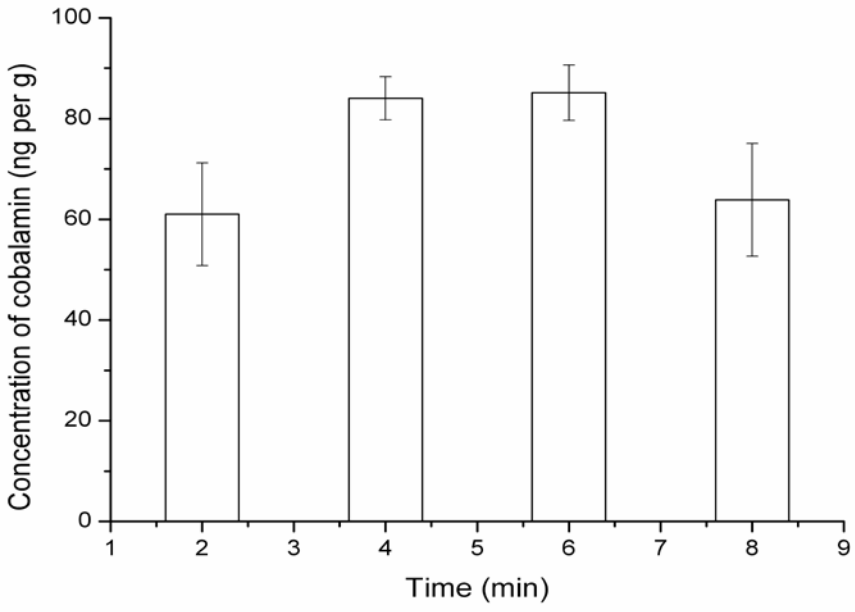


Fig. 4-6 Results of extraction of vitamin B_{12} by microwave treatment

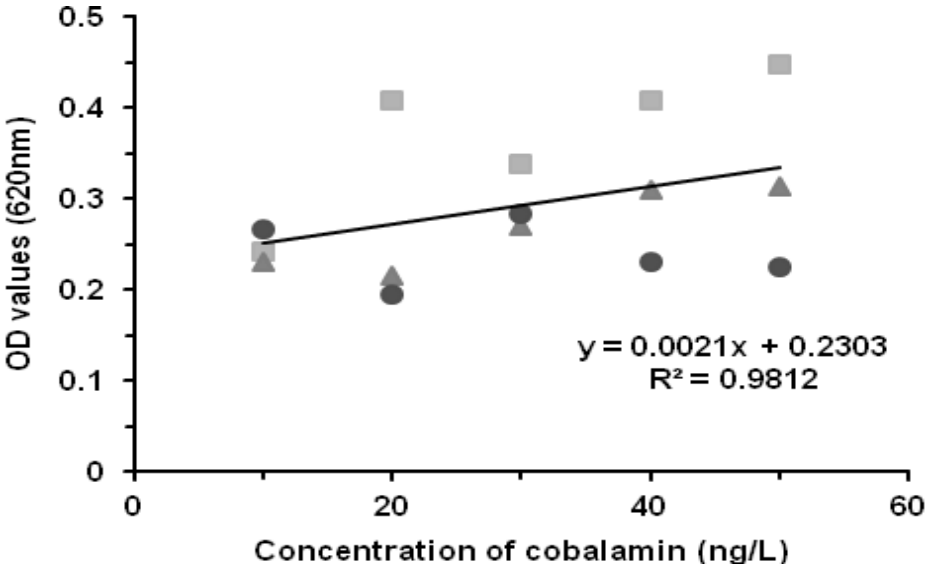


Fig. 4-7 Calibration curve of detection of vitamin B_{12} by microbiological assay

Block, triangle, and circle stand for 3 groups of matrix with a series of cobalamin standard

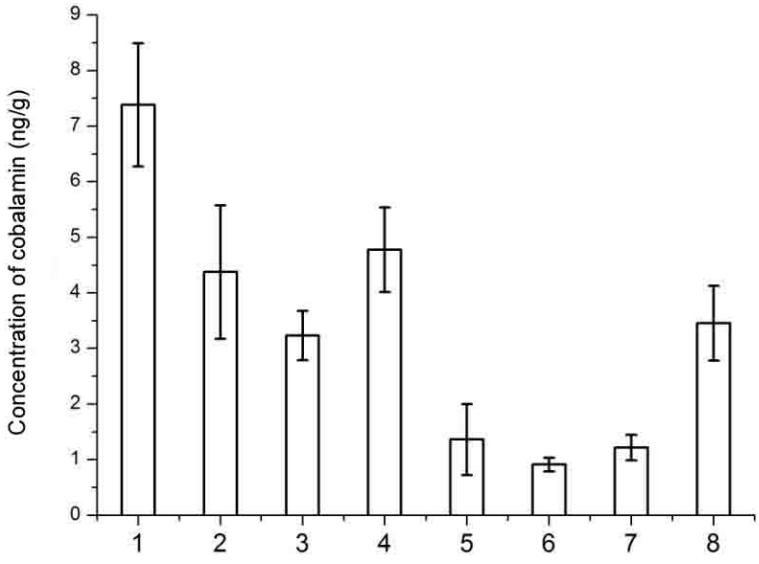


Fig. 4-8 Concentrations of cobalamin in soybean products fermented with various bacteria:

1. *Lactobacillus reuteri* DSM20016, 2. *Lactobacillus* sp. LMH T.10, 3. *Lactobacillus rhamnosus* EK4,

4. *Lactobacillus casei* ssp. *casei*, 5. *Lactobacillus rhamosus* DSM 20021, 6. *Streptococcus* sp. LMH T.11,

7. *Lactobacillus* sp. LMH T.4, and 8. *Lactobacillus* sp. LMH T.12.

4.3.1. Effect of nitrogen source on vitamin B_{12} production

Although our fermentations were performed in tofu which is rich in proteins, nitrogen sources may be still an important factor for growth and reproduction of microorganisms. After fermentations, final pH values were almost the same (Fig. 4-9). Regarding to weak protease activity of *L. reuteri*, some nitrogen supplementations such as peptone were offered. Except casein, others showed no significant differences concerning their cobalamin output (Fig. 4-9). For further experiments, no nitrogen supplementations were used.

4.3.2. Effect of mositure on vitamin B_{12} production

Also water activity has a strong influence on cell growth and productivity. The concentration of vitamin B_{12} after fermentation of tofu in 1:1 ratio of water to tofu was significantly higher than others (Fig. 4-10). A relationship between final pH values and cobalamin production has been observed. As we know, *Lactobacillus reuteri* is a facultatively anaerobic bacterium. Water can create a facultatively anaerobic environment for the growth of cells. Nutrients can also be dissolved in water and diffuse from tofu to cells.

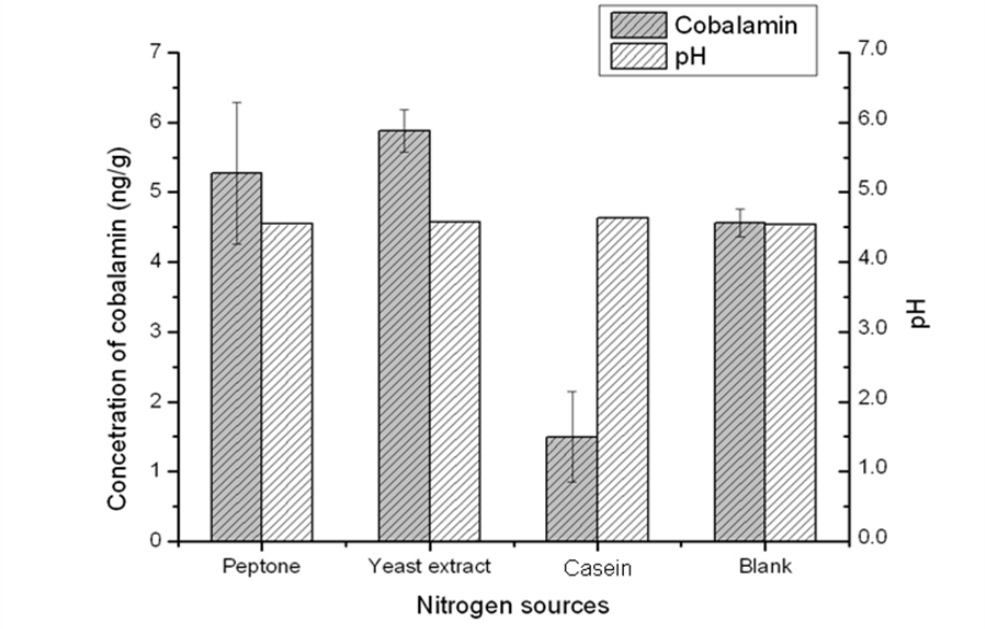


Fig. 4-9 Effects of various nitrogen sources on cobalamin production and pH values

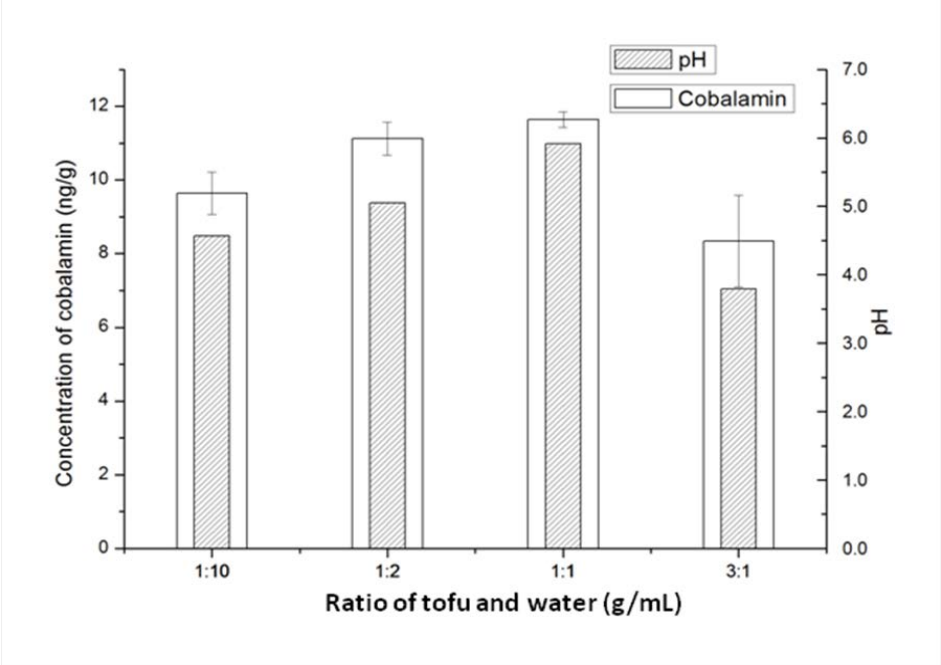


Fig. 4-10 Effects of various mositures on cobalamin production and final pH values

4.3.3. Growth curves and yield curves of cobalamin

Harvest time should be also emphasized. Thus, a growth curve was made to find out the best point to stop fermentation. Fig. 4-11 represents growth curves by an optical density method with different dilutions compared with a corresponding spread plate method. The growth curves from the optical density with 100-fold dilution and colony forming units (CFU) of a corresponding spread plate method showed almost the same trend and configuration. That means that the optical density method can substitute the spread plate method to draw growth curves in further experiments. The correlation coefficient between optical density value with 100-fold dilution and CFU was 0.98. The peak of cobalamin yield appeared after 68 hours and it went down soon (Fig. 4-12). From 45 hours to 54 hours, the cells entered the exponential phase. Starting from 54 hours cells entered the stationary phase. The pH was also observed during the fermentation (Fig. 4-11). The pH values were stable at about 5.7 after 40 hours fermentation. *Lactobacillus reuteri* can produce and accumulate cobalamin at the end of exponential phase and beginning of stationary phase. For further experiments, cobalamin was detected after 3 days of fermentation.

4.3.4. Effects of carbon source on production of vitamin B_{12}

Various carbon sources influence metabolites and the ratio of NADH to NAD^+, thus leading to varying production of vitamin B_{12} to balance oxidation-reduction reactions. Fermentations with different varieties of monosaccharides and polysaccharides (5%) were performed in order to choose an appropriate carbon supplementation for vitamin B_{12} production. Glucose represents the position of the best carbon source compared with others (Fig. 4-13). Final pH values had no definite discrepancy between fermentations with various supplementations. Production of vitamin B_{12} was obviously unrelated to final pH (Fig. 4-13). But fermentations with high production of vitamin B_{12} had obtained a final low pH value. Vitamin B_{12} production of fermentation with glycerol was unexpectedly low. In contrat to this, fermentation with fructose improved production clearly. Nevertheless, glucose was used as carbon supplementation in further fermentations.

4.3.5. Effects of glycerol and fructose on cobalamin production in vitamin B_{12} test broths

Vitamin B_{12} dependent coenzyme involves in a conversion of glycerol to balance the redox reaction. Fructose has also affected the balance of NAD^+ and NADH. These two factors were investigated in a pure medium in order to find out if they have effects or not. The cobalamin production of combination 5 was clearly higher than others (Table 4-3). If we compare the first three combinations with the last three, an unexpected phenomenon can be observed that fermentations with glycerol did not necessarily enhance production of cobalamin. On the other hand, a maximum of cobalamin production was obtained by increasing concentrations of fructose. In brief, a corresponding amount of fructose and glycerol supplementation can definitively enhance production of cobalamin.

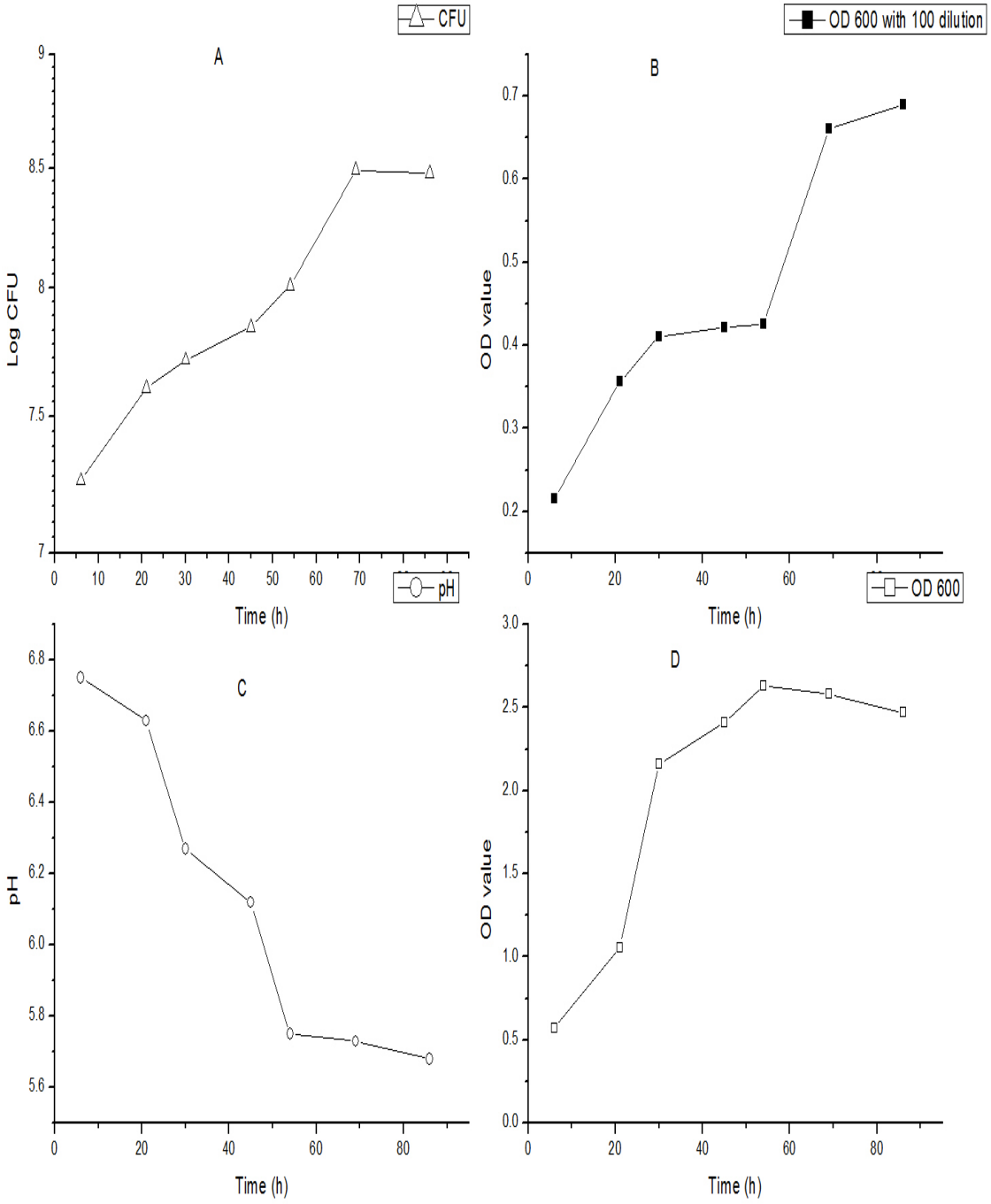


Fig. 4-11 *L. reuteri* growth curves in tofu represented by different methods

Graph A depicts a growth curve made by a spread plate method. Graph B and D indicate growth curves drawn by OD values with and without 100 folds dilution. Graph C shows the change of pH values during growth.

5 % (g/g) glucose supplemented

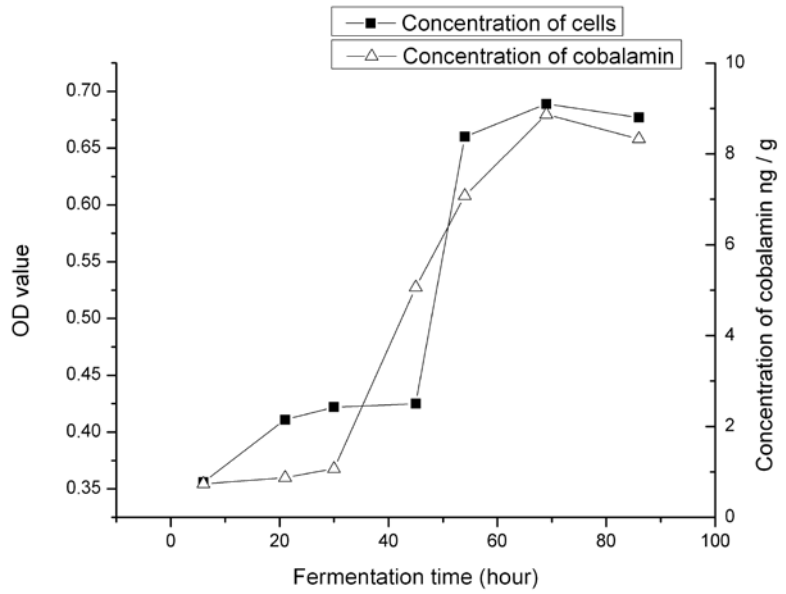


Fig. 4-12 The growth curve of *Lactobacillus reuteri* and the cobalamin yield curve during fermentation
Triangles stand for concentration of cobalamin; blocks strand for concentration of cells
5 % (g/g) glucose supplemented

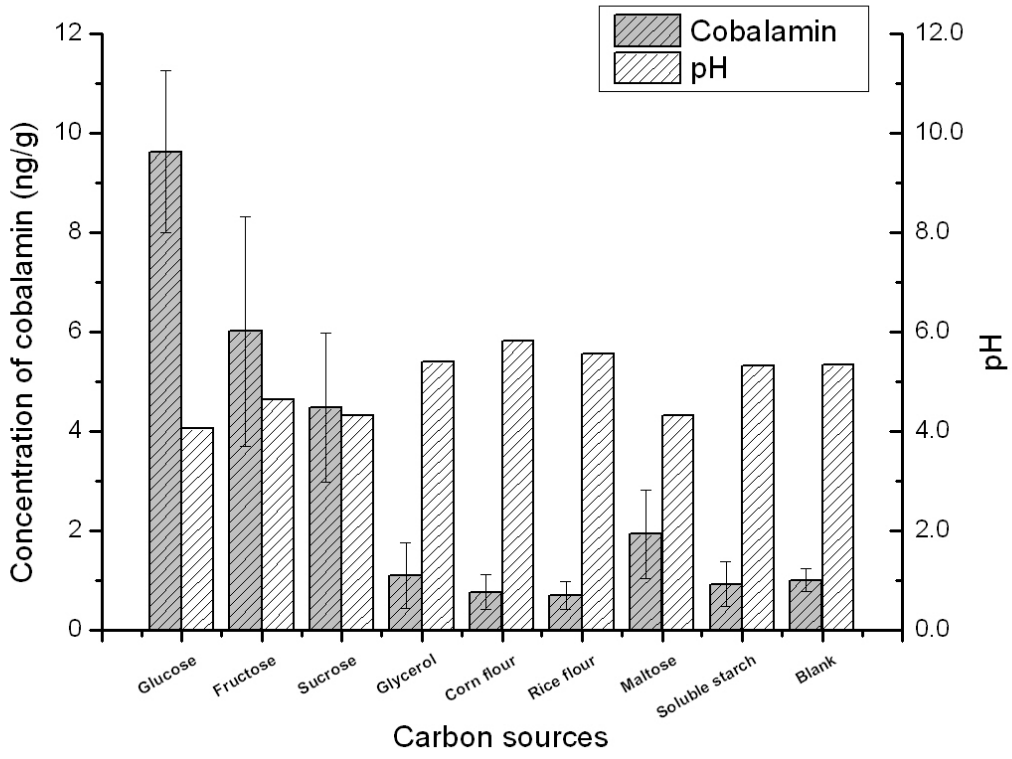


Fig. 4-13 Effects of various nitrogen sources on cobalamin production and pH values
5% (g/g) glucose supplemented
Fermentations for 3 days

4.3.6. Effects of glycerol and fructose on cobalamin production in tofu

Supplementations of glycerol and fructose have a pronounced positive influence on the production of cobalamin, which is related to the production of NAD^+ and NADH. As the glucose content in tofu is low, 20 g per kg of glucose were supplemented. The experiments were designed to find out if supplementations of glycerol and fructose should be used or not. Combination 5 produced up to 13.35 ng per g of cobalamin in tofu (Table 4-4), which was obviously more productive than others. When a corresponding ratio of glycerol and fructose supplementations was met, production of cobalamin was enhanced. A diauxic growth of these fermentations was observed (Fig. 4-14-2 H). As tofu is complex, it also contains other carbohydrates. Besides combination 1, all others reached a high concentration of cells. It could be seen that more cells produced more cobalamin. Without supplementations, cells of combination 1 dropped down after 1 day, but then started to increase a little bit from the 2nd day (Fig. 4-14-2 H). That means the supplementations improved cell growth. After 3 days fermentation, pH values with supplementations were higher than without supplementations (Table 4-4).

After analyzing series of substrates and metabolites, some phenomena were found out. A sudden drop of glucose concentrations was seen between 6 hours to 20 hours (Fig. 4-14-1 A) and then glucose concentrations of various combinations only fell down a little bit. The final glucose concentrations were still higher than 5 gram per Liter. Supplementations of glucose can be reduced in further experiments. Combination 2 and 3 supplemented with 2 g/L glycerol consumed more glucose than others. Production of lactate showed an inverse progress to the trend of glucose (Fig. 4-14-1 B). Combinations with less fructose supplementation produced more lactate and ethanol, and less 1,3-propanediol compared with more fructose supplementations (Fig. 4-14-1 and -2 B, D, F). Glycerol presented an interesting phenomenon (Fig. 4-14-2 E). In combinations 2, 3, 4, and 5 glycerol was consumed completely after 20 hours. Combinations with higher glycerol supplementations produced more acetate and 1,3-propanediol, and less ethanol than concentrations with less glycerol supplementations (Fig. 4-14-1 and -2 C, D, F). Supplementations with more fructose produced less ethanol but more mannitol (Fig. 4-14-1 and -2 D, H). In brief,

glycerol played a main role and fructose played a secondary role in production of acetate, 1,3-propanediol, and lactate. In contrast to this, fructose plays a main role and glycerol is a second factor in production of ethanol and mannitol.

Table 4-3 Results of combinations of glucose, glycerol and fructose in vitamin B_{12} test broths

g/L	Glucose	Glycerol	Fructose	Cobalamin (ng/g)
1	40	0	0	34.15
2	40	0	0.05	41.65
3	40	0	0.1	41.65
4	40	0.04	0.01	20.40
5	40	0.04	0.05	90.15
6	40	0.04	0.1	48.65

Table 4-4 Results of combinations of glucose, glycerol and fructose in tofu

g/kg	Glucose	Glycerol	Fructose	Cobalamin (ng/g)	pH
1	20	0	0	5.09	4.10
2	20	2	0.5	11.20	4.23
3	20	2	2	0.32	4.27
4	20	5	0.5	7.05	4.21
5	20	5	2	13.35	4.28

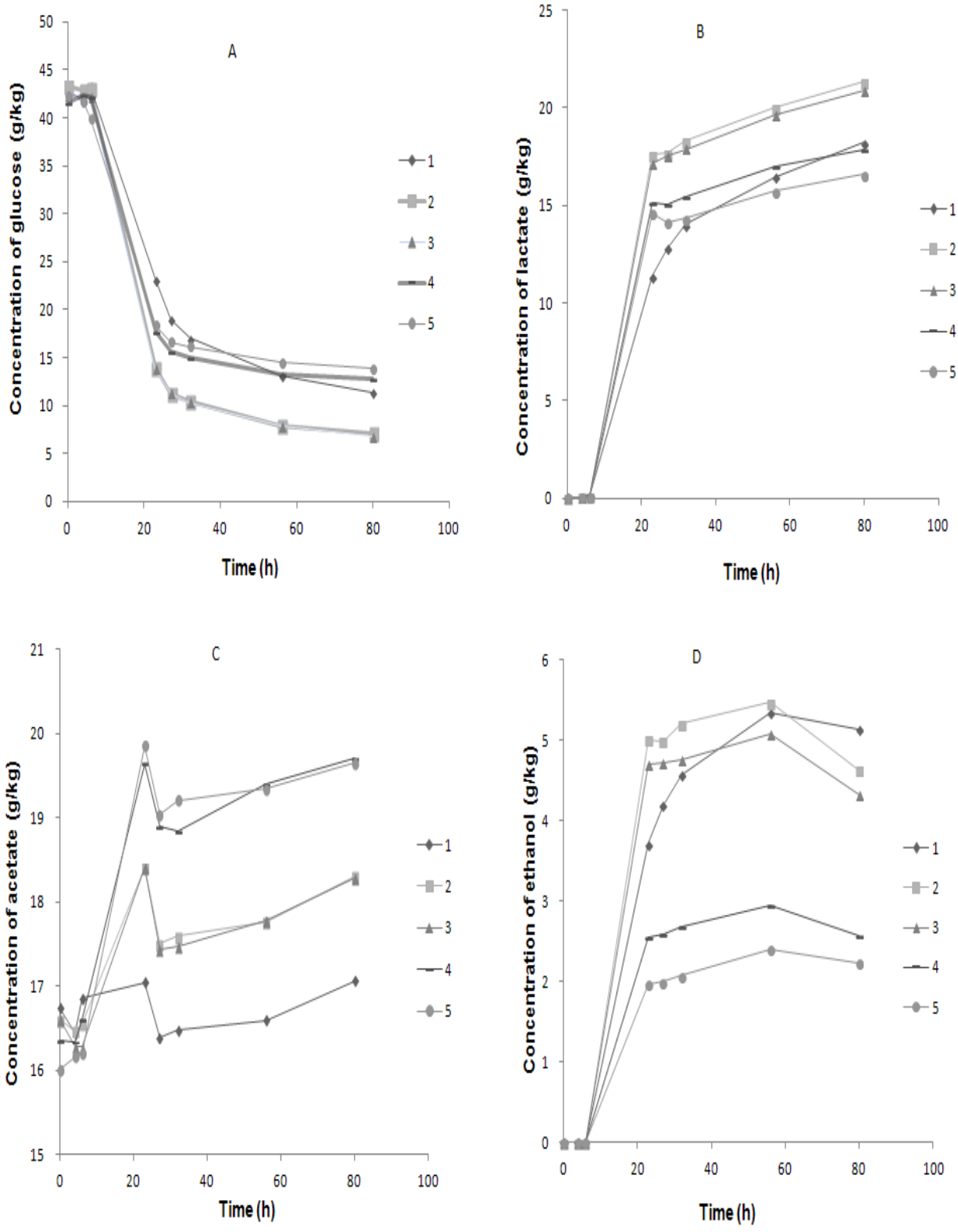


Fig. 4-14-1 Results of production of metabolites, substrates consumption, and growth curves of various combinations of glucose, glycerol and fructose in tofu. Graph A means glucose comsuption. Graph B stands for lactate production. Graph C represents acetate production. Graph D means ethanol production.

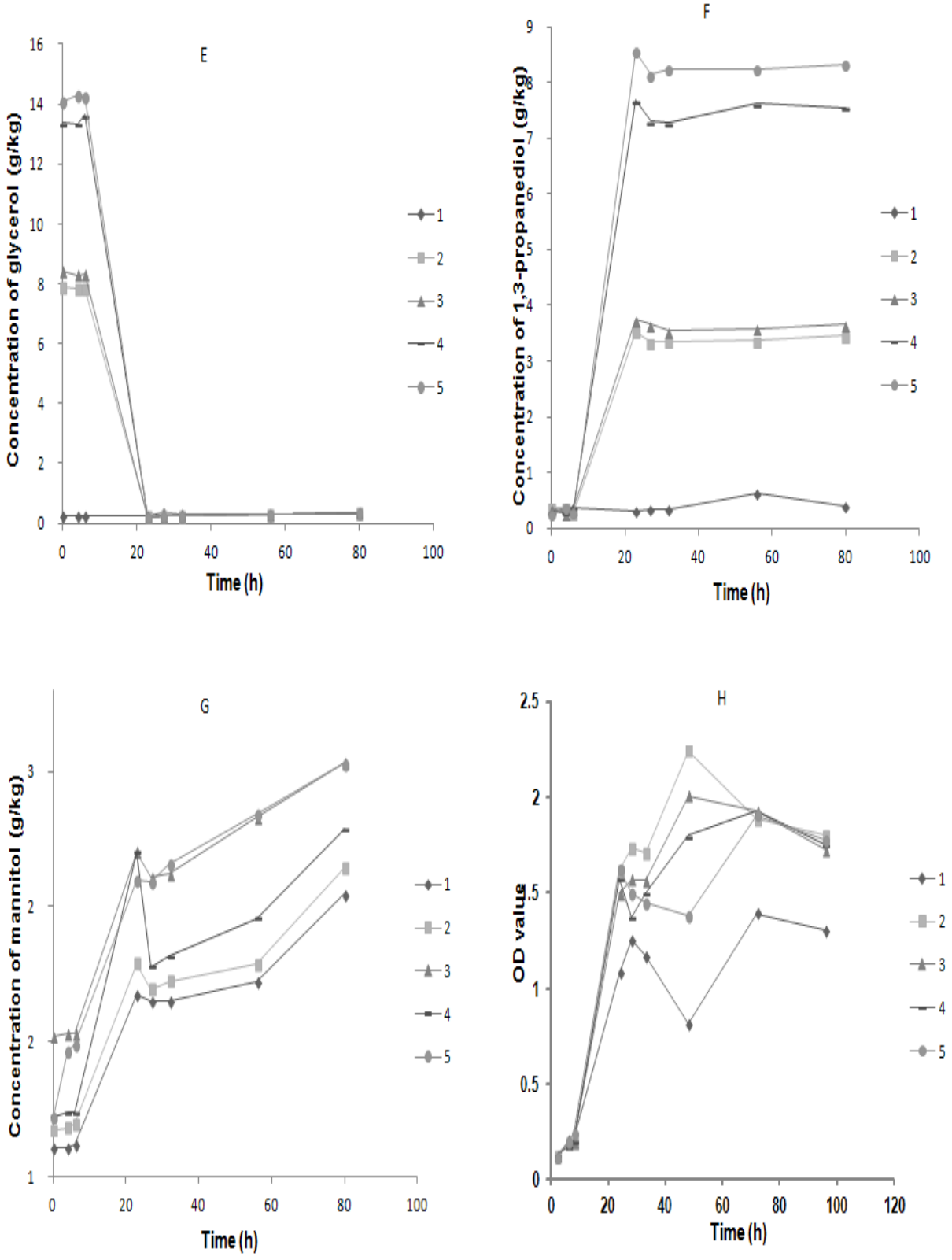


Fig. 4-14-2 Results of production of metabolites, substrates consumption, and growth curves of various combinations of glucose, glycerol and fructose in tofu.. Graph E, F and G represent production of glycerol and 1,3-propanediol and concentration of mannitol. Graph H shows growth curves of cells at OD 600

4.3.7 FFD experiments

A 2^{5-1} fractional factorial design requested 16 experiments and other four experiments at the center of design were repeated four times in order to analyze the variance. Every main effect was aliased with four-factor interaction, and two-factor interaction was aliased with three-factor interaction. The results of FFD are depicted in Table 4-6 and Table 4-7. After fermentation, concentrations of vitamin B_{12} in every gram of wet soybean ranged from 0.19 to 15.01 ng g^{-1}. Increasing concentrations of glucose ($p < 0.001$) had a dramatically negative influence on the yield of vitamin B_{12}, whereas the increase of glycerol ($p < 0.05$) had a positive effect. Interaction effects of glucose and $CoCl_2$, glycerol and fructose, and glycerol and $CoCl_2$ had a negative influence on the response. However interactive effects of fructose and $CoCl_2$, and fructose and Dmbi showed positive effects on the response. A concentration of glucose of 18.5 g/kg and glycerol of 0.75 g/kg produced more vitamin B_{12} than glucose concentration of 21.5 g/kg and glycerol concentration of 0.25 g/kg. Negative interactive effects of glucose and $CoCl_2$, glycerol and fructose, and glycerol and $CoCl_2$ were caused by low pH values that inhibited the propagation of cells and synthesis of cobalamin. The phenomena of positive interactions of fructose and $CoCl_2$, and fructose and Dmbi on vitamin B_{12} production had been validated by adding fructose into samples, which enhanced the yield 1.2-1.8 fold. Low concentrations of fructose improved production of cobalamin. Other factors had no significant effect on the production of vitamin B_{12}. The value of the regression coefficients were calculated and the first order equation can be written down as follows,

$$Y_{VB12} = 6.49 - 1.785\ X_1 + 0.818\ X_2 - 0.575\ X_3 + 0.195\ X_4 + 0.719\ X_5 - 0.899\ X_1*X_4 - 1.843\ X_2*X_3 - 0.937\ X_2*X_4 + 2.178\ X_3*X_4 + 1.434\ X_3*X_5 \quad \text{(Equ. 4-2)}$$

Regeression analysis results of FFD experiments in Table 4-6 illustrated that glucose and glycerol were the two most important factors for vitamin B_{12} producion and accumulation by the probability level of 99.99% and 95.80% respectively. The other three factors were not found to be significant at the probability level of 95%.

The coefficient R^2 of the model equation at 0.963 indicated that the model explained 96.3 % of the variability in the data. The value of F-test at 11.589 confirming the statistical significance of

the model equation indicated that the model was adequate to the data at the probability level of 99%.

The pH value and OD 600 were not related with the cobalamin production. The pH values were ranging from 4.4 to 4.82.

4.3.8 Steepest ascent experiments

According to results of FFD, a steepest ascent design was conducted. Fructose, $CoCl_2$, and Dmbi were not significant at the probability level of 95%. But all of them had very strong interactive effects with others on the response. As a result of that, the concentration of $CoCl_2$ was fixed at 2.5 mg/kg and the concentration of Dmbi at 3.5 mg/kg which can afford enough precursors of cobalamin.

Table 4-6 Experiment results of FFD

Run	X_1	X_2	X_3	X_4	X_5	Cobalamin observed (ng/g)	Cobalamin expected (ng/g)	pH	OD 600
1	1	1	-1	-1	1	11.194	11.045	4.80	1.76
2	-1	1	-1	-1	-1	15.006	14.247	4.48	1.814
3	0	0	0	0	0	6.667	6.490	4.66	1.611
4	0	0	0	0	0	7.578	6.490	4.59	1.55
5	1	-1	1	1	-1	3.768	5.413	4.44	1.678
6	-1	1	1	-1	1	5.923	6.493	4.59	1.66
7	1	-1	-1	1	1	1.369	-0.041	4.56	1.763
8	1	-1	-1	-1	-1	4.930	5.279	4.59	1.595
9	-1	-1	1	-1	-1	2.734	2.363	4.47	1.643
10	-1	1	1	1	-1	7.622	6.857	4.82	1.596
11	-1	-1	-1	-1	1	4.326	5.621	4.52	1.637
12	0	0	0	0	0	6.543	6.490	4.65	1.69
13	1	1	-1	1	-1	4.193	4.837	4.63	1.637
14	-1	1	-1	1	1	7.777	8.775	4.57	1.531
15	1	-1	1	-1	1	5.434	4.897	4.46	1.863
16	1	1	1	1	1	5.937	5.795	4.47	1.684
17	-1	-1	-1	1	-1	6.255	6.757	4.44	1.613
18	1	1	1	-1	1	3.873	4.721	4.66	1.508
19	1	1	1	-1	-1	0.188	0.415	4.59	1.569
20	0	0	0	0	0	8.116	6.490	4.63	1.581

$X_1 = (x_1 - 20)/1.5$, $X_2 = (x_2 - 0.5)/0.25$, $X_3 = (x_3 - 2.5)/1.5$, $X_4 = x_4 - 1.5$, $X_5 = (x_5 - 1.75)/1.75$. x_1, x_2, x_3, x_4, and x_5 stand for natural variables of glucose (g/kg), glycerol (g/kg), fructose (g/kg), $CoCl_2$ (mg/kg),and Dmbi (mg/kg).

As mentioned before, a low concentration of fructose enhanced the production. So the concentration of fructose was fixed at 1.5 g/kg. In the view of the two vital important factors, decreasing concentrations of glucose and increasing concentrations of glycerol had a positive contribution to production of vitamin B_{12}. The changing directions of both varieties and experimental design are shown in Table 4-8. The concentration of glucose was decreasing at interval by 1 g/kg and the concentration of glycerol was increasing serially by 0.25 g/kg. Run 5 went to a peak of vitamin B_{12} yield. Parameters of run 5 were chosen to be the starter condition to do further optimizations. Changes of glucose and glycerol in this field did not dramatically influence pH and cells.

Table 4-7 Results of FFD regression analysis for cobalamin

Term	Regression analysis for Cobalamin		
	Coefficient	t-value	Significant level
Intercept	6.490	21.483	0.000
X_1	-1.785	-5.167	0.001***
X_2	0.818	2.369	0.042**
X_3	-0.575	-1.682	0.127
X_4	0.195	0.564	0.586
X_5	0.719	2.102	0.065*
X_1*X_4	-0.899	-2.628	0.027**
X_2*X_3	-1.843	-5.335	0.000***
X_2*X_4	-0.937	-2.739	0.023**
X_3*X_4	2.178	6.304	0.000***
X_3*X_5	1.434	4.191	0.002***
		R^2=0.963	F= 11.589 > $F_{10, 9, 0.01}$= 5.257

$X_1 = (x_1 - 20)/1.5$, $X_2 = (x_2 - 0.5)/0.25$, $X_3 = (x_3 - 2.5)/1.5$, $X_4 = x_4 - 1.5$, $X_5 = (x_5 - 1.75)/1.75$. x_1, x_2, x_3, x_4, and x_5 stand for natural variables of glucose (g/kg), glycerol (g/kg), fructose (g/kg), $CoCl_2$ (mg/kg), and Dmbi (mg/kg).

4.3.9 Further optimization of the medium by RSM

Throughout a series of experiments, the neighborhood of optimal response had been approached. For a further optimization by RSM, factors of glucose and glycerol were chosen. To find out the optimal response in the soybean product, a CCD experiment design was conducted. The levels of both varieties, the experimental plan, and the results aew presented in Table 4-9.

Table 4-8 Results of the ascent and corresponding response

Run	X_1	X_2	Cobalamin (ng/g)	pH	OD 600
1	12. 5	2	11.557	4.90	1.228
2	13. 5	1.75	10.298	4.88	1.267
3	14. 5	1.5	12.162	4.83	1.230
4	15. 5	1.25	8.991	4.86	1.173
5	16. 5	1	15.210	4.86	1.238
6	17. 5	0.75	13.516	4.87	1.240
7	18. 5	0.5	12.423	4.87	1.128

X_1 and X_2 stand for natural variables of glucose and glycerol (g/kg).

Results of CCD experiment were fitted with a second-order response surface model. Analysis of results of regression of CCD is presented in Table 4-10. A second-order polynomial equation can be formed as follows.

$$Y = 15.221830 - 0.051115\ X_1 - 1.020486\ X_2 - 1.760958\ X_1*X_1 + 0.082500\ X_2*X_1 - 0.596530\ X_2*X_2 \quad \text{(Equ. 4-3)}$$

According to the equation, the coefficients of X_1^2 and X_2^2 are negative, which means they are open downward parabolas. This equation indicats a maximum point. The result of analysis released that the surface response model is significant, as it is evidenced by the high F-test ($F= 6.07 > F_{5, 7, 0.025}=5.2852$) and a low probability. The quadratic regression model is appropriate and can adequately explain the variation observed in vitamin B_{12} production. The R^2 of 0.963 indicats that 96.3% variability in the response can be explained by the model. It is proven that the model is fitting to describe the response of vitamin B_{12} production and to predict a response value inside of ranges. The contour plot of the model equation is presented in Fig. 4-15. The rather broad plateau region illustrats that vitamin B_{12} production is changed relatively little when concentrations of factors are varied.

A maximum point of the model can be obtained, at 16.41 g/kg of glucose and 0.7 g/kg of glycerol, from derivative of the equation. A maximum response of 15.66 ng/g of vitamin B_{12} was predicted by the model. With the purpose of confirming predicted results of the model, experiments with conditions of this maximum point were repeated three times. A value of 16.33 ± 0.58 ng/g was

reached, which was even higher than the prognosis value. The validity of the response model and an existing optimal point of fermentations were verified by a good correlation between these two results.

Table 4-9. Results of the central composite design

Run	X_1	X_2	Cobalamin observed (ng/g)	Cobalamin expected (ng/g)	pH	OD 600
1	-1	-1	15.35	14.02	4.73	1.184
2	1	-1	14.96	13.75	4.69	1.205
3	-1	1	11.51	11.81	4.7	1.089
4	1	1	11.45	11.88	4.7	1.086
5	-1.414	0	11.25	11.79	4.78	1.08
6	1.414	0	11.28	11.65	4.68	1.116
7	0	-1.414	13.86	15.47	4.76	1.178
8	0	1.414	13.30	12.60	4.75	1.014
9	0	0	15.12	15.22	4.68	1.203
10	0	0	15.34	15.22	4.69	1.173
11	0	0	15.33	15.22	4.72	1.214
12	0	0	15.12	15.22	4.66	1.208
13	0	0	15.21	15.22	4.67	1.21

$X_1 = (x_1 - 16.5)/3.5$, $X_2 = (x_2 - 1)/0.5$. x_1 and x_2 stand for the natural variables of glucose (g/kg) and glycerol (g/kg).

Table 4-10 Results of CCD regression analysis for cobalamin

Term	Regression analysis for		
	Coefficient	t-value	Significant level
Intercept	15.221830	33.83	<0.0001
X_1	-0.051115	-0.14	0.8900
X_2	-1.020486	-2.86	0.0242**
X_1*X_1	-1.760958	-4.60	0.0025**
X_2*X_1	0.082500	0.16	0.8744
X_2*X_2	-0.596530	-1.56	0.1634
		R^2=0.8125	F= 6.07 > $F_{5, 7, 0.025}$=5.2852

$X_1 = (x_1 - 16.5)/3.5$, $X_2 = (x_2 - 1)/0.5$. x_1 and x_2 stand for the natural variables of glucose (g/kg) and glycerol (g/kg).

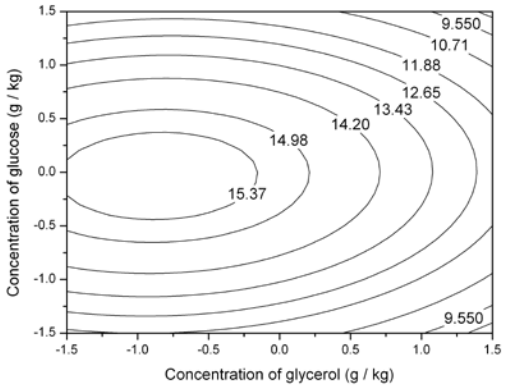


Fig. 4-15 Contour plot of the model equation fitted to the data of the central composite design experiment. On the X_1 and X_2 axises, concentrations of glucose and glycerol are given in their coded forms as listed in Table 4-9 respectively.

4.4. Cofermentation

4.4.1. Cofermentations in vitamin B_{12} test broth by *Lactobacillus reuteri* and *Propionibacterium* spp.

As 1.6 µg per 100 gram of tofu is not satisfying to the meet of recommend daily intake of vitamin B_{12}, further experiments were first conducted in vitamin B_{12} test broths to find that cofermentations with *Lactobacillus reuteri* and *Propionibacterium freudenreichii* at 37 °C produced the highest cobalamin concentration among these combinations (Table 4-11). Both of cofermentations produced more cobalamin than any single fermentation. The pH of fermentation with propionibacteria was above 5.0, below which the growth of propionibacteria will be inhibited. Fermentation at high temperatures consumed more glucose (Fig. 4-16a A) and produced more lactate (Fig. 4-16a B). The production of acetate fluctuated from 20 hours to 80 hours, maybe due to evaporation of acetate (Fig. 4-16a C). Co-fermentations produced more propionic acid after 40 hours than the single fermentation with *P. freudenreichii* (Fig. 4-16a D). Production of ethanol was higher and earlier at high temperatures than at low temperatures (Fig. 4-16a E). The growth of *L. reuteri* was faster than others but finally they met each other (Fig. 4-16a F). The cell concentrations in combination 2 and 3 were higher in contrast to others (Fig. 4-16a F). That may

explain the abnormal phenomenon of high production of cobalamin with high production of lactate and ethanol in combination 2. Further experiments of co-fermentations will be conducted in tofu.

Table 4-11 Results of co-fermentation at different temperatures in vitamin B_{12} assay broth

Run	*Lactobacillus reuteri*	*Propionibacterium freudenreichii* spp.	Temperature (°C)	pH	Cobalamin (ng/g)
1		*P. freundenreichii* ssp. *shermanii*	30	5.56	37.90
2	*L. reuteri*	*P. freudenreichii* ssp. *freudenreichii*	37	4.30	86.66
3	*L. reuteri*		37	4.31	36.46
4		*P. freudenreichii* ssp. *freudenreichii*	30	5.43	34.66
5	*L. reuteri*	*P. freudenreichii* ssp. *freudenreichii*	30	4.85	60.78
6	*L. reuteri*	*P. freundenreichii* ssp. *shermanii*	37	4.47	40.87
7	*L. reuteri*	*P. freundenreichii* ssp. *shermanii*	30	4.96	28.98

4.4.2. Cofermentation at different temperatures in tofu by *Lactobacillus reuteri* and *P. freudenreichii* ssp. *freudenreichii*

All results are represented in Fig. 4-16-1b and Fig. 4-16-2b. Diauxic growth curves and patterns can be observed again. The cell concentrations of co-fermentations are higher than in single fermentation. A peak of cobalamin production by the cofermentation at 30 °C appears at the 7th day. Peaks of other two fermentations appear at the 3rd day. Surprisingly, all these pH values are above 5.0 (Fig. 4-16-1b C). This phenomenon may be resulting from depletion of glucose supplementations. All supplementations of glucose in cofermentations were comsumed, but not in single fermentation (Fig. 4-16-1b D). Concentrations of ethanol, lactate and acetate of cofermentation are higher than in single fermentation (Fig. 4-16-2b E, F, G).

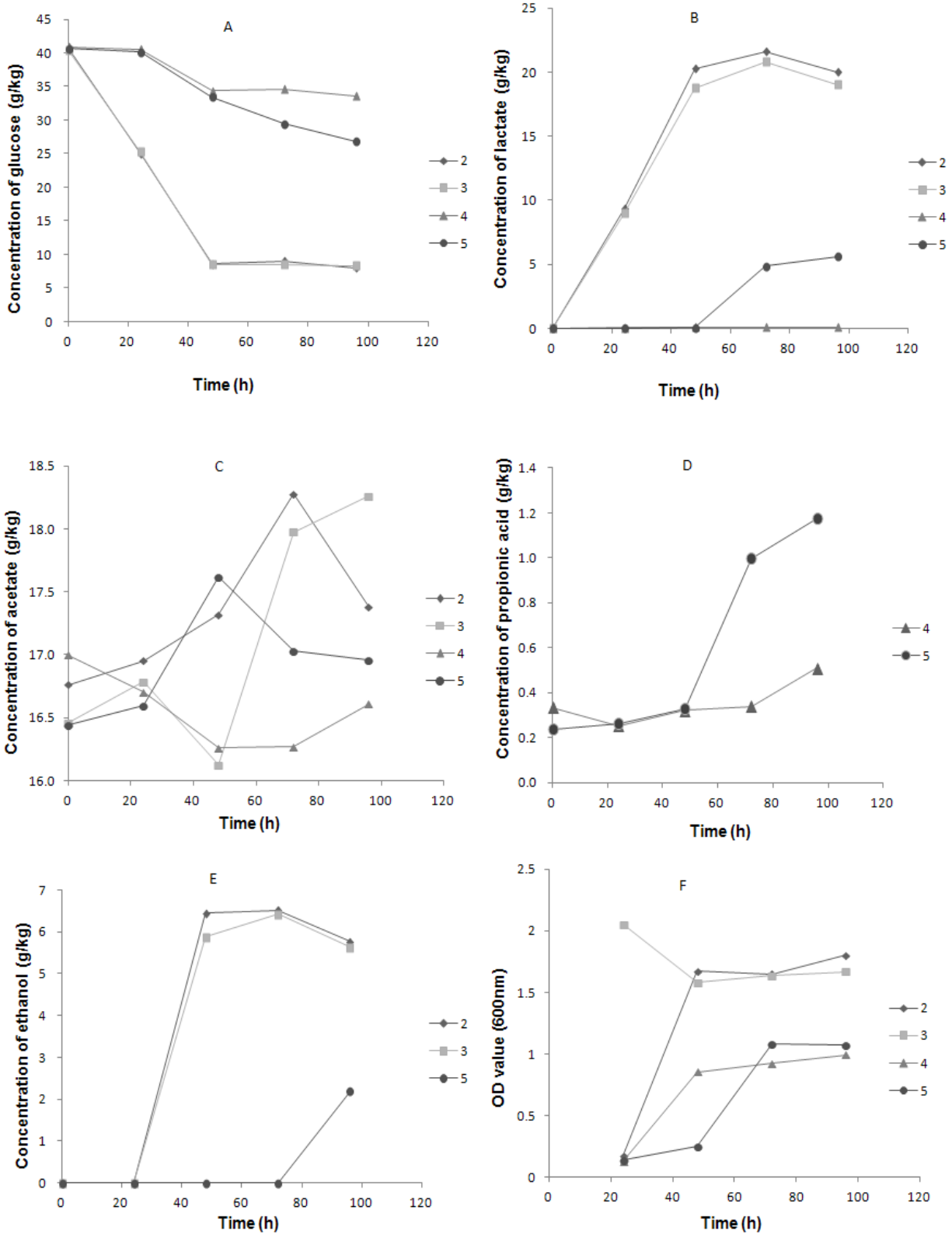


Fig. 4-16a Results of production of metabolites, substrate consumption and growth curves of various combinations of cofermentations at different temperatures in vitamin B_{12} assay broths. Graph A means glucose comsuption. Graph B stands for production of lactate. Graph C represents production of acetate. Graph D means production of propionic acid. Graph E means production of ethanol.

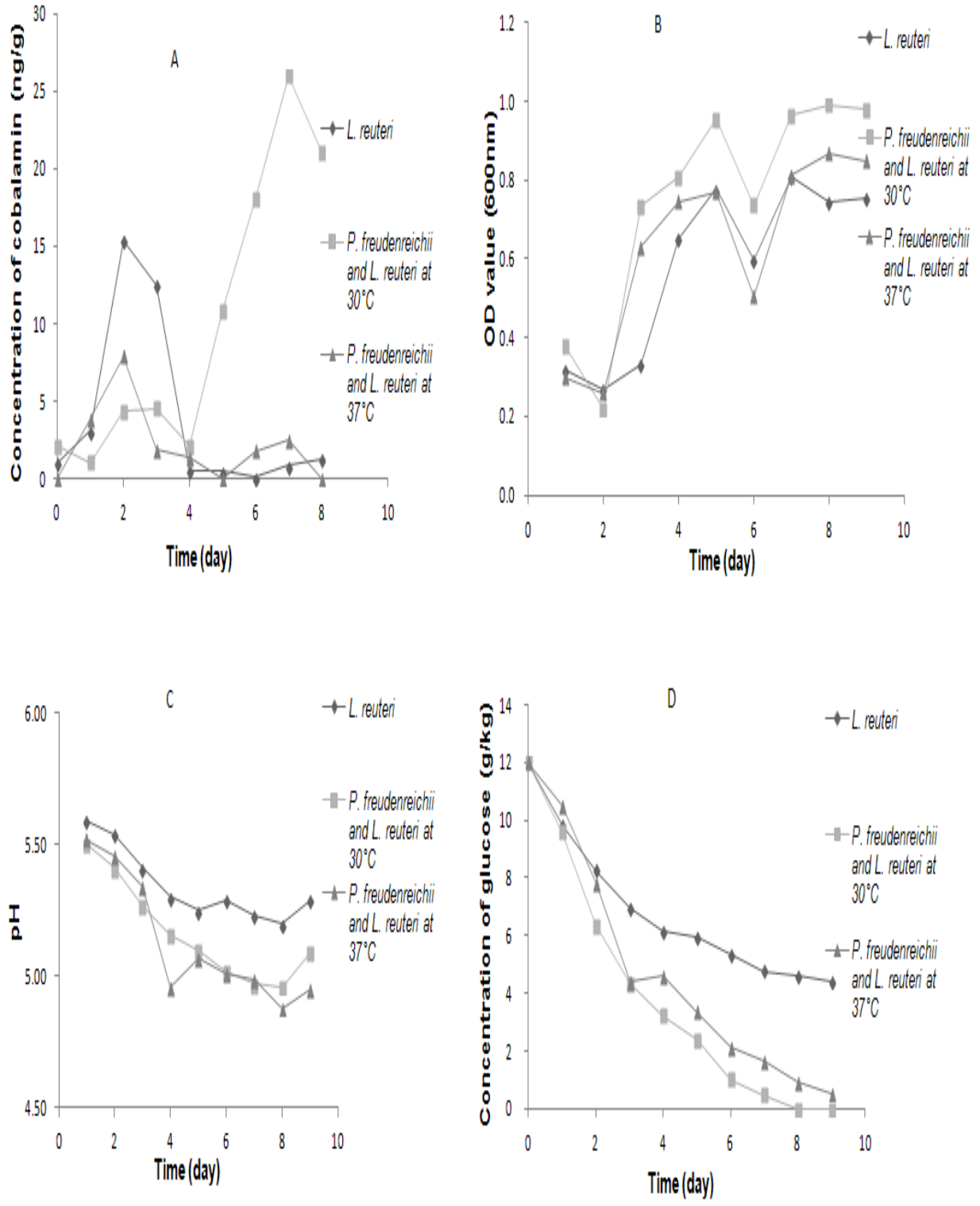


Fig. 4-16-1b Results of production of metabolites, substrate consumption and growth curves of single and cofermentation at different temperatures. Graph A means production of cobalamin. Graph B stands for growth curves. Graph C represents changing of pH values. Graph D means glucose consumption.

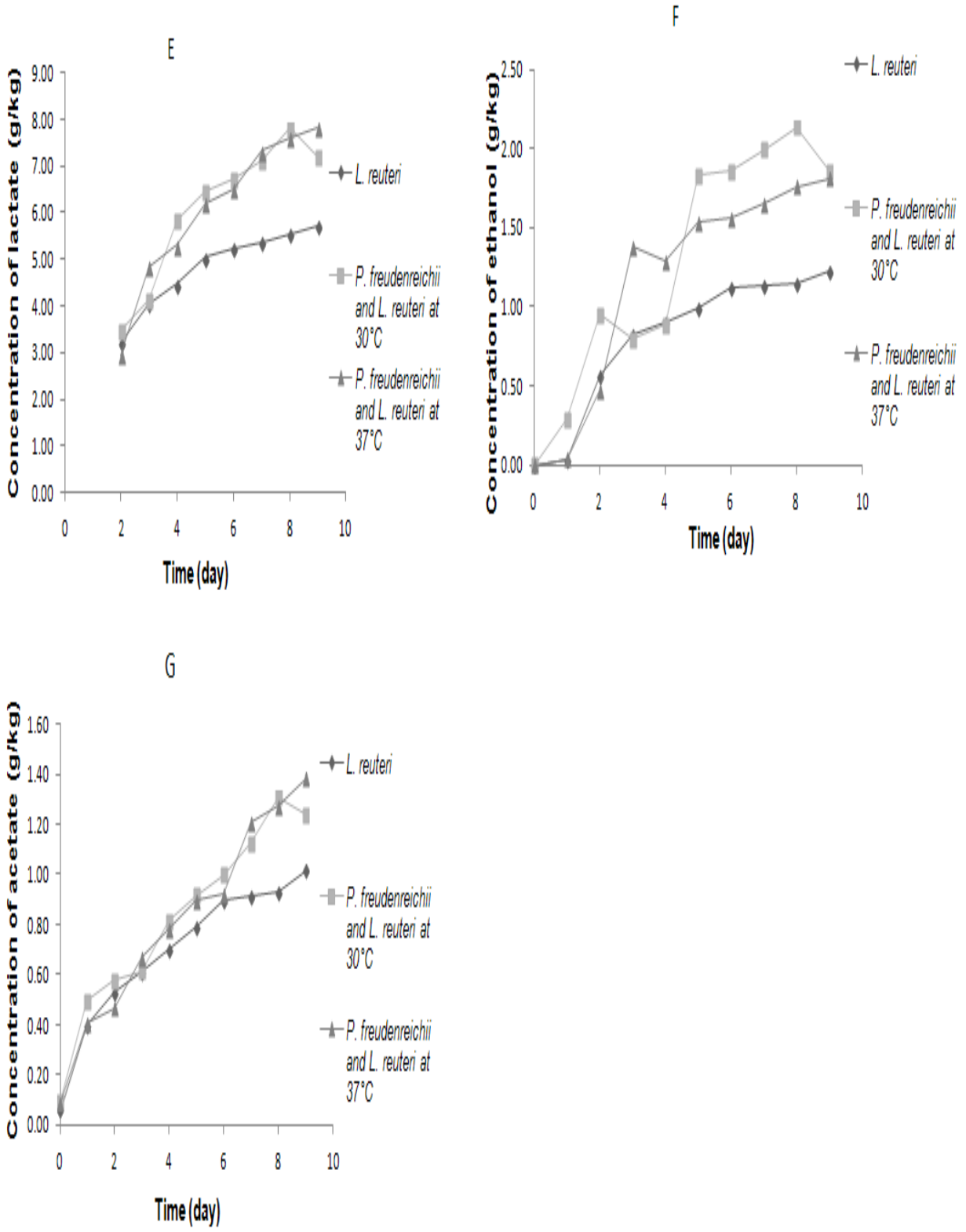


Fig. 4-16-2b Results of production of metabolites, substrate consumption and growth curves of single and cofermentation at different temperatures. Graph E, F, G mean production of lactate, ethanol and acetate.

According to the results, cofermentations resulted in minor lactate concentrations per glucose, as lactate may be used as a carbon source for propionibacteria. The cofermentation at 30°C produced the lowest amount of acetate per glucose, but the highest concentrations of ethanol. That indicated that both of microorganisms adapted to the environment and contributed to the production of cobalamin. Through these experiments, a solid conclusion can be drawn that cofermentation in tofu at 30 °C in 7 days produces up to 1.5-fold more cobalamin than single fermentation, when the end of second exponential phase was reached.

4.4.3. Cofermentation supplemented with glycerol and glucose at different temperatures

Cobalamin produced by a single fermentation with propionibacteria in tofu was not detected by us. The reason may be that propionibacteria do not absorb enough carbohydrates. *Lactobacillus reuteri* can generate a final metabolite, lactate, which can be used as carbohydrate by propionibacteira. However other supplementations were still tried to enhance the production of cobalamin. Glycerol was used to balance the redox as before.

After experiments, we found that the fermentation at 30 °C entirely synthesized more cobalamin than the corresponding fermentation at 37 °C. Furthermore the combination with 0.5 g of glucose at 30 °C produced cobalamin from 50.1 ng per g on the 3rd day up to 64.9 ng per g on the 7th day (Fig. 4-17). An interesting result of pH was observed. The pH values of both fermentations supplemented with 0.5 g/kg of glucose were always above 5.3 (Fig. 4-18-1 A), which was adapted by both microorganisms to grow and proliferate. The pH values of fermentations supplemented with glycerol ranged from 4.9 to 5.4 (Fig. 4-18-1 A). With more supplementations of glucose, the pH dropped down quickly and was under 5.0 (Fig. 4-18-1 A). This can explain that fermentation with 5 g/kg of glucose at 30 °C primarily produced lots of cobalamin and then decreased obviously. Glucose had been consumed in all fermentations except the fermentation with 0.5 g/kg glycerol at 30 °C (Fig. 4-18-1 B), which only consumed half of the glucose.

Production of mannitol and succinate was affected by temperature (Fig. 4-18-1 C and D). Less supplementations of glucose led to a high yield of mannitol. Meanwhile glycerol can inhibit

production of mannitol. The increase and production of succinate of fermentation at 30 °C were higher than at 37 °C (Fig. 4-18-1 D). Apart from fermentations supplemented with more glucose, which produced up to 7 g/kg of lactate, others were under 1.5 g/L (Fig. 4-18-2 E). Particularly for the fermentation supplemented with 0.5 g/kg glucose at 37 °C, the production of lactate decreased clearly. Only a fermentation supplemented with 5 g/kg of glucose at 30 °C produced up to 2.5 g/L acetate, two times as much as others (Fig. 4-18-2 F). Acetate can repress growth of both microorganisms. Propionic acid also inhibits growth of microorganisms. The fermentation at 30 °C produced more propionic acid, especially supplemented with 5 g/kg glucose and 0.5 g/kg glycerol, than others (Fig. 4-18-2 G).

We compared all concentrations of metabolites on the last day with values of glucose consumption (Table 4-12). The fermentation supplemented with 0.5 g/kg of glucose at 30 °C produced less lactate, less acetate and more propionic acid and mannitol (Table 4-12), which can explain the reason of high production capability of cobalamin. An unexpected result of the fermentation with 5 g/kg of glucose at 37 °C was observed with a very low amount of propionic acid. A tentative assumption can be drawn that a high temperature and a high amount of supplementations of glucose may block the growth of microorganisms and also inhibit cobalamin production. The conditions of 30°C, 0.5 g/kg of glucose, and 7 days fermentation were used in further experiments.

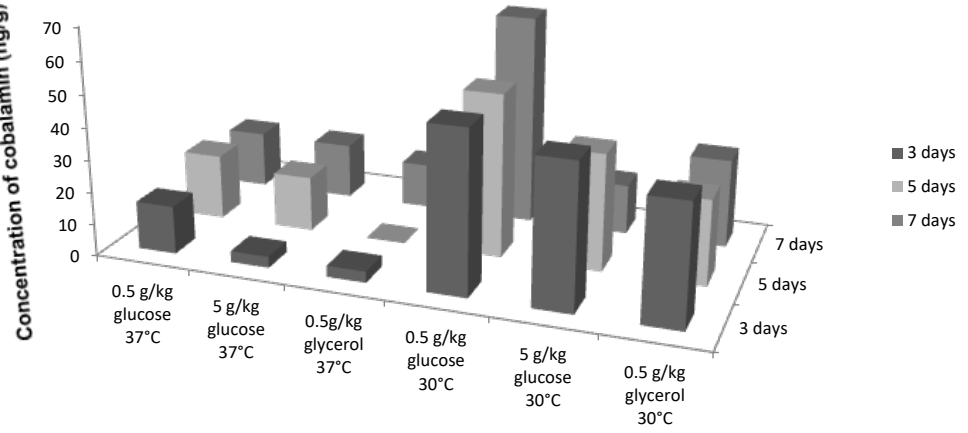


Fig. 4-17 Results of different carbohydrates and temperatures of cobalamin production on the 3rd, 5th and 7th day.

Table 4-12 Production of different final metabolites comparing with values of glucose comsuption

	Mannitol	Succinate	Lactate	Acetate	Propionic acid
0.5 g/kg glucose 37°C	1.254	0.059	0.361	0.660	0.417
5.0 g/kg glucose 37C	0.146	0.005	0.569	0.074	0.040
0.5g/kg glycerol 37°C	0.962	0.044	0.889	0.755	0.346
0.5 g/kg glucose 30°C	0.840	0.087	0.552	0.668	0.553
5.0 g/kg glucose 30°C	0.052	0.010	0.565	0.189	0.327
0.5 g/kg glycerol 30°C	0.000	0.244	2.420	1.186	6.750

4.4.4. Effects of various supplementations

Concerning the pathway of cobalamin synthesis, some precursors were introduced. Riboflavin is a precursor of Dmbi, which binds with cobalt by Coα lower axial ligand. Betaine and methionine can transfer methyl to cobalamin. Cobalt is the central atom of cobalamin. Glycine, succinate and glutamate can be installed into delta-aminolevulinic acid. 1-amino-2-propanol, which is decarboxylated from threonin, can be attached to cobyric acid to form adenosylcobinamide. Propanediol and succinate can promote production of cobalamin. Succinate can be not only used as a precursor but also involves into the metabolism of propionic acid fermentation. Fructose and glucose were used as substitute carbohydrates to improve cobalamin production.

We found that betaine, Dmbi, lactose, glycine, a low concentration of fructose, and a low concentration of cobalt chloride had a positive effect on cobalamin production (Fig. 4-19 A). Only the pH value of fermentation with 10 g/kg of lactose was under 5.0 (Fig. 4-19 B). The pH values of high cobalamin production were all above 5.3, which is a good environment for microorganisms. A negative effect appeared sometimes, when concentrations of cobalt chloride were increased. Cobalamin production of fermentations supplemented with fructose, trimethygylcine, Dmbi, and glycine were 1.5-fold as much as the fermentation with no supplementation. However, cobalamin production of fermentations supplemented with riboflavin and Dmbi did not show a great difference. Therefore, we tried to replace Dmbi with riboflavin in next experiments and also to investigate other factors.

4.4.5. Effects of glycine and fructose on cobalamin production

All of these supplementations showed a great difference of cobalamin production between low and high concentrations in the experiments mentioned above. Further single factor experiments were conducted under the assumption that they did not have strong synergies with each other, even with other factors.

4.4.5.1. Effects of glycine on cobalamin production

After a gradient test, combination 2 with glycine of 5 g/kg was respectively recognized as the best concentration of supplement for glycine (Fig. 4-20-1a A). The changing of pH values (Fig. 4-20-1a B) also indicated that a high concentration of glycine prohibited growth of microorganisms.

Glycine supplementations under 5 g/kg stimulated and accelerated consumption of glucose (Fig. 4-20-2a E). Adversely, 10 and 15 g/kg of glycine supplementations inhibited all physiological parameters, due to the growth inhibition, except production of mannitol that may be used to balance electron equilibrium. Combinations 1, 2, and 5 produce more lactate, acetate, propionic acid and ethanol than others (Fig. 4-20-2a G, H, I, and J). The final low concentration of lactate of combination 1 and the high concentration of propionic acid may be resulting from an activity of propionibacteria.

Fructose as supplementation did not influence the growth of microorganisms (Fig. 4-20-1b C and D). Low concentrations of fructose, however, boomed the propagation of *P. freudenreichii*.

The trend and diagram of glucose consumption and mannitol production were similar (Fig. 4-20-2b E and F). Beyond our image, fructose supplementations could lower the productin of acetate, ethanol, and lactate (Fig. 4-20-2b G, H, and J). The final production of propionic acid was stimulated during increasing supplementations of fructose (Fig. 4-20-2b I). High cobalamin production can be interpreted by the suppression of production of acetate and lactate.

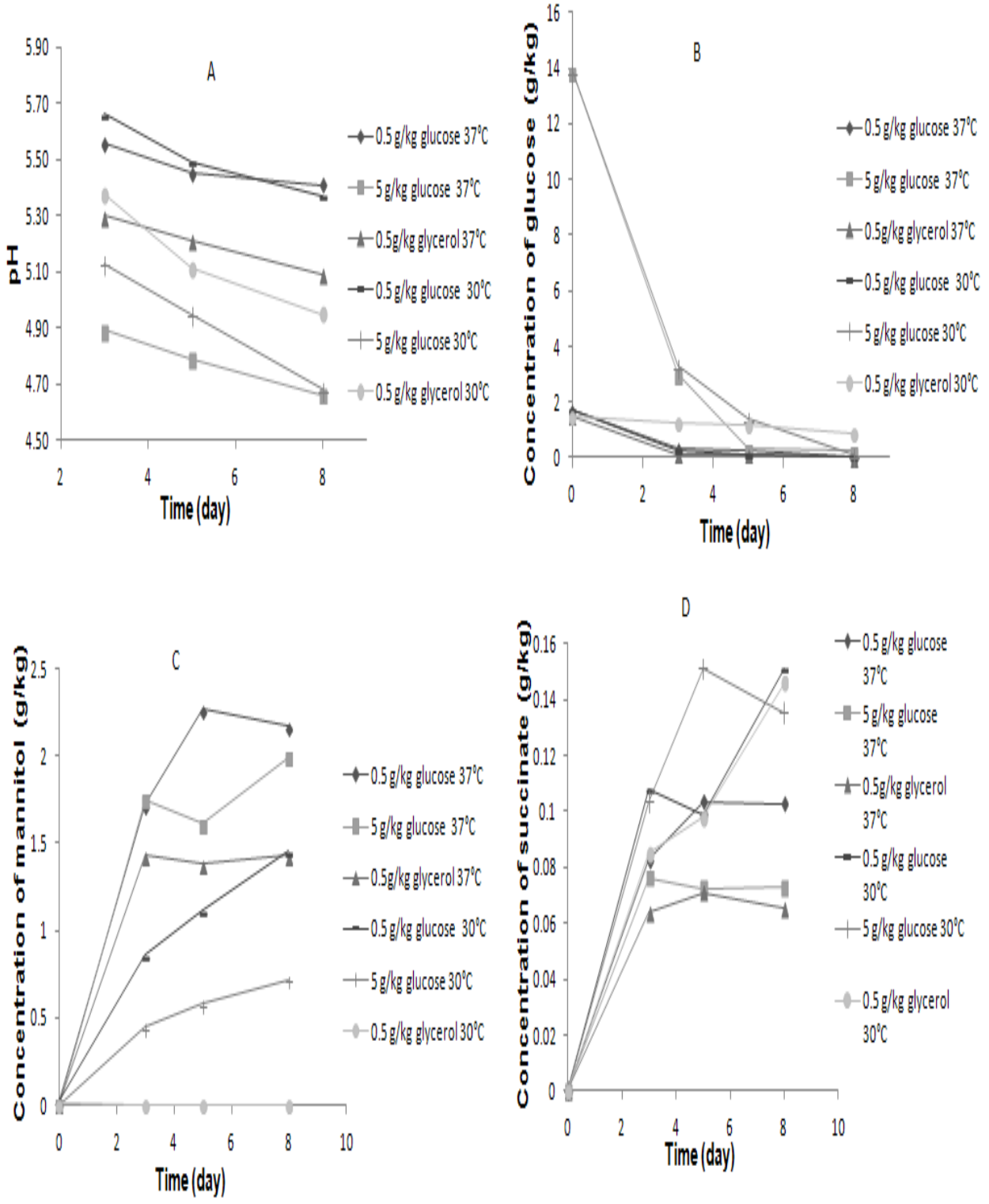


Fig. 4-18-1 Results of pH values and production of different metabolites using different carbohydrates and temperatures. Graph A means changing of pH values. Graph B stands for glucose consumption. Graph C represents production of mannitol. Graph D means production of succinate.

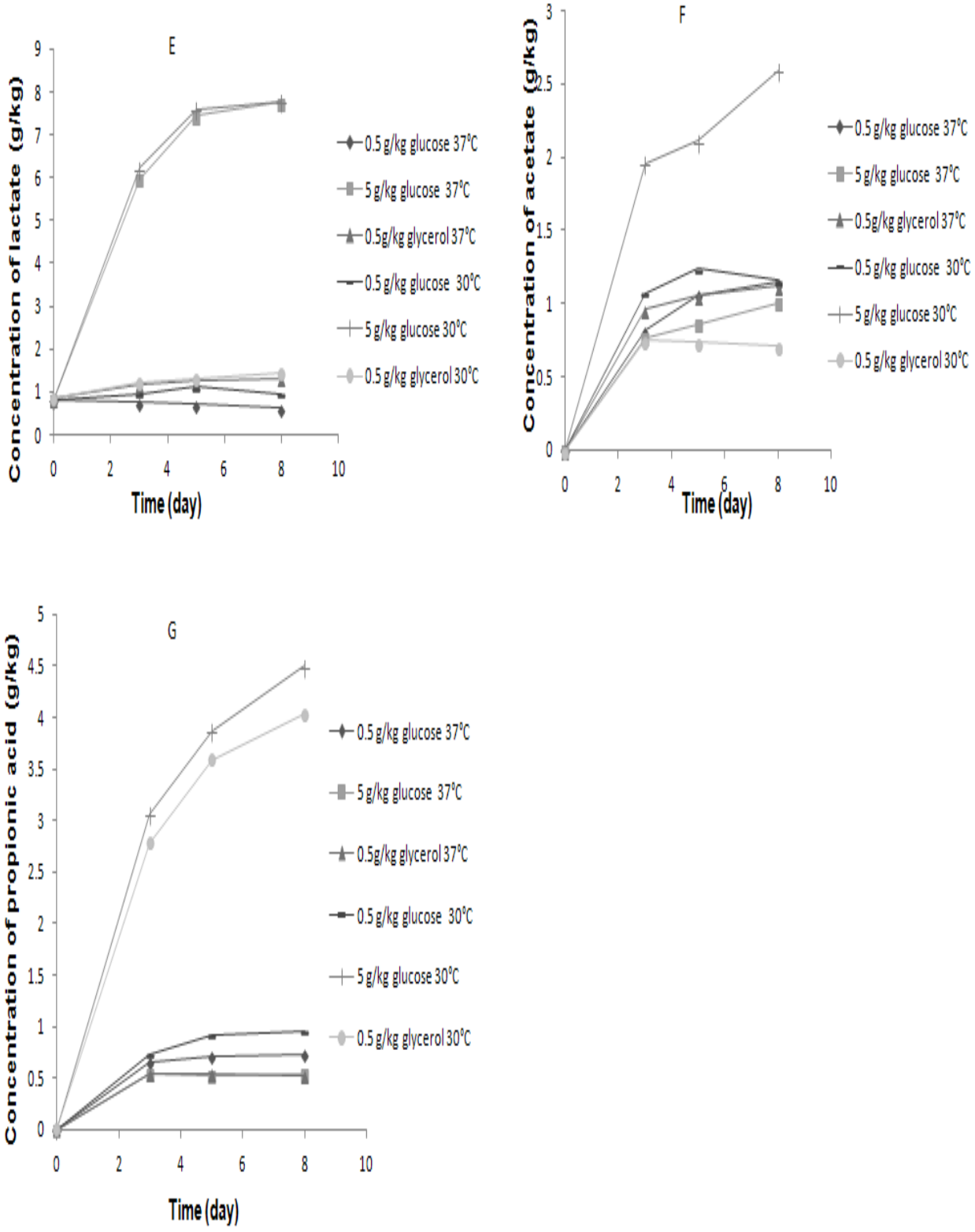
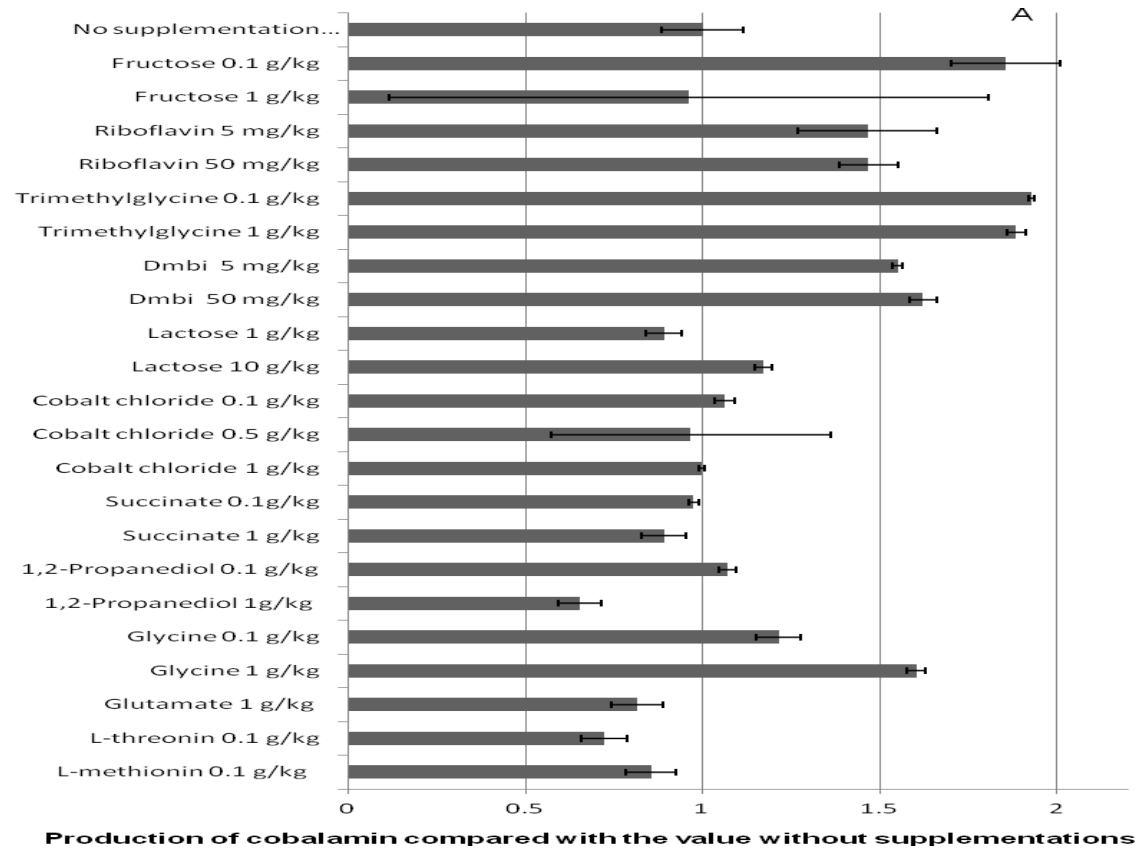


Fig. 4-18-2 Results of pH values and production of different metabolites using different carbohydrates and temperatures. Graph E means production of lactate. Graph F and G represtent production of actate and propionic acid.



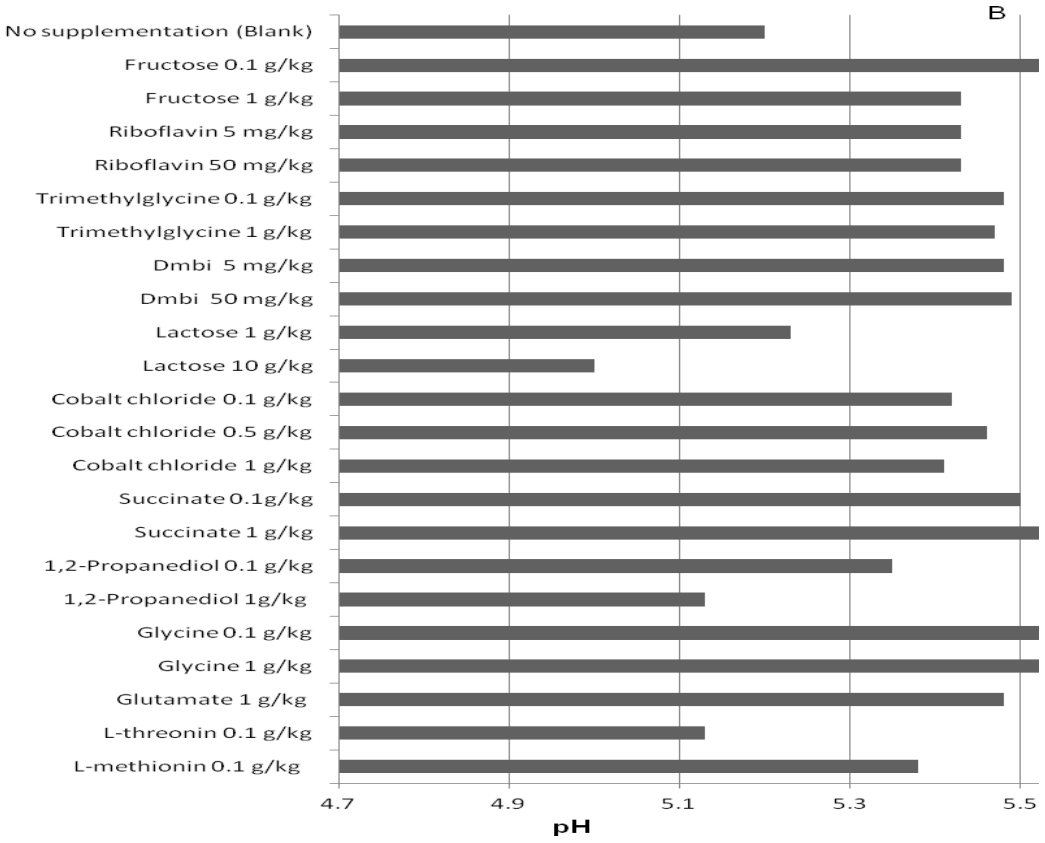


Fig. 4-19 Results of cobalamin production and final pH with various supplementations

Table 4-13 Code for various supplements

Combination code	Supplements
1	Glycine 1 g/kg
2	Glycine 5 g/kg
3	Glycine 10 g/kg
4	Glycine 15 g/kg
5	No supplement
6	Fructose 0.05 g/kg
7	Fructose 0.1 g/kg
8	Fructose 0.5 g/kg

Table 4-14 Experiment results of FFD

Run	X_1	X_2	X_3	pH	OD 600nm	Cobalamin Observed (ng/g)
1	-1	-1	-1	5.80	1.62	56.60
2	-1	-1	1	5.81	1.704	44.99
3	-1	1	-1	5.88	1.561	56.77
4	-1	1	1	5.87	1.572	53.25
5	1	-1	-1	5.75	1.911	49.46
6	1	-1	1	5.93	2.139	35.72
7	1	1	-1	5.88	1.619	42.51
8	1	1	1	5.90	1.753	45.01
9	0	0	0	5.89	1.789	49.22
10	0	0	0	5.86	1.501	49.13

X_1 = (X1 -200)/100, X_2 = (X2 -5000)/2500, X_3 = (X3 -500)/250, X1, X2, and X3 stand for natural variables of riboflavin (μg/kg), CoCl2 (μg/kg), and Betaine (mg/kg).

Nevertheless cell concentrations were lower compared with others, which may be caused by inhibition of oxygen, difference of ingredients in different batches of tofu or inhibiting of these supplementations. Granting these reasons, both supplementations were added only in fermentations with natural substances to avoid the interferences from these supplementations to other experiments.

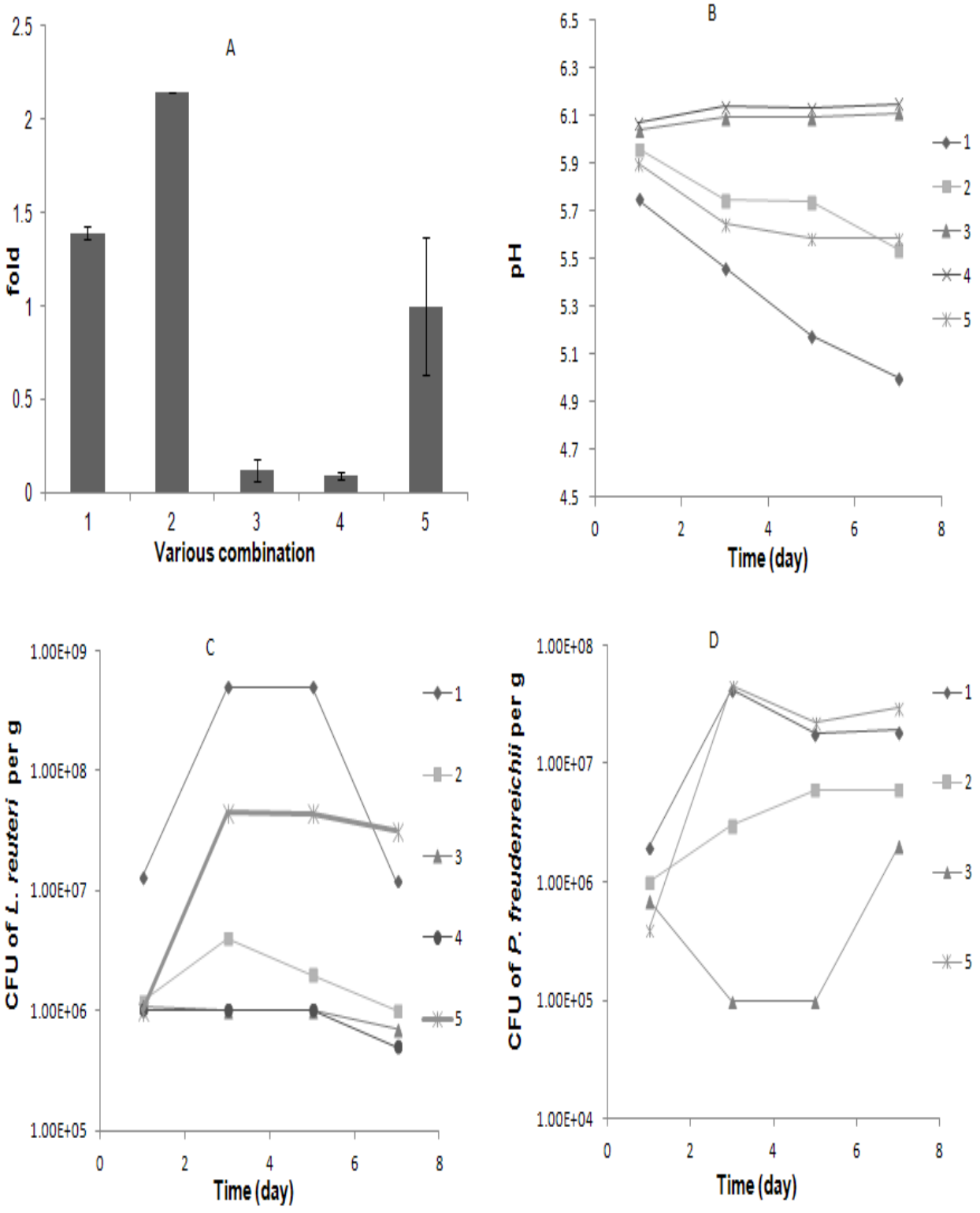


Fig. 4-20-1a Results of cobalamin production, growth curves of both microorganisms and final pH with various concentrations of glycine supplementation. Graph A means cobalamin production of various glycine supplementations with cobalamin production without supplementation. Graph B means the changing of pH values. Graph C and D indicats growth curves of *L. reuteri* and *P. freudenreichii*.

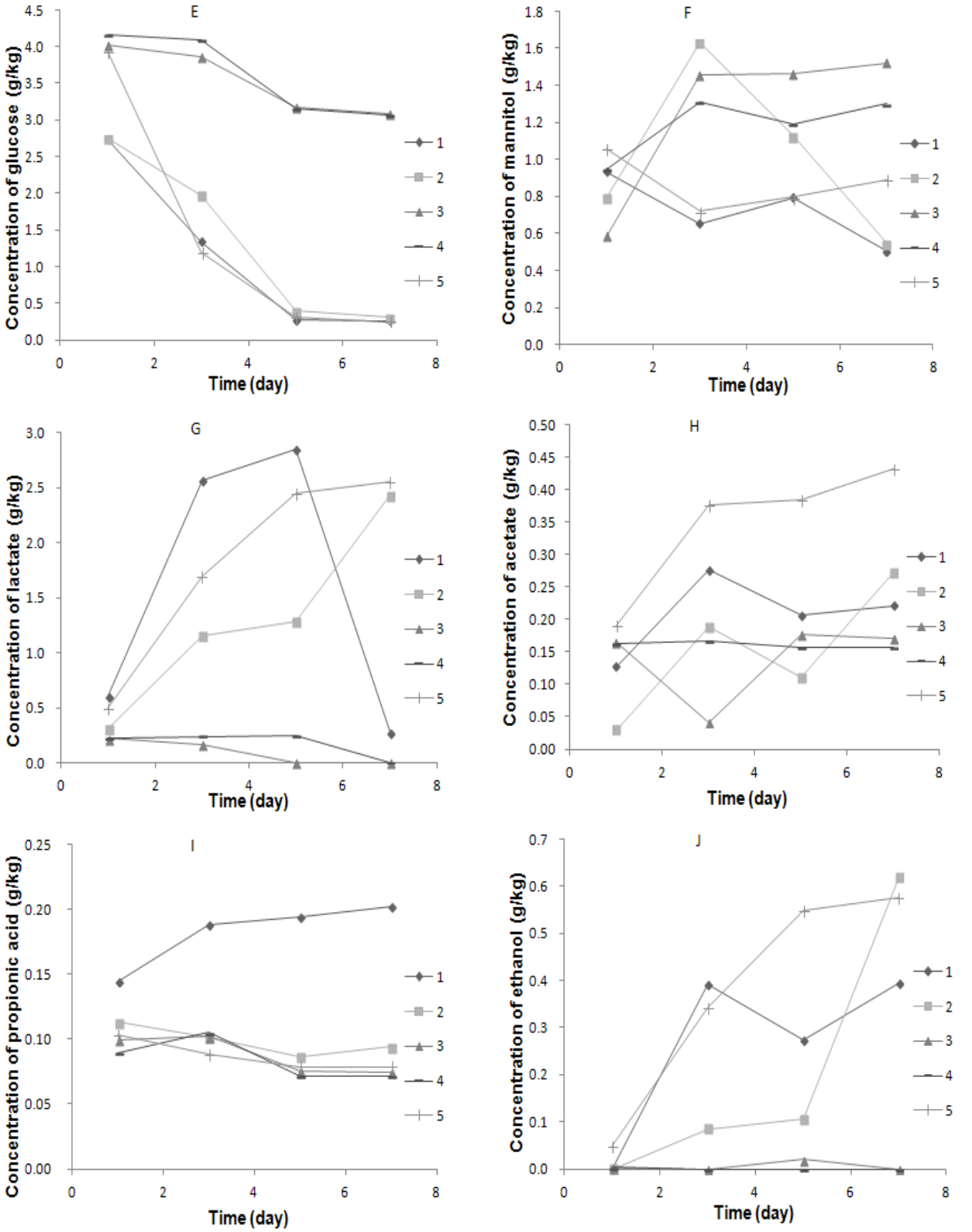


Fig. 4-20-2a Results of cobalamin production, growth curves of both microorganisms and final pH with various concentrations of glycine supplementation. Graph E means consumption of glucose. Graph F, G, H, I, and J respectively stand for production of mannitol, lactate, actate, propionic acid, and ethanol.

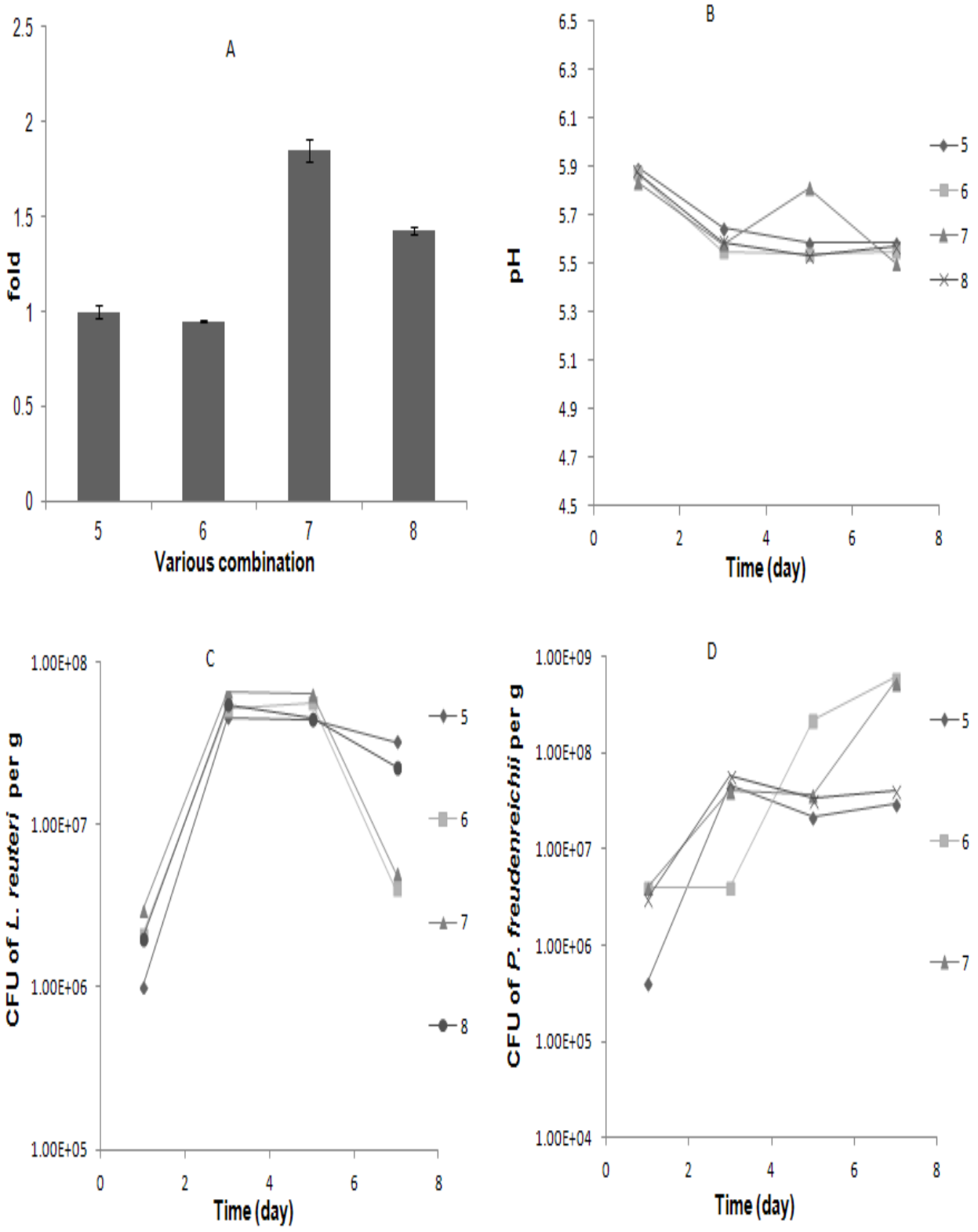


Fig. 4-20-1b Results of cobalamin production, growth curves of both microorganisms and final pH with various concentrations of fructose supplementations. Graph A means cobalamin production of various fructose supplementations with cobalamin production without supplementation. Graph B means the changing of pH values. Graph C and D indicats growth curves of *L. reuteri* and *P. freudenreichii*.

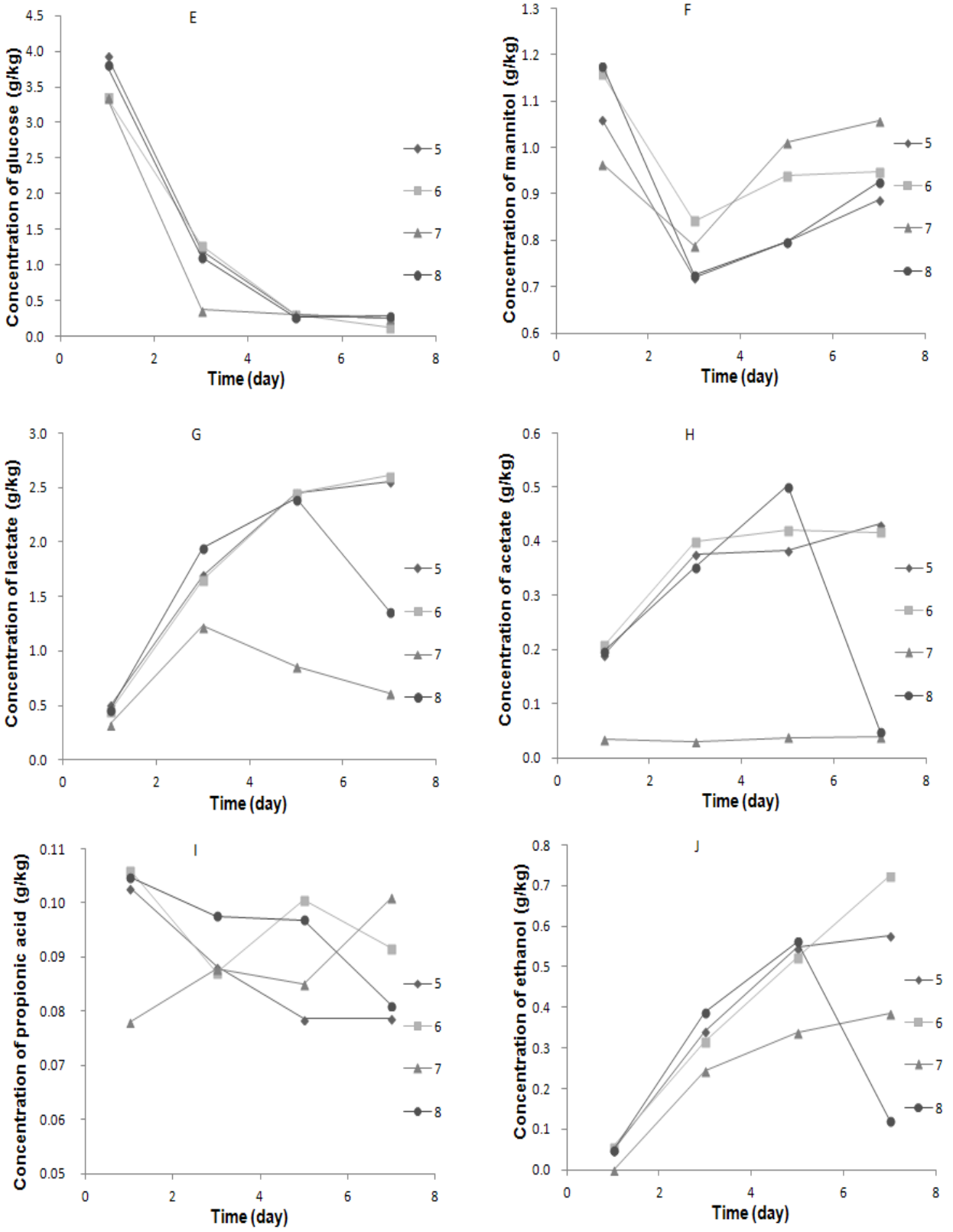


Fig. 4-20-2b Results of cobalamin production, growth curves of both microorganisms and final pH with various concentrations of fructose supplementations. Graph E means consumption of glucose. Graph F, G, H, I, and J respectively stand for production of mannitol, lactate, actate, propionic acid, and ethanol.

4.4.6. Effects of riboflavin, Dmbi, and days of anaerobic fermentation

As described in the introduction, Dmbi, converted from riboflavin, is an important and key step to synthesize cobalamin, or adenosylcobinamine will be converted into other analogues. Oxygen interrupts synthesis of Dmbi and cobalamin. Riboflavin can be converted into Dmbi and produce the same level of cobalamin after 4, 5 and 6 days respectively of anaerobic fermentation (Fig. 4-21). After 5 and 6 days of anaerobic fermentation, production of cobalamin had reached a peak and started to decrease. After these experiments, riboflavin was recognized as a substitute of Dmbi that is expensive and 5 days of anaerobic fermentation is the more efficient and economic procedure.

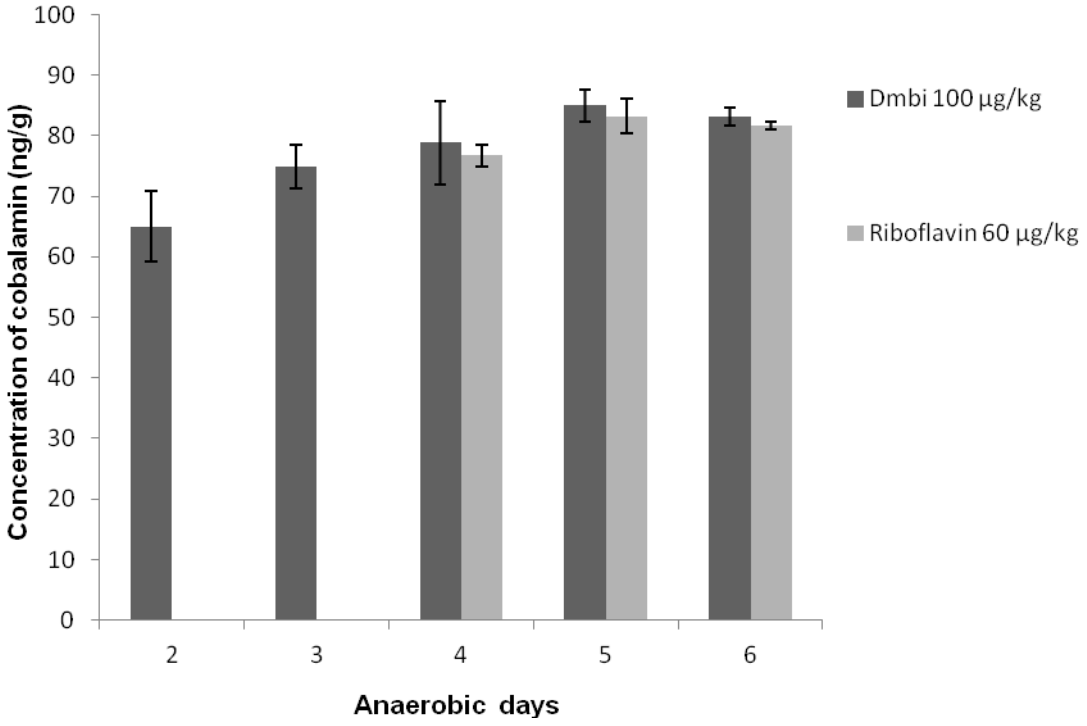


Fig. 4-21 Results of effects of riboflavin, Dmbi, and anaerobic days on cobalamin production. The black block means supplementations with 100 µg/kg of Dmbi and grey block represents supplementation with 60 µg/kg of riboflavin.

4.4.7. Effects of $CoCl_2$ on cobalamin synthesis

After factors selection experiments, cobalt chloride had an effect on cobalamin synthesis. But

production dropped down during concentrations of cobalt were increasing. A further check was conducted. When concentrations of cobalt deceased down to 1 mg/kg, production was significantly higher than before (Fig. 4-22). High concentrations of cobalt supplementations led to a decrease of pH. This concentration of cobalt chloride was used to do further optimization experiments.

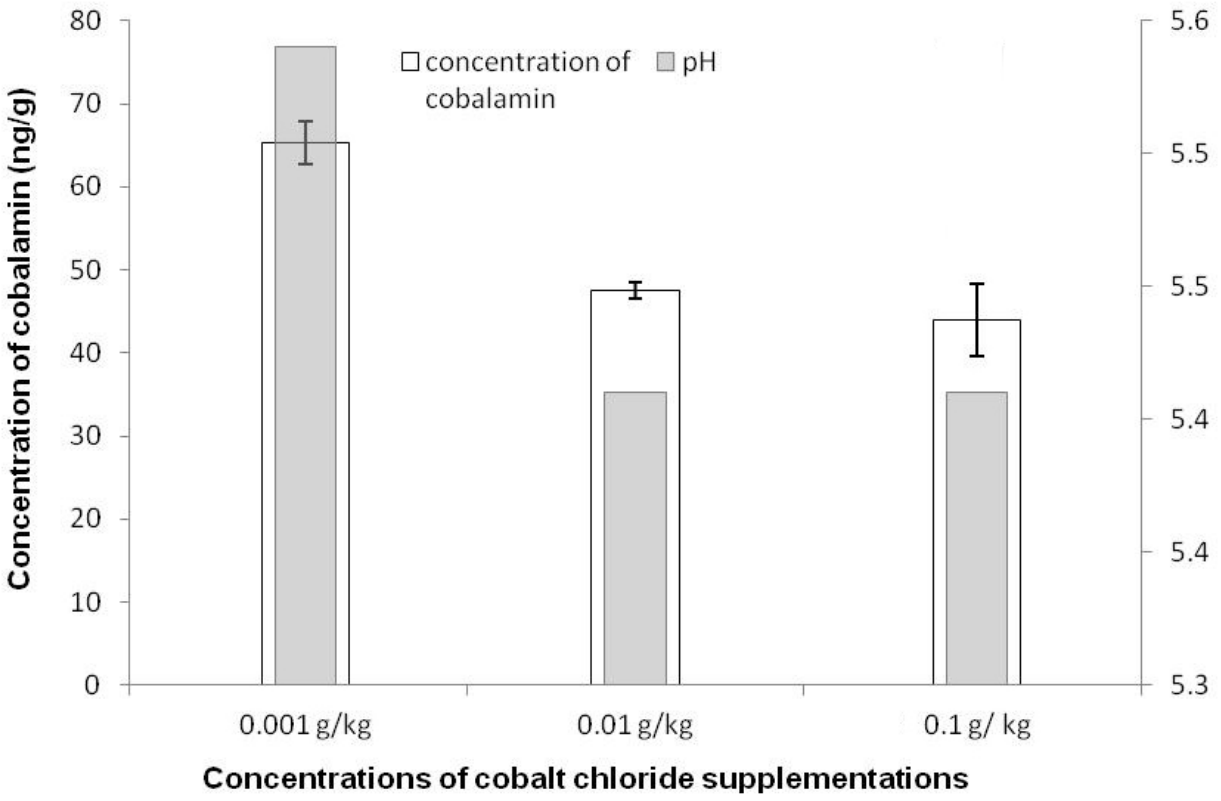


Fig. 4-22 Results of effects of cobalt on cobalmin production. White columns with black lines mean concentration of cobalamin. Grey conlumns mean pH values.

4.4.8. Full factorial design experiments of $CoCl_2$, riboflavin, and betaine

A 2^3 fractional factorial design requested 8 experiments and other four experiments at the center of design were repeated four times to analyze the variance. The results of FFD are shown in Table 4-14 and Table 4-15. Production of vitamin B_{12} in every gram of wet tofu ranged from 42.51 to 56.60 ng/g. The increase of riboflavin ($p < 0.05$) had a dramatically negative influence on the yield of vitamin B_{12}. Cobalt ($p = 0.46$) and betaine ($p = 0.12$) have no clear effect on production of

cobalamin. The value of the regression coefficients were calculated and the first order equation can be written down as follows,

$Y_{VB12} = 48.04 – 4.864\ X_1 + 1.345\ X_2 – 3.298\ X_3$ (Equ. 4-4)

Regeression analysis results of FFD experiment in table 4-15 illustrate that only one factor, riboflavin, plays an important role in vitamin B_{12} production at a probability level of 95.67%. Other three factors were not found to be significant at the probability level of 90%.

The coefficient R^2 of the model equation at 0.766 indicates that the model only explains 76.6 % of the variability in the data. The value of F-test at 4.36 confirming the statistical significance of the model equation indicats that the model is adequate to the data at the probability level of 91%.

The pH value was not related with cobalamin production. But pH values varied from 5.75 to 5.93, which is higher than results of cobalt. Riboflavin led to an increase of pH. However, the correlation between OD 600 and cobalamin was -0.71. That means cell concentrations in this situation have a negative effect on cobalamin production. This was the first time that we found that increasing of cells suppressed cobalamin production.

4.4.9. Riboflavin single factor design

Relying on above results, an optimization was conducted by decreasing the concentration of riboflavin at an interval of 20 µg/kg. We could easily figure out that the supplementation with 80 µg/kg of riboflavin made a biggest contribution to cobalamin production (Fig. 4-23). Definitely, 80 µg/kg of riboflavin was chosen for further experiments.

4.4.10. Prolongation of riboflavin and oxygen supply

Cobalamin is also an inhibiting regulator to cobalamin synthesis by connecting with a riboswitch. In that case, we tried to postpone the conversion from adenosylcobinamin to adenosylcobalamin to alleviate an inhibited function of cobalamin. An experiment was designed and conducted to figure out the best time for adding of riboflavin. After analysis of ANOVA, adding riboflavin after 2 days was significantly different with others (Fig. 4-24). This was used in further

experiments. The cobalamin production dropped down in fermentations of adding riboflavin after 3 and 4 days, because microorganisms may distribute some sources to synthesize riboflavin first under scarcity of riboflavin.

Table 4-15 Results of FFD regression analysis for cobalamin

Term	Regression analysis for Cobalamin		
	Coefficient	t-value	Significant level
Intercept	48.040	28.84	0.0000
X_1	-4.864	-2.92	0.0433**
X_2	1.345	0.81	0.4646
X_3	-3.298	-1.98	0.1189
	R^2=0.766	F= 4.36	P = 0.0943

X_1 = (X1 -200)/100, X_2 = (X2 -5000)/2500, X_3 = (X3 -500)/250, X1, X2, and X3 stand for natural variables of riboflavin (μg/kg), $CoCl_2$ (μg/kg), and Betaine (mg/kg).

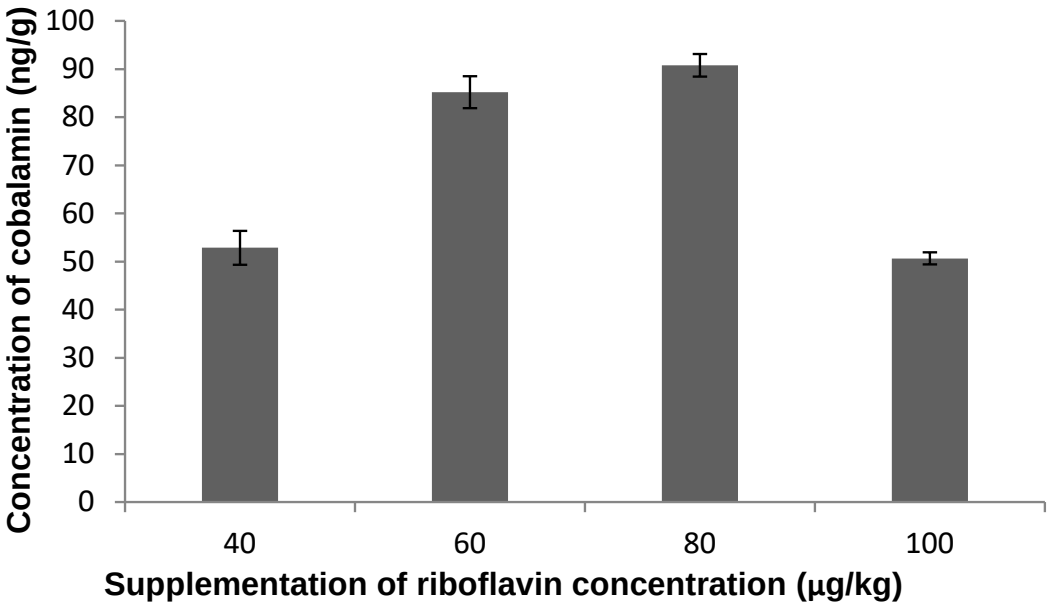


Fig. 4-23 Effects of different concentrations of riboflavin on cobalamin production

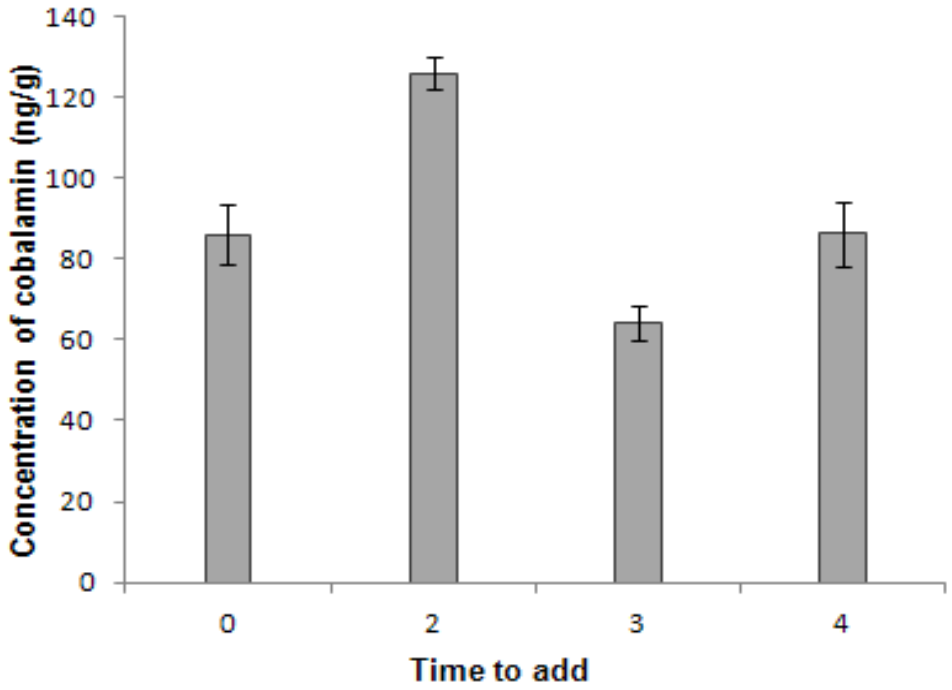


Fig. 4-24 Effects of adding time of riboflavin on cobalamin production

4.4.11. Effects of oxygen supplementations on cobalamin production

Oxygen influences CobT enzyme, conversion of riboflavin to Dmbi, production of propionic acid, and also growth of microorganisms. The supply of oxygen was designed to improve the conversion of riboflavin and to decrease the concentration of propionic acid (Miyano et al. 2000). As a result of experiments (Fig. 4-25a), oxygen had a significant influence on the growth of *L. reuteri*. Oxygen boomed the growth of *P. freudenreichii* due to low concentration of propionic acid (Fig. 4-25c). Compared with fermentations without oxygen supplementations, fermentations with oxygen supplementation contained less ethanol and lactate (Fig. 4-25c). In contrast, more acetate was detected. Regarding to propionic acid, fermentations with oxygen supplementation contained less than others. All these data meant all fermentations diminished the production of propionic acid. However, all experiments led almost to the same pH value. Fermentations with oxygen for the last 2 days got as much cobalamin as standing fermentations (Fig. 4-25b). ANOVA showed that there are no differences between them. Otherwise cobalamin production of fermentations with oxygen supplementation for 1 and no supplementation was reduced clearly. Aerobic fermentations for last the 2 days with proper oxygen supplementation was important for the production of cobalamin.

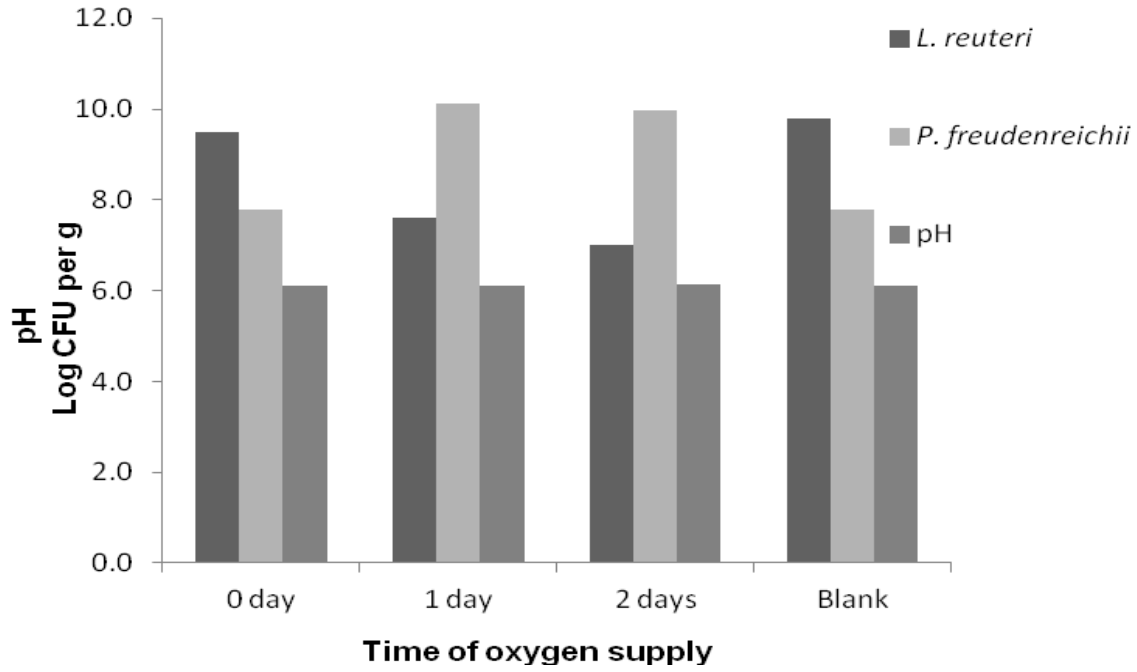


Fig. 4-25a Effect of oxygen on final cell concentrations of microorganisms and pH values

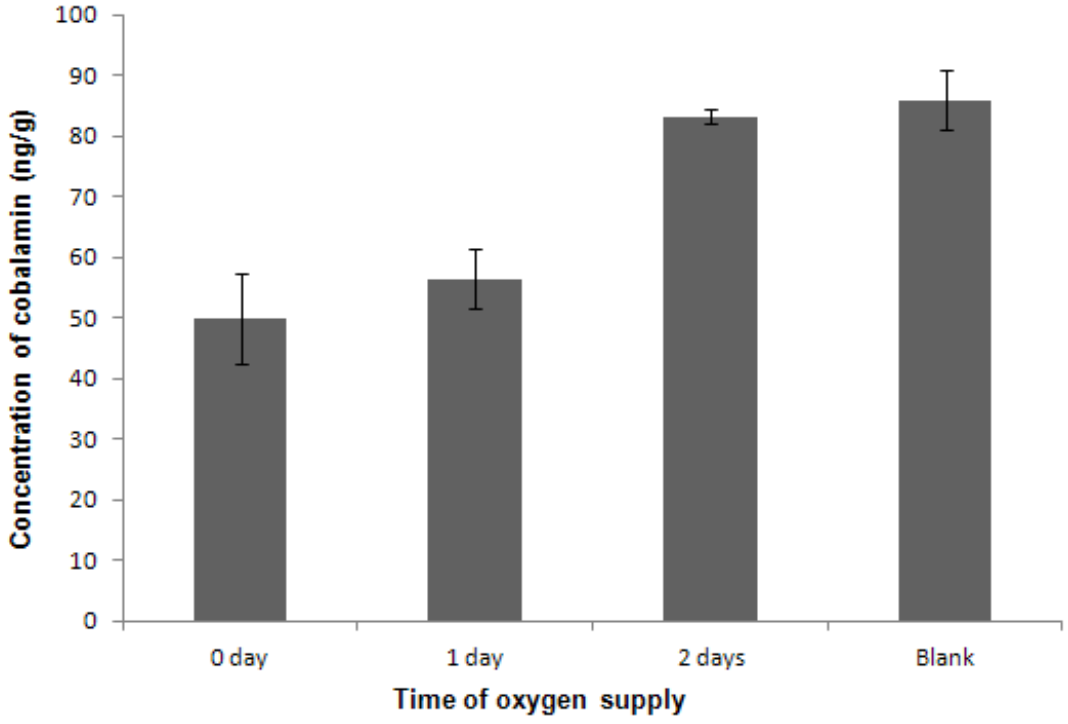


Fig. 4-25b Effects of oxygen on cobalamin production

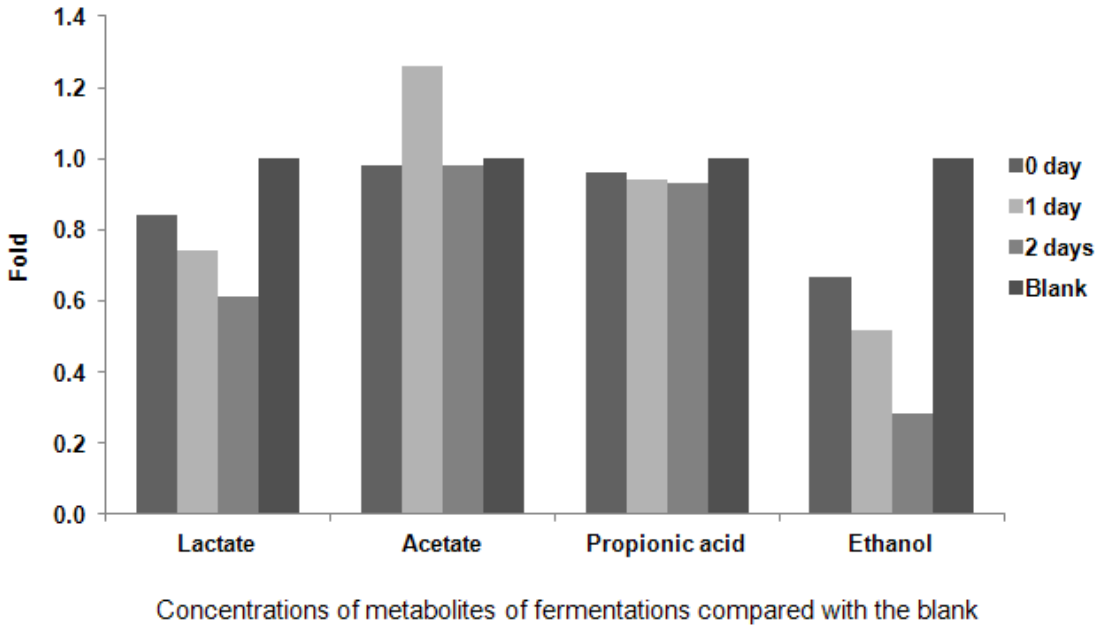


Fig. 4-25c Final metabolites of different oxygen supply fermentations compared with the fermentation without oxygen supply

4.4.12. Effects of different initial pH values on the production of cobalamin

The initial pH value has a prevailing function on the growth of microorganisms, final metabolites and also production of cobalamin. Initial pH values of 6.5 and 7.0 obviously increased final numbers of both bacteria compared with others (Fig 4-26a). But no differences of final pH values were found. Initial pH values of 6.5 and 7.0 also produced more cobalamin than others (Fig. 4-26b). By analysis of T-test, no difference ($p = 0.61$) was found between them. All metabolites are compared with corresponding metabolites of an initial pH value of 7.0 (Fig. 4-26c). Fermentations with an initial pH value of 7.0 produced more ethanol, and propionic acid. Adversely, all others produced more acetate than fermentation with initial pH values of 6.5 and 7.0. The final concentrations of lactate were not so different. The initial pH value had a positive impact on metabolism and cell growth. Generally, the initial pH value of fermentations was adjusted to between pH 6.5 and 7.0 in order to produce more cobalamin.

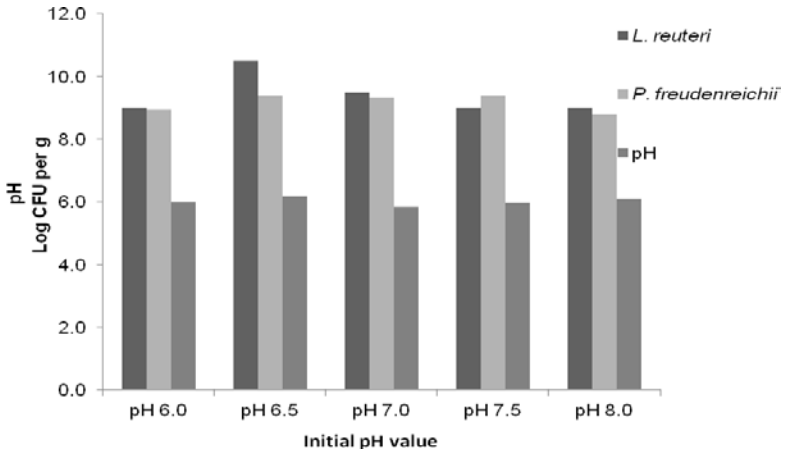


Fig. 4-26a Effects of different initial pH values on final concentrations of microorganisms and final pH values

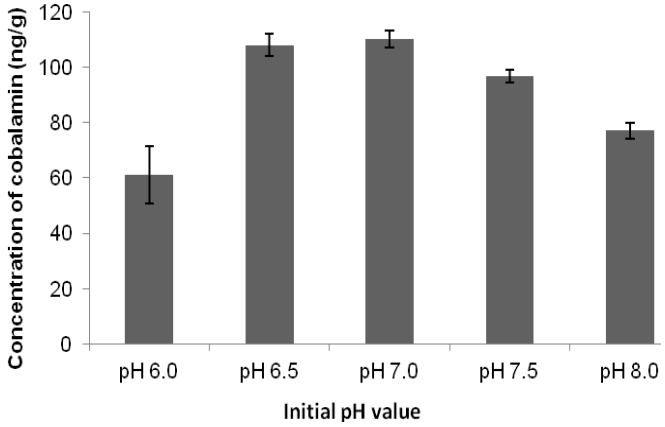


Fig. 4-26b Effects of different initial pH values on cobalamin production

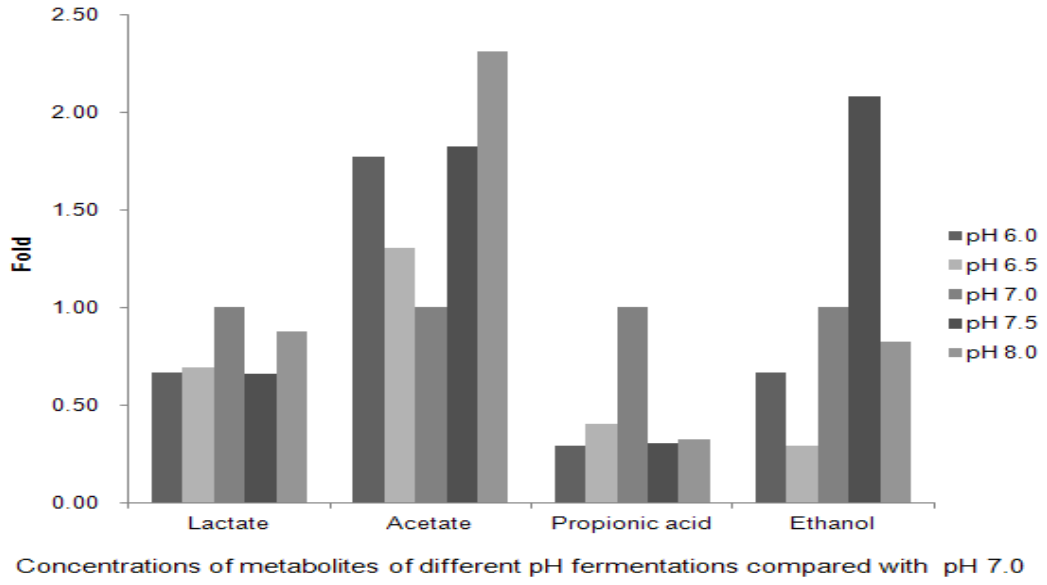


Fig. 4-26c Final metabolites of fermentations with different initial pH values compared with the fermentation with the initial pH value of 7.0

4.4.13. Effects of temperatures on production of cobalamin

All microorganisms have their own optimum growth temperatures. For cofermentations, we should find a temperature fitting for both microorganisms. The final cell concentrations of both bacteral strains declined with the increasing of temperatures (Fig. 4-27a). However, temperature had no influence on final pH. The highest cobalamin production was found in the fermentation at 30 °C. Ethanol production was decreasing with increase of temperatures (Fig. 4-27c). The acceleration evaporation by high temperatures explained this phenomenon. Final metabolites from fermentation at 30 °C and 35 °C were almost the same. 30 °C was the optimum cobalamin production temperature.

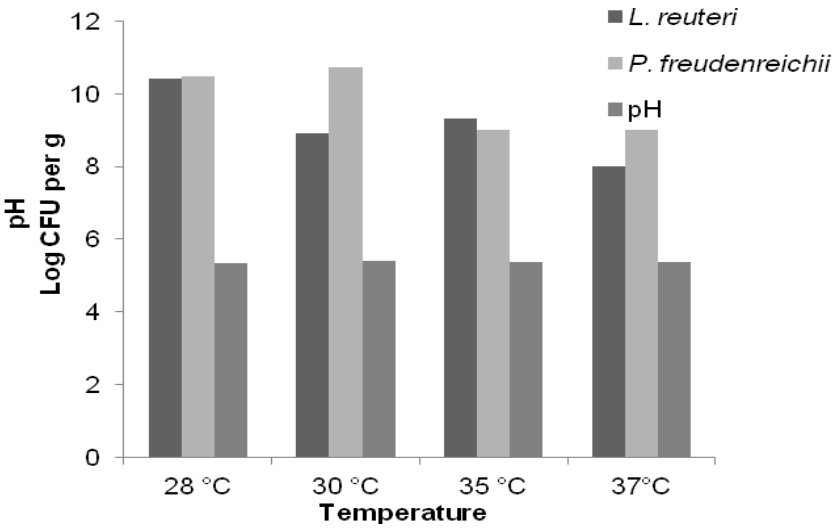


Fig. 4-27a Effects of temperatures on final concentrations of microorganisms and final pH values

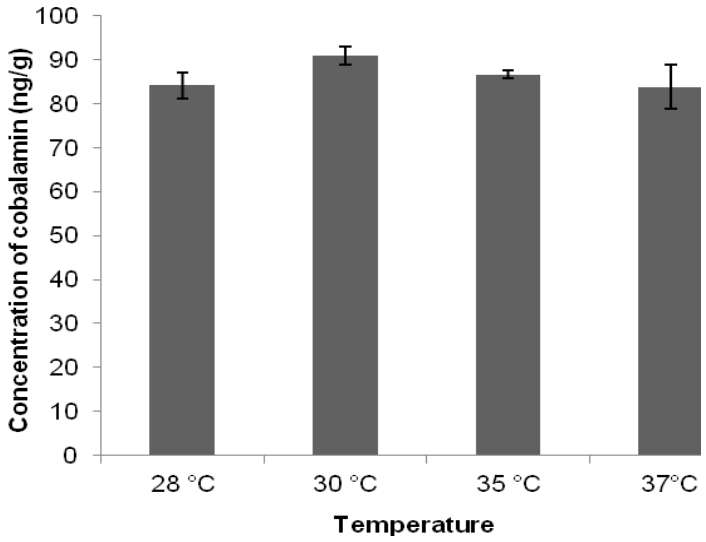


Fig. 4-27b Effects of temperatures on cobalamin production

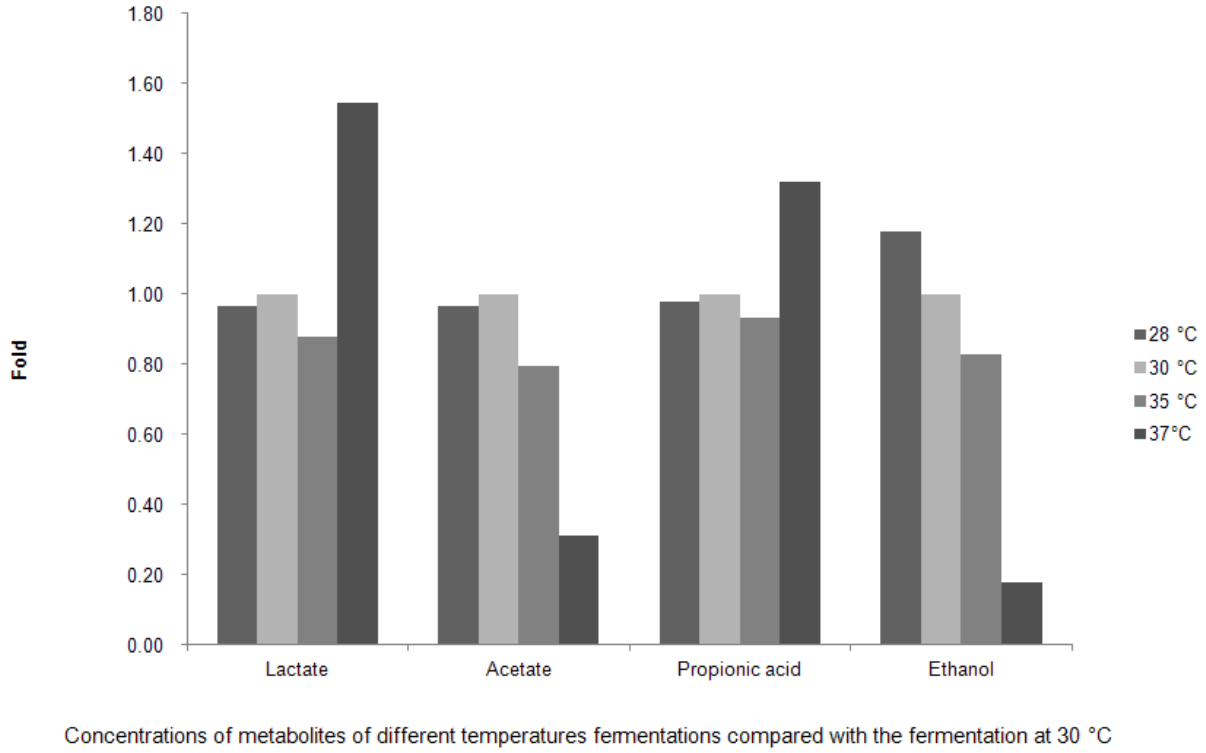


Fig. 4-27c Final metabolites of fermentations with different temperatures compared with the fermentation at 30 °C

4.4.14. Effects of different inoculum titers on production of cobalamin

Two steps were involved into cobalamin production. The first step was increment of bacterial cells producing cobalamin. The second step was the yielding step, which needed optimum conditions. The first design was made by three combinations: C1 means fermentations inoculated with 1 mL of *L. reuteri* and *P. freudenreichii*. C2 means fermentations inoculated with 0.5 mL of *L. reuteri* and *P. freudenreichii*. C3 means fermentations inoculated with 0.1 mL of *L. reuteri* and *P. freudenreichii*. The growth trend of all fermentations looked similar (Fig. 4-28a A and B). The descending trends of pH values of combinations C2 and C3 were similar, but different from combination C1. Production of cobalamin in all fermentations was over 80 ng/g, but combination C2 reached the highest value among them (Fig. 4-28a D). These results came from good distribution of sources between two steps by proper inoculation value. The inoculum volume would be fixed at 0.5 mL precultures (about $5*10^7$).

Table 4-16 Combinations of various time and inoculation experiments

	Inoculum densities of *L.reuteri* (CFU per mL)		
P. freudenreichii adding time (day)	1.00E+06	1.00E+07	1.00E+08
0	L0.1	L0.2	L0.3
1	L1.1	L1.2	L1.3
2	L2.1	L2.2	L2.3
3	L3.1	L3.2	L3.3
No propionibacteria	L1	L2	L3

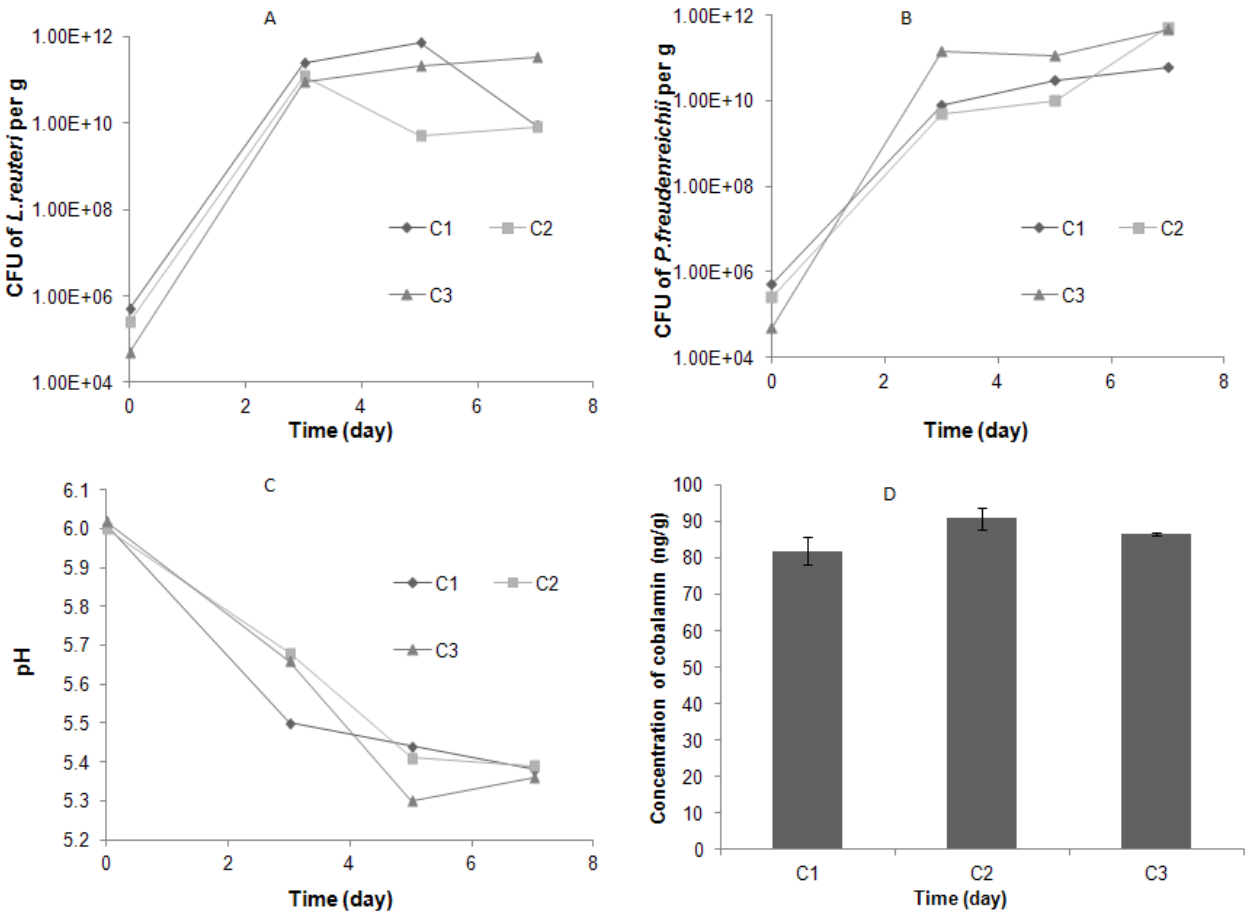


Fig. 4-28a Effects of different inoculum densities on growth curves of *L. reuteri* (Graph A), *P. freudenreichii* (Graph B), pH values (Graph C) and cobalamin production (Graph D).

An idea was drawn from a traditional cheese making procedure. Firstly *L. reuteri* was inoculated at different densities, and then after several days *P. freudenreichii* was inoculated to the fermentation. The combinations are illustrated in Table 4-16. Only L 0.3 produced as much cobalamin as we reached before (Fig, 4-28b). Others produced less than 20 ng/g of cobalamin. In

combinations of L 2.2 and L 3.3, no cobalamin was detected. These phenomena could be interpreted by the information listed below.

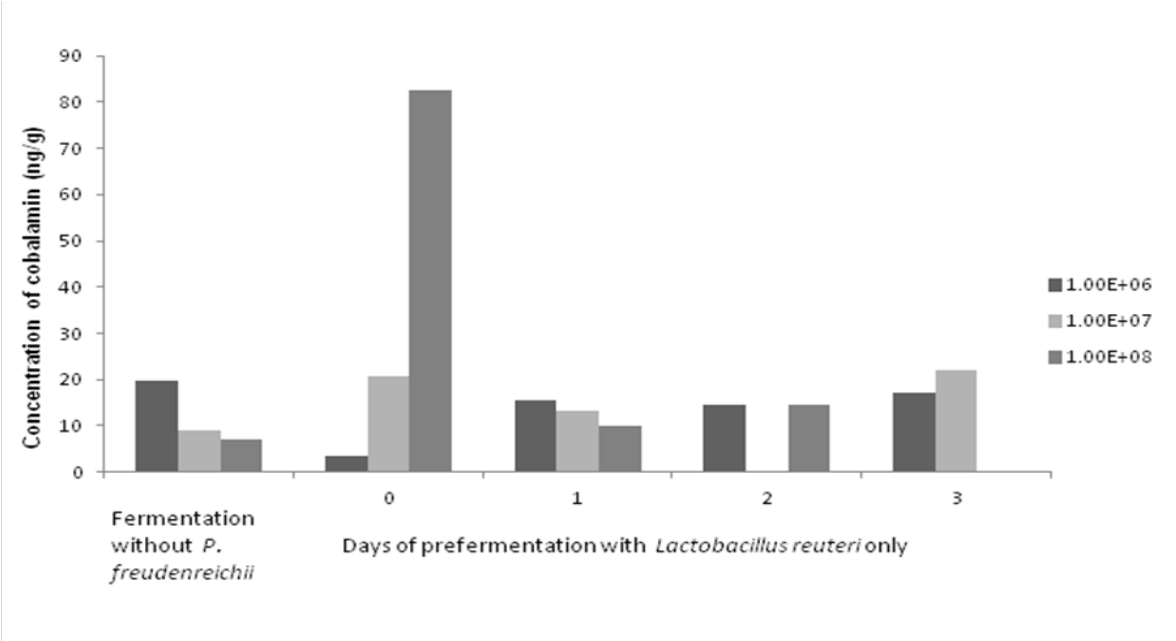


Fig. 4-28b Effects of different inoculum densities of *L. reuteri* and different time to add *P. freudenreichii* on cobalamin production Black column means an inoculum density of $1*10^6$ CFU/kg of L. reuteri. Grey column means an inoculum density of $1*10^7$ CFU/kg of *L. reuteri*. Dark grey column means an inoculum density of $1*10^8$ CFU/kg of *L. reuteri*.

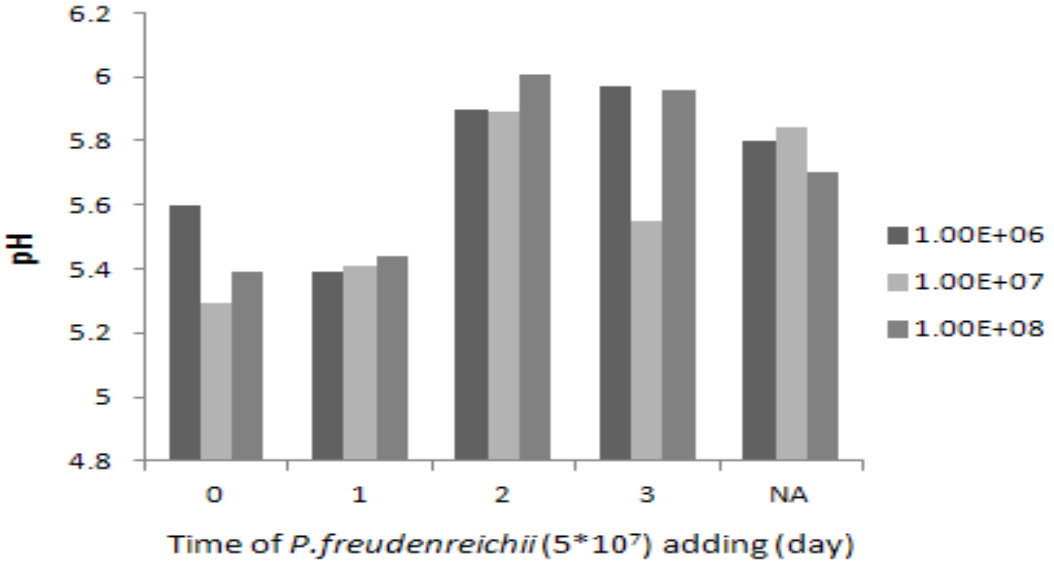
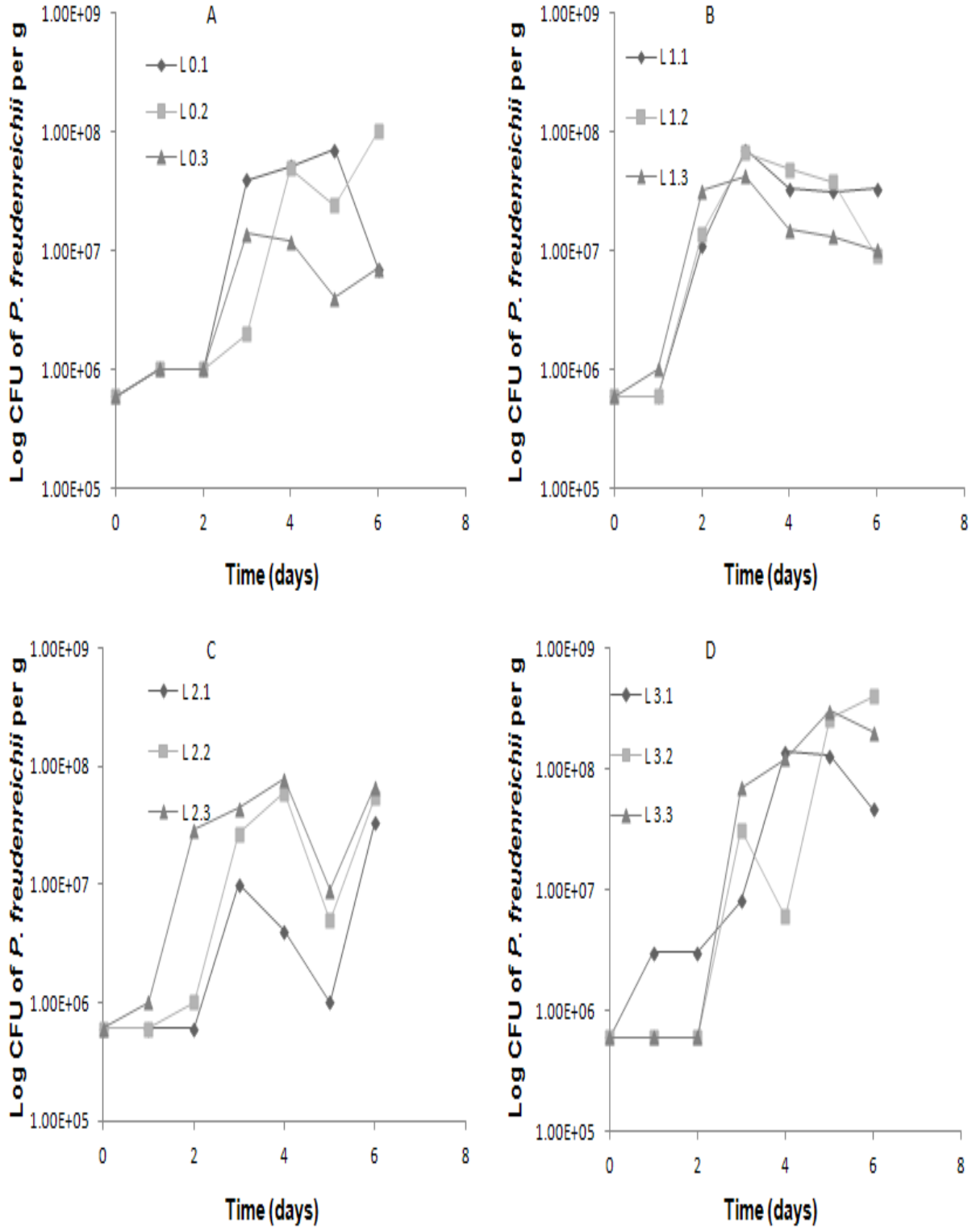
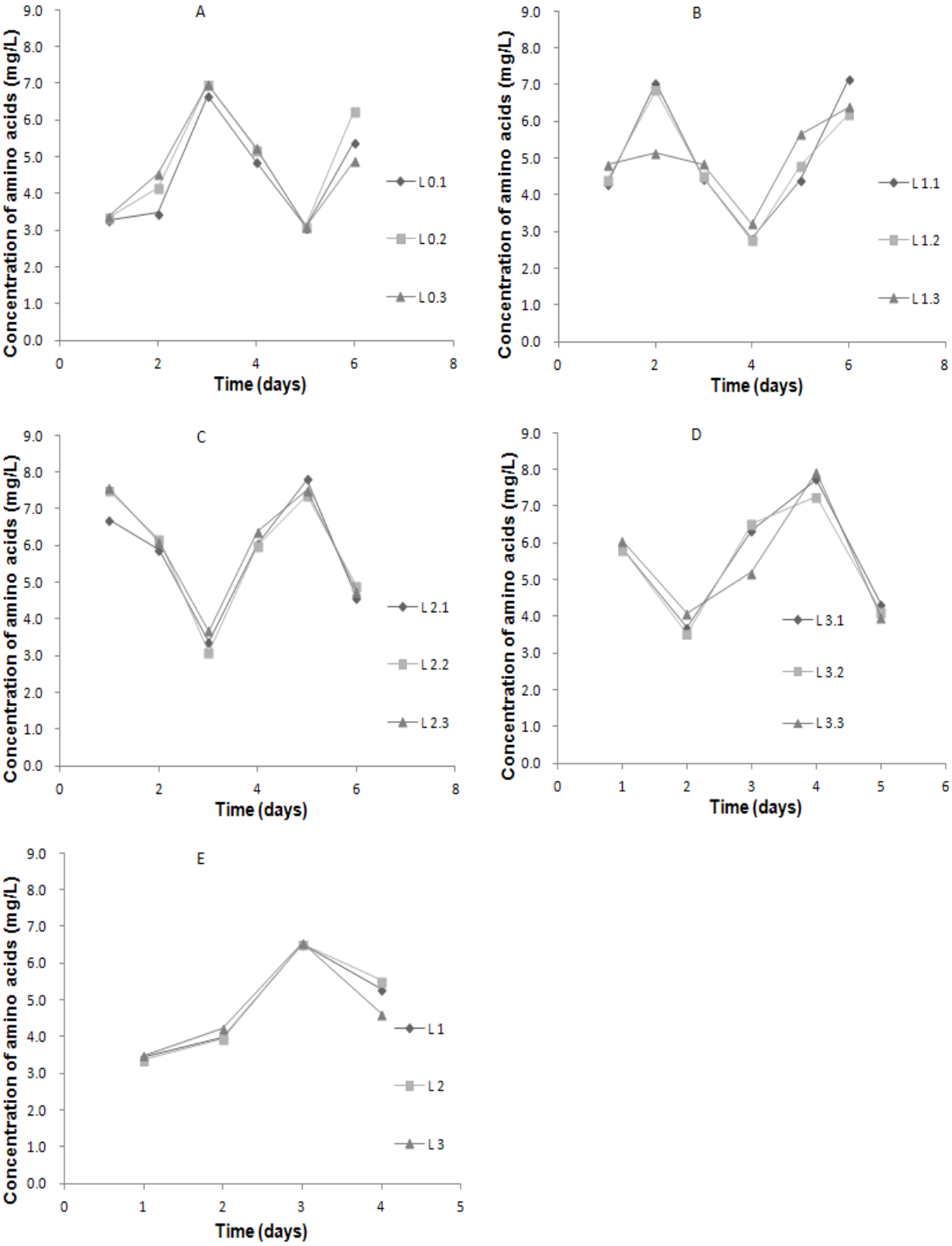


Fig. 4-28c Effects of different inoculum densities of *L. reuteri* and different time to add *P. freudenreichii* on final pH values Black column means a inoculum density of $1*10^6$ CFU/kg of *L. reuteri*. Grey column means a inoculum density of $1*10^7$ CFU/kg of *L. reuteri*. Deep grey column means a inoculum density of $1*10^8$ CFU/kg of *L. reuteri*.



Fig, 4-28d Effects of different inoculum densities of *L.reuteri* and different time to add *P. freudenreichii* on growth of *P. freudenreichii*



Fig, 4-28e Effects of different inoculum densities of *L. reuteri* and different time to add *P. freudenreichii* on concentration of free amino acids in the fermentation surpernant.

The final pH values of fermentations with inoculation of *P. freudenreichii* after 2 or 3 days of fermentation were higher than after 1 day and cofermentation starting from beginning (Fig. 4-28c). Inoculations of *P. freudenreichii* at L0.1, L0.2 and L0.3 grew slower than others (Fig. 4-28d). Shortage of amino acids would be a critical factor for growth of *P. freudenreichii* (Fig. 4-28e A). Graph A and C in Fig. 4-28d show a clear diauxic growth. According to Fig. 4-28e, preliminary fermentations with *Lactobacillus* could offer amino acids to *P. freudenreichii.* The earlier *L.reuteri* was added, the quicker amino acids were produced and consumed (Fig. 4-28e). More amino acids were produced, after concentrations of amino acids decreased down to ca. 3 mg/kg. In conclusion, 0.5 mL of both of precultures ($5*10^7$ cells of *L. reuteri* and $1*10^8$ of cells of *P. freudenreichii*) was used to inoculate together at beginning for following fermentations.

4.5. Scaling up

4.5.1. 1 kg batch fermentations with various concentrations of glucose supplementation

To improve cobalamin production and set up a reference for fed batch experiments, batch experiments were conducted. We found that the batch fermentation with 5 g/kg of glucose supplementations led to the highest concentration of cobalamin among these experiments (Fig. 4-29-2 I). The speed of decrease of pH values and final pH values were positively related to glucose supplementation concentrations (Fig. 4-29-1 A). Growth curves of *L. reuteri* looked definitely diauxic (Fig. 4-29-1 B). Except the fermentation supplemented with 7 g/kg of glucose, *P. freudenreichii* growth curves in other batches showed a continuous exponential growth. The consumption of glucose was fast at the first 2 days and stayed steady for the next 1 day (Fig. 4-29-1 D). Then from 3rd day glucose was consumed faster till the end. Fig. 4-29-2 E and F illustrate the lactate and acetate production. All of them were always increasing till the end. Concentration of propionic acid was fluctuant (Fig. 29 G) due to oxygen. Ethanol could not be detected before the 4th day and then increased suddenly (Fig. 4-29-2 H).

4.5.2. Fed batch experiments

Depending on results of batch fermentations with 5 g/kg of glucose, we found that on the 1st day and 3rd day the rest concentration of glucose decreased dramatically. Hence a series of fed batch

experiments was designed. F1 means that 4 grams of glucose were added to fermentations after 4 days to offer energy and nutrition to bacteria to do further production. With the same target, F2 means 4 grams of glucose were added to fermentations after 5 days. F3 means 4 grams of glucose were added to fermentations after 6 days. F4 means 1 gram of glucose was added every day to fermentations and F5 means 2 gram of glucose were added every 2 days to fermentations. After experiments, a much lower final pH values was obtained (Fig. 4-30-1 A). The pH values decreased more slowly than batch experiments, and less cells of both microorganisms were produced (Fig. 4-30-1 A, B and C).

That may be caused by the fact that oxygen inhibited growth of *L. reuteri*. In consequence, less lactate was produced, which can enhance the growth of *P. freudenreichii*. In these experiments anaerobic containers were opened several times to messure paramenters. This time glucose was consumed faster compared with batch fementations and glucose was nearly consumed completely (Fig. 4-30-1 D). More lactate was produced than batch fementation (Fig. 4-30-2 E). There are no big differences in acetate and propionic acid concentrations compared with batch experiments (Fig. 4-30-2 F and G). But F4 was more active in production of acetate and propionic acid. In these experiments ethanol was earlier detected than before and production was higher. All these fermentations generated more cobalamin, over 200 ng/g (Fig. 4-30-2 I), than batch experiments. These fed batch fermentations were used for cobalamin production in tofu.

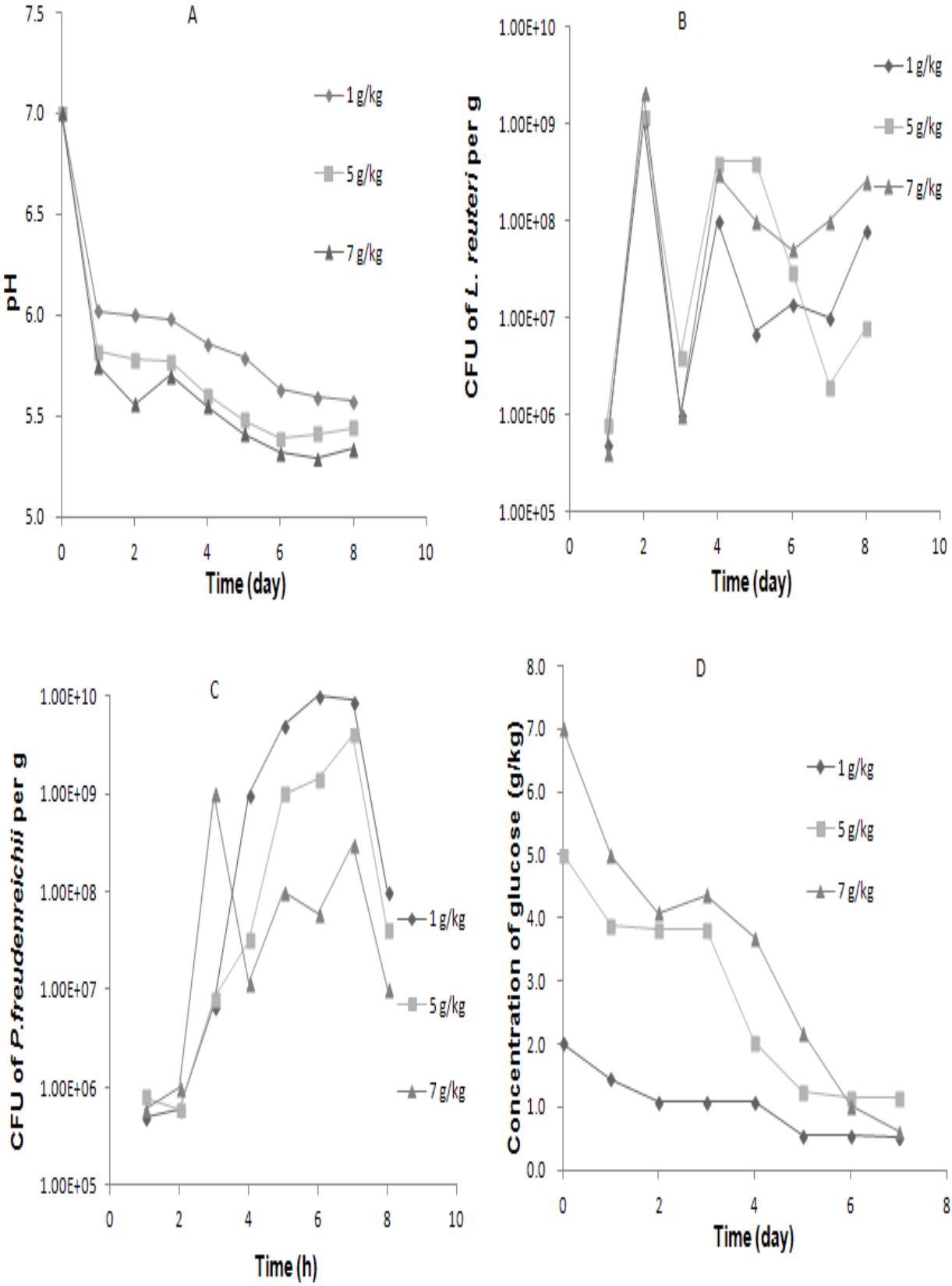


Fig. 4-29-1 Effects of batch experiments on pH values (A), growth of *L. reuteri* (B), growth of *P. freudenreichii* (C), consumption of glucose (D)

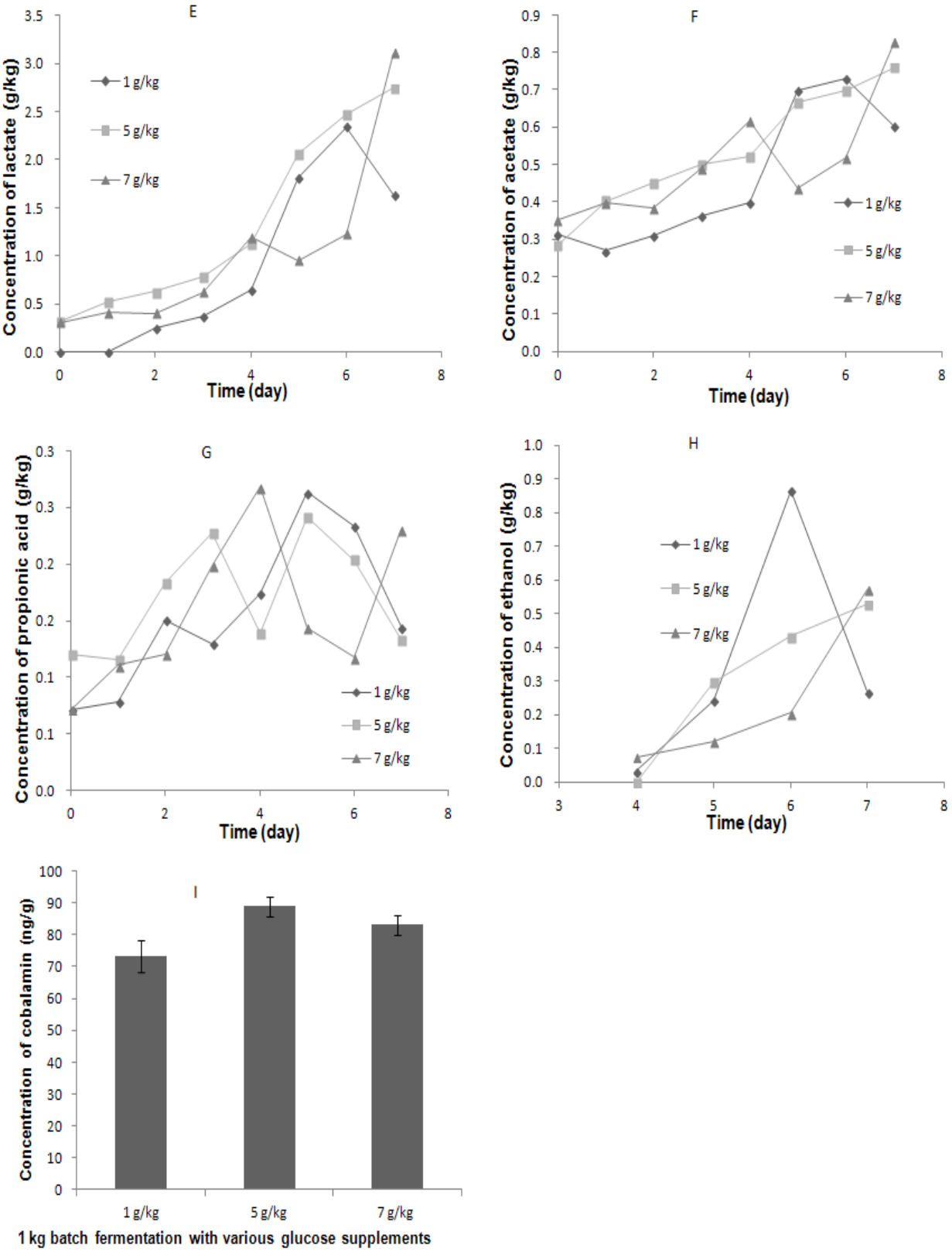


Fig. 4-29-2 Effects of batch experiments on pH values production of lactate (E), acetate (F), propionic acid (G), ethanol (H) and cobalamin production (I).

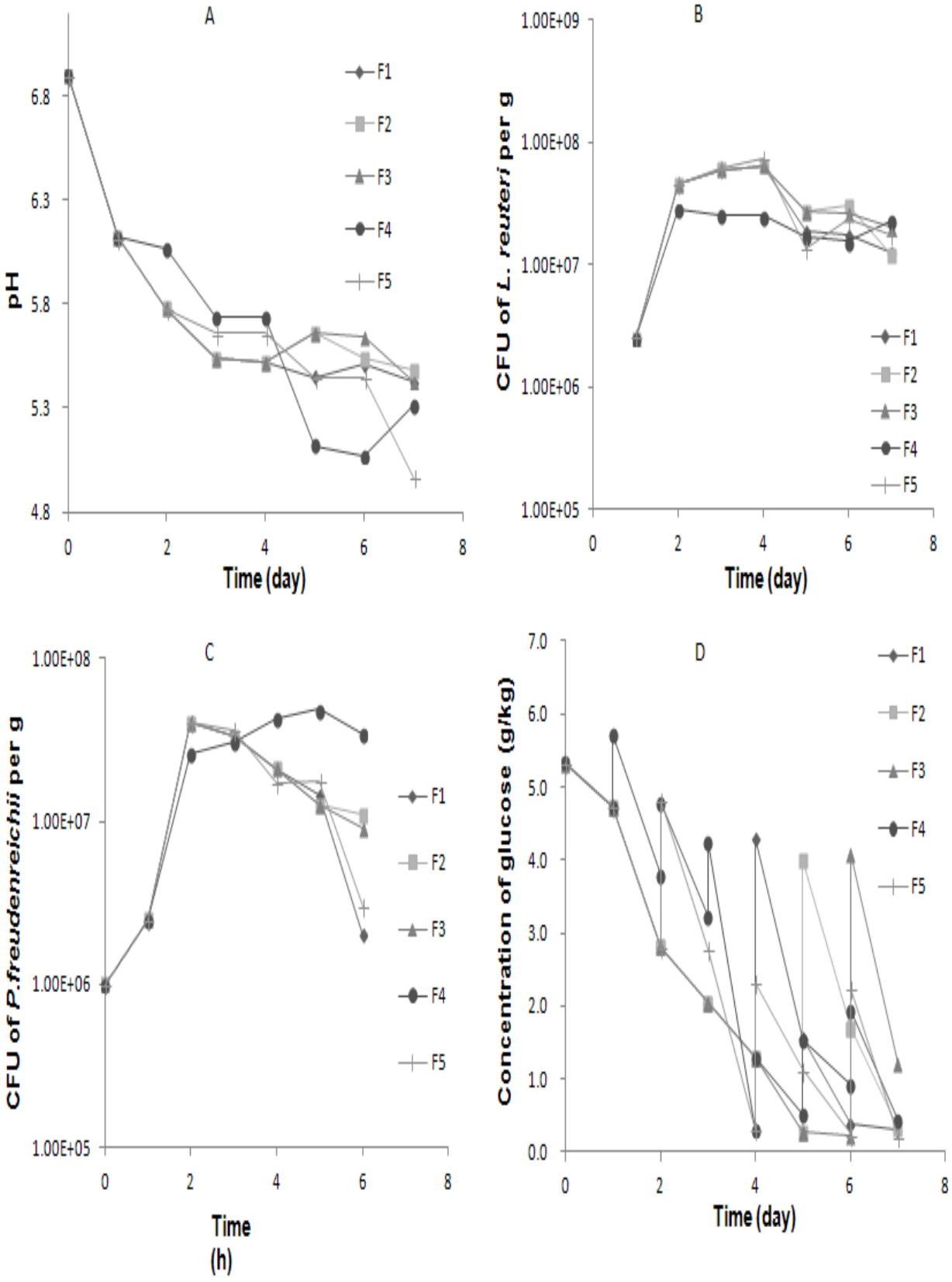


Fig. 4-30-1 Effects of various concentrations of glucose supplementations in 1 kg fed batch experiments on pH values (A), growth of *L. reuteri* (B), growth of *P. freudenreichii* (C), consumption of glucose (D).

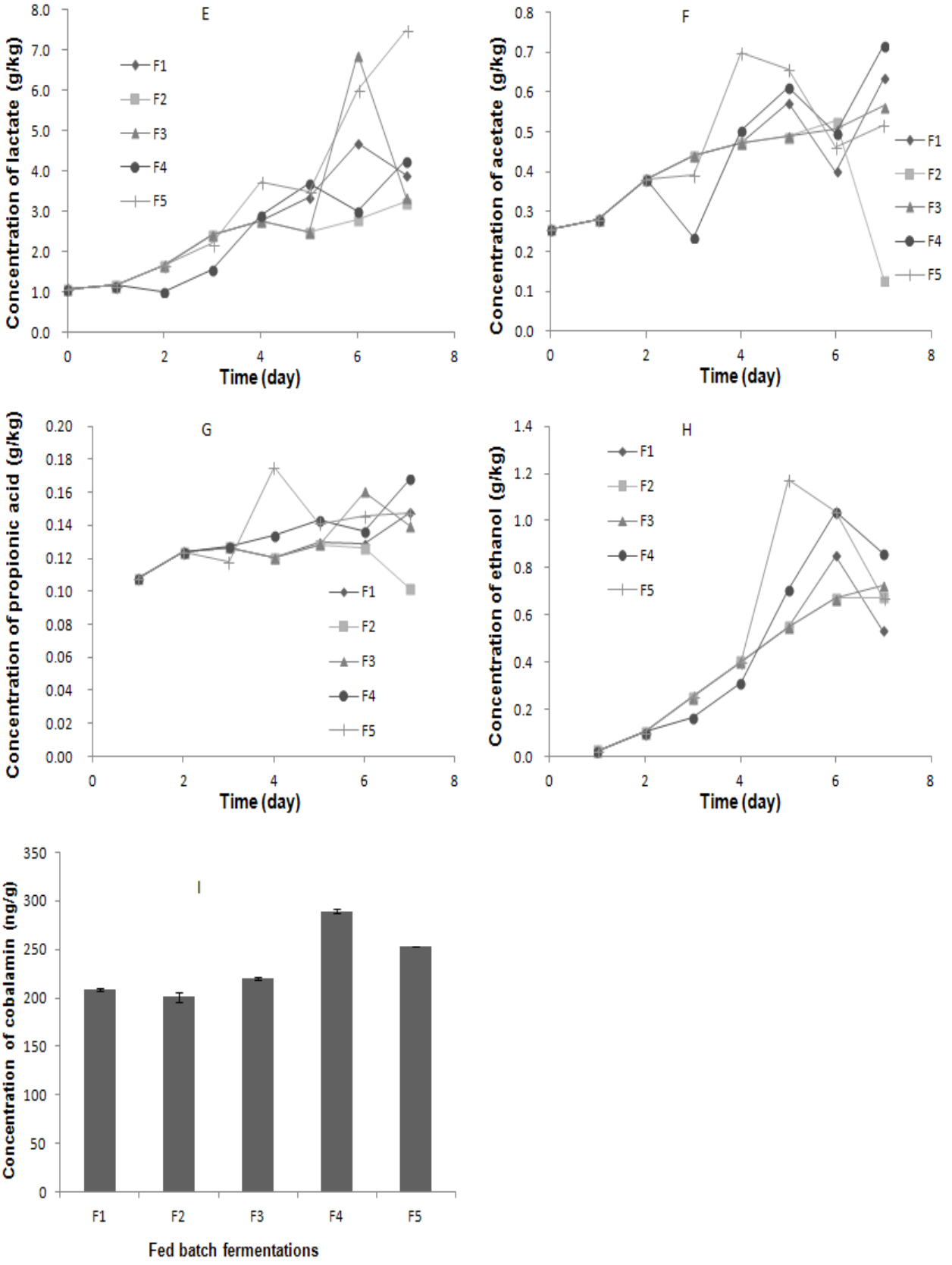


Fig. 4-30-2 Effects of various concentrations of glucose supplementations in 1 kg fed batch experiments on production of lactate (E), acetate (F), propionic acid (G), ethanol (H), and cobalamin production (I).

4.6. Preculture and culture optimization

4.6.1. Comparison of CFU and pH in tofu and fermentation liquid

The submerged solid fermentations consist out of two phases: one is tofu and the other is the supernatant. Tofu is the solid phase that affords nitrogen and other sources. Supernatant can diffuse nutritious substances and isolate oxygen. As concentration mentioned in methods, samples of tofu were first diluted 10 times and then pH was measured. The pH values measured in tofu were higher than in supernatant (Fig. 4-31). The correlation between them is 0.96. In further experiments pH in supernatant was used to calculate pH in tofu. After 3 days of fermentation the numbers of bateria in tofu and supernatant were almost in a similar level (Fig. 4-32). We also compared cell count of bacteria in tofu and supernatant under anaerobic and aerobic conditions. Cell counts of tofu were higher under aerobic conditions than under anaerobic conditions (Fig. 4-33).

4.6.2. Culture optimization

With regards to pathway of cobalamin synthesis, there are two different ways from formation of uroporphyrinogen III. One is further approaching to cobalamin and the other is approaching to synthesize heme. Heme is known as a negative regulator to uroporphyrinogen III synthesis. We assumed that strains surviving in a high concentration of heme may have a capability of high cobalamin production, and production of synthesis enzymes of heme may be suppressed.

L. reuteri and *P. freudenreichii* were respectively incubated in vitamin B_{12} test broths and vitamin B_{12} test broths were supplemented with 10 mg/L of heme for 20 generations. Strains incubated in heme for 20 generations produced at lest 1.5 fold more cobalamin than normal strains (Fig. 4-34 and 4-35). But normal strains in media with heme produced more. Heme helps bacteria to remove the stress from oxygen. On the contrary, strains incubated in heme produced more cobalamin, may be because of low capability of heme synthesis enzyme production after optimization.

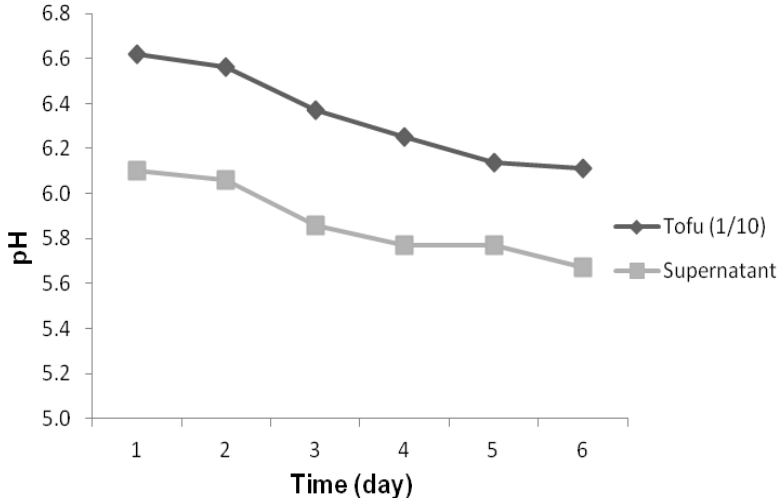


Fig. 4-31 pH values in tofu and supernatant
Tofu was diluted 10 folds and then pH was measured.

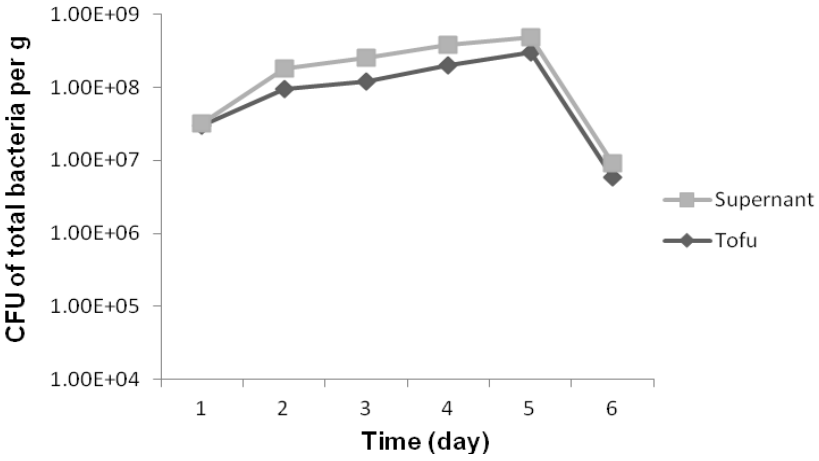


Fig. 4-32 Growth curves of total bacteria in tofu and supernatant

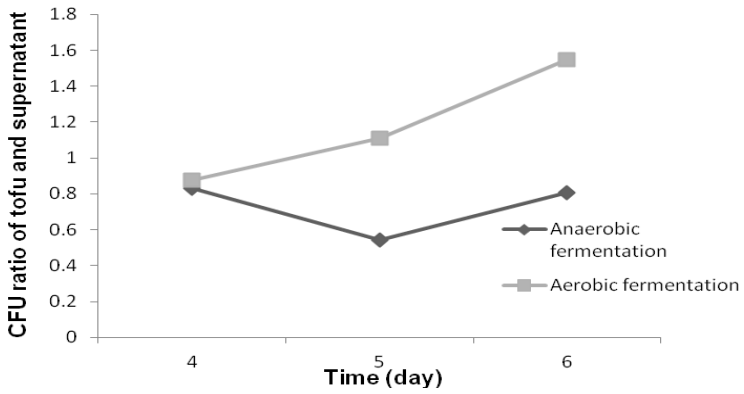


Fig. 4-33 Ratio of bacteria numbers in tofu and supernatant in the last three days under anaerobic and aerobic conditions

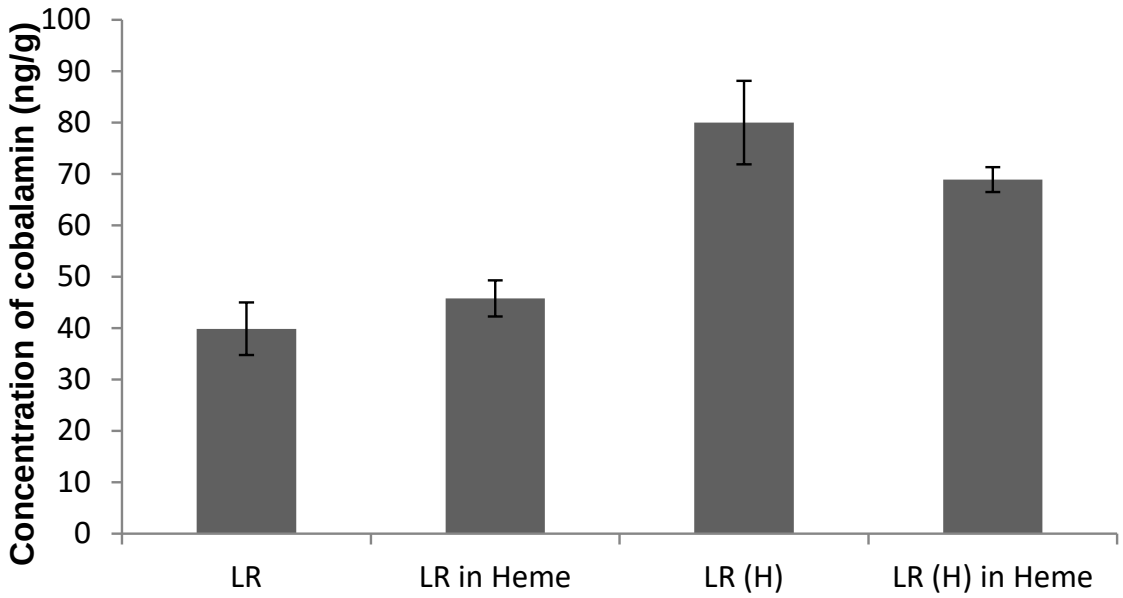


Fig. 4-34 Production of cobalamin in normal and breeded *L. reuteri* in vitamin B_{12} test assay with and without heme. Heme means vitamin B_{12} test broths with heme. LR and PF respectively mean *L. reuteri* and *P. freudenreichii* incubated in vitamin B_{12} test broths for 20 generations. LR (H) and PF (H) mean *L. reuteri* and *P. freudenreichii* incubated in vitamin B_{12} test broths with heme for 20 generations.

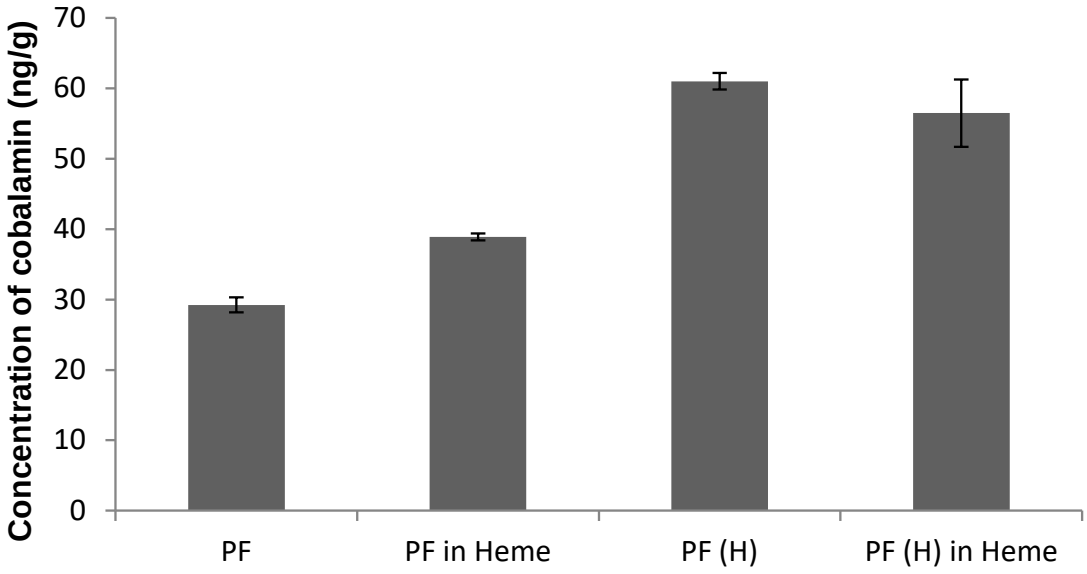


Fig. 4-35 Production of cobalamin in normal and breeded *P. freudenreichii* in vitamin B_{12} test assay with and without heme

Heme means vitamin B_{12} test broths with heme. Heme means vitamin B_{12} test broths with heme.

LR and PF respectively mean *L. reuteri* and *P. freudenreichii* incubated in vitamin B_{12} test broths for 20 generations. LR (H) and PF (H) mean *L. reuteri* and *P. freudenreichii* incubated in vitamin B_{12} test broths with heme for 20 generations.

4.6.3. Precultures preparation

Waste from tofu making is a good source of carbon and nitrogen. Two 2^{4-1} fractional factorial designs requested 8 experiments and other four experiments at the center of design were repeated four times to analyze the variance. The results of FFD are shown in Table 4-17 and Table 4-18.

4.6.3.1. Optimization of *L. reuteri*

The increasing of maize extract ($p < 0.05$) had a positive influence on the yield of cells. Meat extract, peptone, and yeast extract have no clear effect on the production of cobalamin.

Regeression analysis results of FFD experiment in Table 4-17 illustrates that only one factor, maize extract, plays a critical role in yield of cells and accumulation at the probability level of 95.89%. Other three factors were not found to be significant at the probability level of 90%.

The coefficient R^2 of the model equation at 0.5379 indicates that the model cannot explain the variability well. The value of F-test at 2.04 confirming the statistical significance of the model equation indicates that the model is adequate to the data at a probability level of 80%. According to Fig. 4-36, the highest concentration of *L. reuteri* was found in fermentation with 1 g/L of maize extract and 0.4 g/L of meat extract. This medium was used in preculture of *L. reuteri*.

4.6.3.2. Optimization of *P. freudenreichii*

The increasing of meat extract had a positive influence on the yield of cells of *P. freudenreichii*. Maize extract, peptone, and yeast extract have no clear effect on the production of cobalamin.

Regeression analysis results of FFD experiment in Table 4-18 illustrates that only one factor, meat extract, plays an important role in yield of cells and accumulation at a probability level of 93.43%. Other three factors were not found to be significant at the probability level of 90%.

The coefficient R^2 of the model equation at 0.59 indicates that the model cannot explain the variability well. The value of F-test at 2.57 confirming the statistical significance of the model equation indicated that the model was adequate to the data at the probability level of 87%.

According to Fig. 4-37, the highest concentration of *P. freudenreichii* was found in fermentation with 20 g/L of meat extract and 0.5 g/L of maize extract. These media were used in cell preparations.

Table 4-17 Results of FFD regression analysis for OD600 values of *L. reuteri*

Term	Regression analysis for OD600 values of *L. reuteri*			
	Coefficient	t-value	Significant level	
Intercept	0.5660	37.55	<0.0001	
X_1	0.0103	0.56	0.5917	
X_2	0.0181	0.98	0.3590	
X_3	-0.0146	-0.79	0.4543	
X_4	0.0461	2.50	0.04111**	
		R^2=0.538	F= 2.04	P = 0.1934

X_1, X_2, X_3, X_4, and X_5 (g/L) stand for natural variables of meat extract, peptone, yeast extract, and maize extract.

Table 4-18 Results of FFD regression analysis for OD600 values of *P. freudenreichii*

Term	Regression analysis for OD600 values of *P. freudenreichii*			
	Coefficient	t-value	Significant level	
Intercept	0.609	66.41	<0.0001	
X_1	0.024	0.85	0.0657*	
X_2	0.010	1.85	0.4259	
X_3	0.021	1.18	0.1074	
X_4	0.013	1.18	0.2769	
		R^2=0.59	F= 2.57	P = 0.1308

X_1, X_2, X_3, X_4, and X_5 (g/L) stand for natural variables of meat extract, peptone, yeast extract, and maize extract.

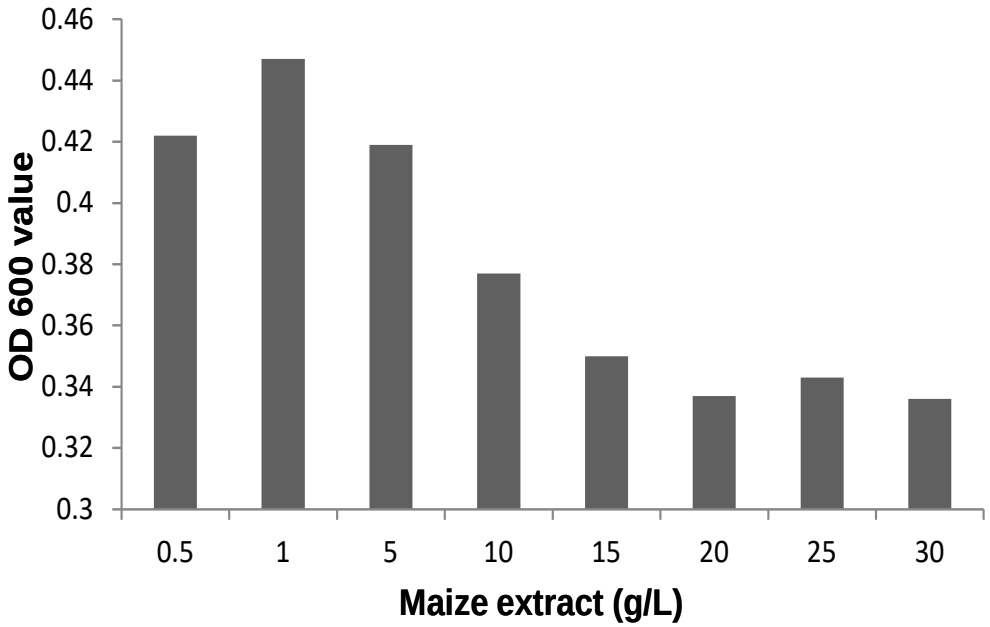


Fig. 4-36 Effects of various maize extract concentrations on cells concentration of *L. reuteri*

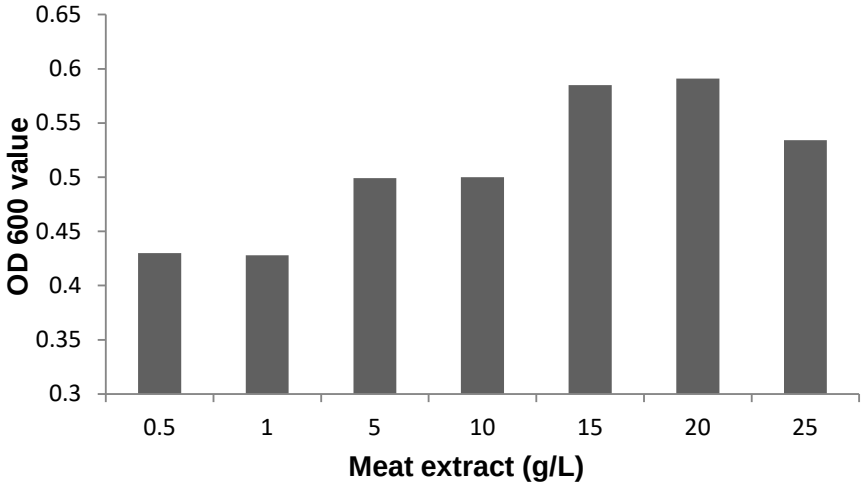


Fig. 4-37 Effects of various meat extract concentrations on cells concentration of *P. freudenreichii*

4.7. Fermentation with natural substrates

To replace pure chemical substances with natural substrates, we searched food with proper concentrations of riboflavin, cobalt, betaine, and glycine. Banana was used as carbohydrate source. Spinach was recognized as sources of cobalt and betaine. Seaweed was used as source of glycine. Some researchers regard seaweed as a source of cobalamin.

However based on our analysis no true cobalamin was determined in seeweed we used. After an orthogonal experiment in Table 4-19 and by a distance analysis method, banana plays the prevailing function on cobalamin production and the others also have a positive effect on cobalamin production. The run 1 produced 179.38 ng/g of cobalamin. Run 3 produced a lower concentration of lactate than others (Fig. 4-38 B). Fig. 4-38 A illustrates that production of propionic acid in all fermentations was almost similar. But production of propionic acid in run 1 earlier led to a high concentration. Concentrations of propionic acid were 5 to 10 times higher than fed batch fermentation. Also lactate concentrations were lower than after fed batch fermentations. The fermentations may fix more free water to the solid phase so that it reduced the evaporation of metabolites and also blocked oxygen uptake into this system. The pH value of run 2 declined much faster than others (Fig. 4-38 D). That may be resulting from increasing supplementations of banana and reduced supplementations of seaweed and spinach. Otherwise all the pH values and final pH values were higher than those of fed batch fermentation. Banana as a

substitute of glucose does not have a good diffusing capacity. This time we only observed growth curves of both microorganisms on 3rd, 5th, and 7th day (Fig. 4-38 C and E). All growth curves were stable, except run 2 which was a little different with all above experiments. This may be caused by the fluidity of fermentations. The nutrients cannot be supplied to bacteria easily and also diffusion of metabolites is difficult. But natural substrates may contain other factors that can promote production of cobalamin.

Table 4-19 Results of L4 (23) orthogonal experiments

Runs	Banana	Spinach	seaweed	cobalamin ng/g
1	1	1	1	179.38
2	1	-1	-1	154.75
3	-1	1	-1	94.20
4	-1	-1	1	92.39
I	167.07	136.79	135.89	
II	93.29	123.57	124.48	
Distance	73.78	13.22	11.41	

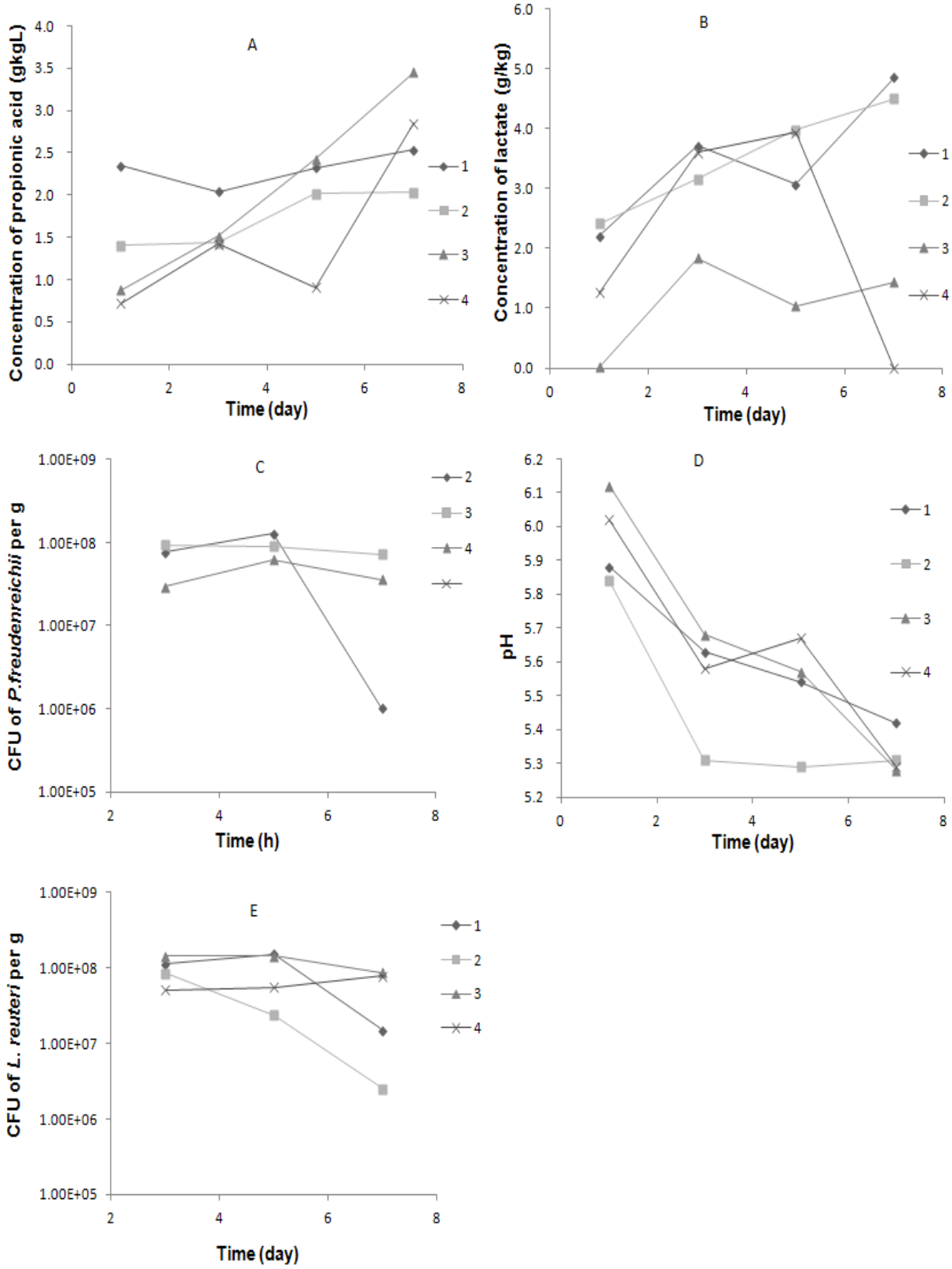


Fig. 4-38 Production of metabolites, growth curves of *P. freudenreichiii* and *L. reuteri*, and pH values in various combinations of fermentations with natural substrates Graph A and B show production of propionic acid and lactate. Graph C and E mean growth curves of *P. freudenreichiii* and *L. reuteri*. Graph D indicates pH values.

4.8. Interaction coefficients

A Lotka Volterra model known as an ecological predator-prey model was employed to describe the competition relationship between both microorganisms. The interaction coefficients that describe the antagonistic activities were obtained by fitting the modified Lotka Volterra model with least square methods. The coefficients of a and b mean the interspecific competition paramenters of *Propionibacterium freudenreichii* on *Lactobacillus reuteri* and vice versa.

With the exception of F5 and pH 6.5, other fermentations with a high production of cobalamin did not show strong antagonistic effects between both microorganisms. With the increasing concentration of glycine and decreasing concentration of fructose, the interaction coefficients of b were simultaneously sinking, which means both of them played an inducing role in effects of *P. freudenreichii* suppressing *L. reuteri*. The experiment of oxygen supply for 1 day acquired huge negative value of interaction coefficients. That may be explained that oxygen to some extent became the main inhibitor for the growth of both microorganisms.

Interaction coefficients were increasing from positive figures to negative figures during fermentations with different initial pH values from 6.0 to 8.0. That means a high initial pH value was beneficial for growth of both bacteria. No big difference of interactions was found in fermentations with different temperatures. In batch fermentations, less glucose supplementation showed only moderate effects on interaction coeffecients between both bacteria. The biggest value of interaction coefficient was found in F5 (b of 133.13).

Table 4-20 Results of interaction coefficients of different fermentations

Items	Interaction coefficients	
	a	b
Glycine 1 g/kg	-0.1050	57.8000
Glycine 5 g/kg	0.3330	1.9500
Glycine 10 g/kg	-0.0179	-0.0179
Blankl	0.2080	1.0700
Fructose 0.05 g/kg	-0.5637	0.2778
Fructose 0.1 g/kg	0.0049	0.2791
Fructose 0.5 g/kg	0.0861	1.7484
Oxygen supply for 0 days	-33.5287	-23.5816
Oxygen supply for 1 days	-0.1195	-0.6374
Oxygen supply for 2 days	0.0059	-0.0146
pH 6.0	0.3497	0.7528
pH 6.5	10.4190	12.8018
pH 7.0	-7.8402	-7.9293
pH 7.5	-9.3029	-13.3751
pH 8.0	-7.1238	-6.5552
28 °C	-0.0816	-0.0710
30 °C	-0.1257	-0.0588
35 °C	2.1073	2.1073
37 °C	2.2235	3.3456
Glucose 1 g/kg	-0.0231	-0.0006
Glucose 5 g/kg	-0.247	-0.0721
Glucose 7 g/kg	-0.8128	-21.5701
F1	-0.2711	14.5773
F2	-0.3368	8.1217
F3	-0.3353	8.2962
F4	0.1368	2.0598
F5	-0.3241	113.1301

5. Discussion

5.1. Analysis method

Every method has its own benefits and detriments to be applied to detect cobalamin in complex matrixes such as baby milk, tablets, and so on. It is known that various analytical procedures for vitamin B_{12} determination may lead to highly variable results for a given sample. Watanabe (2007) demonstrated that the values measured by a microbiological assay were 6 to 8 folds higher than those determined by the chemiluminescent method. Hence, it is hardly possible to compare the individual value of vitamin B_{12} presented here to those from previous published results via different determined methods and to make any absolute comparison intentionally. But differences and similarities in the cobalamin production trends and magnitudes of the present work and previous published work can be compared and noted. Upon the reasons, the microbiological assay and HPLC method were applied for fermented tofu and compared to set up a highly adapted method in cobalamin detection.

5.1.1. Microbiological assay

The results via a microbiological assay in tofu represented extremely huge errors at 10 and 40 ng/L of a standard recovery experiment (Table 4-2) and an extraordinarily low determination limit (Fig. 4-7) compared to HPLC method in our present work. Errors in such a matrix could be caused by deoxyribonucleoside with abilities to partly replace cobalamin, or folate as an inhibitor at high concentration and high amount of serine (Hoffmann et al. 1949; Shorb and Briggs 1948). Another realistic problem of cross interaction was also proposed from analogues in the microbiological assay (Schneider and Stroinski 1987). Several cyanocobamides can be detected as cyanocobalamin by *Lactobacillus delbrueckii*. Cyanocobamides with nucleoside moieties of 5-methylbenzimidazole and benzimidazole, in particular, have 92% and 100% activities respectively compared with cyanocobalamin. However, adenosylcobalamin has no biological activity on *Lactobacillus delbrueckii.* Our work (data not shown) also demonstrats a correlation less than 0.7 between the results detected via microbiological assay and HPLC, confirming the previous findings.

5.1.2. HPLC

To solve the aforementioned problem, advanced or modified methods were applied to determine cobalamin in fermented food. Although the microbiological assay is still frequently used in samples from submerged fermentations (Akasaka et al. 2004; Bullerman and Berry 1966), a modified biological method was used to detect cobalamin in solid fermented food, tempeh, by Denter and Bisping (1994). A spectrophotometrical method was conducted at a wavelength of 367 nm to determine cyanocobalamin dimer from disrupted cells (Miyano et al. 2000; Ye et al. 1996). Simultaneously an advanced method for HPLC with capacities of a high precision under ideal conditions was developed with different detectors and sample preparation methods. Researchers (Gauch et al. 1992; Wongyai 2000) stated that a HPLC method with a determination limit at 0.05 µg was performed in tablets and milk. Heudi et al. (2006) demonstrated that 10 ng of cobalamin was detected by their HPLC method with an immunoaffinity extraction (Heudi et al. 2006). Even lower determination limits in tablets were reached by using a fluorescence detector (Li et al. 2000) and an ESI-MS detector (Luo et al. 2006). However none of them was applied for a fermentation matrix, even not a solid fermentation matrix. Our work represented a calibration range from 0.5 µg to 10 µg and 5 ng as determination limit that is enough and satisfying with requirements of samples from solid fermentations.

No matter what kind of methods was chosen, the heat stability of cobalamin is considered to be a vital critical factor for determinations. As cobalamin is produced intracellularly by bacteira and some exists as a coenzyme form, different treatments were introduced and applied to disrupt cells and split cobalamin from enzymes. In our work, cobalamin had a superior property of heat stability even at 100 °C for 80 min (Fig. 4-1). Nevertheless, Blitz et al. (1956) demonstrated that vitamin B_{12} was unstable in the presence of an abundant amount of thiamine and niacinamide, but was relatively stable at lower concentrations. Unfortunately, tofu normally contains ca. 6.26-6.85 µg/g thiamine (Fernando and Murphy 1990). Concentrations of cobalamin out of samples released with different treatments all represented a dramatic drop after a peak was reached (Fig. 4-5 and Fig. 4-6). It would be perhaps more appropriate to suggest that cobalamin extracellularly released was more unstable due to the fact that it is accessible to niacinamide and thiamin in tofu.

SPE can not only purify the samples, but also concentrate vitamin B_{12}. According to Fig. 4-2, the conclusion can be drawn that at pH 7.0 most of the cyanocobalamin can be detected, but the recovery is not very high. Some factors involved in the phenomenon were very crucial. First, vitamin B_{12} is a polyacidic base with a pKa of 3.3 (Hill et al. 1965), which can be easily ionized, even under a neutral condition. When protonated, cyanocobalamin appears to be more susceptible to photoactivation (Ahmad et al. 1992). It has been reported that cynobalamin is very sensitive to light below pH 6.0 and above pH 9.0. Furthermore, other kinds of vitamins such as thiamine and niacinamide contribute to the destruction of vitamin B_{12} (Blitz et al. 1956). Indicative conclusion could be made that the solution should be adjusted to pH 7.0 before passing the SPE.

The results of recovery experiments which have been repeated 5 times performed by adding standards at different concentrations into tofu (Table 4-1) were only ca. 70%. A similar result was reported by some researchers (Arella et al. 1996), who found that the recoveries of vitamin B_1 and B_2 in chocolate powder were approximately 50% and 75% by liquid chromatography, which has been confirmed by routine analysis carried out in French official laboratories (Laboratoire Interregional de la DGCCRF) (Arella et al. 1996) for food controls. They concluded that most probably the vitamins and compounds out of chocolate formed a molecular complex (Arella et al. 1996). Dried soybeans contain 50% protein, 27% fat, and other substances such as carbohydrates and minerals (Liu et al. 1995). These complex ingredients may also have a similar effect.

The microwave treatment is faster than others but 25% of cobalamin could not be detected (Fig. 4-6). Thereof, both of graphs (Fig. 4-5) indicate that ultrasonic and boiling methods are not only efficient but also lead to high yields. Literature data indicated that ultrasonic method is much better suited than the boiling method concerning cell disruption. As we used a solid state fermentation, solid substrate not only supplies nutrients to the culture but also serves as a carrier material for the bacteria. Bacteria cannot be completely destroyed by ultrasonic wave, because substrates will absorb energy partially. On the other hand side ultrasonic method only needs half of the time that is needed by boiling method. For the lab work, the ultrasonic and boiling disruption are a good choice.

5.2. Microorganisms

The capability of cobalamin synthesis exists in abundant microorganisms, as its primary function is to ferment small molecules with the purpose of generating both oxidizable compounds and electron sinks to equivalent the redox reaction. Due to the high productive ability of cobalamin and rapid and mass growth in industrial conditions, *Pseudomonas denitrificans*, *Propionibacterium shermanii* (Bullerman and Berry 1966), *Propionibacterium fruedenreichi* (Van Wyk et al. 2012), and *Propionibacterium acidipropionici* (Quesada-Chanto et al. 1994) were employed in industries. Unlike other genera, the genus *Propionibacterium* would be assumedly preferred by food industries, as it has attained the GRAS status from the United States Food and Drug Administration. However in our single fermentation work cobalamin produced by *P. shermanii* and *P. freudenreichii* growing on tofu could not be found. A possible interpretation may be due to the low content of carbohydrates in tofu. Carbohydrate is a main energy source of this genus. Low capabilities of proteases (Britz and Riedel 1991) cannot release and produce enough nutrients to support the rapid and mass growth of *Propionibacterium* sp..

A surprising result was that *L. reuteri* was found to have a higher capability of cobalamin productivity in tofu than *Propionibacterium* sp.. In a previous report, Taranto et al. (2003) showed the production of cobalamin in a sugar-glycerol cofermentation in vitamin B_{12} medium. This prototrophic species may be a good candidate to increase the cobalamin content in fermented foods not only in tofu. Other lactic acid bacteria, such as *Lactobacillus plantarum* that was reported to produce 13 ng/g of cobalamin (Madhu et al. 2003), did not obtain good results in tofu as substrate as all were less than 5 ng/g of cobalamin. A hypothetical explaination was put forward while bearing in mind that the content of tofu with a low concentration of carbohydrates and a high concentration of lipids may explain the results. Microorganisms would economize limited carbohydrates under barren conditions to meet the growth and survival of microorganisms with more ATP generation, but not to balance the redox reaction as primary function. Meanwhile, glycerol that can be converted into HPA accompanied with the conversion of NADH to NAD^+ can be degraded from lipid to induce the production of cobalamin. The full proteolytic system (Rollan and Font de Valdez 2001) including proteinases and peptidases, which is able to hydrolyze

proteins into small peptides and amino acids, is an additional advantage for this genus living in tofu.

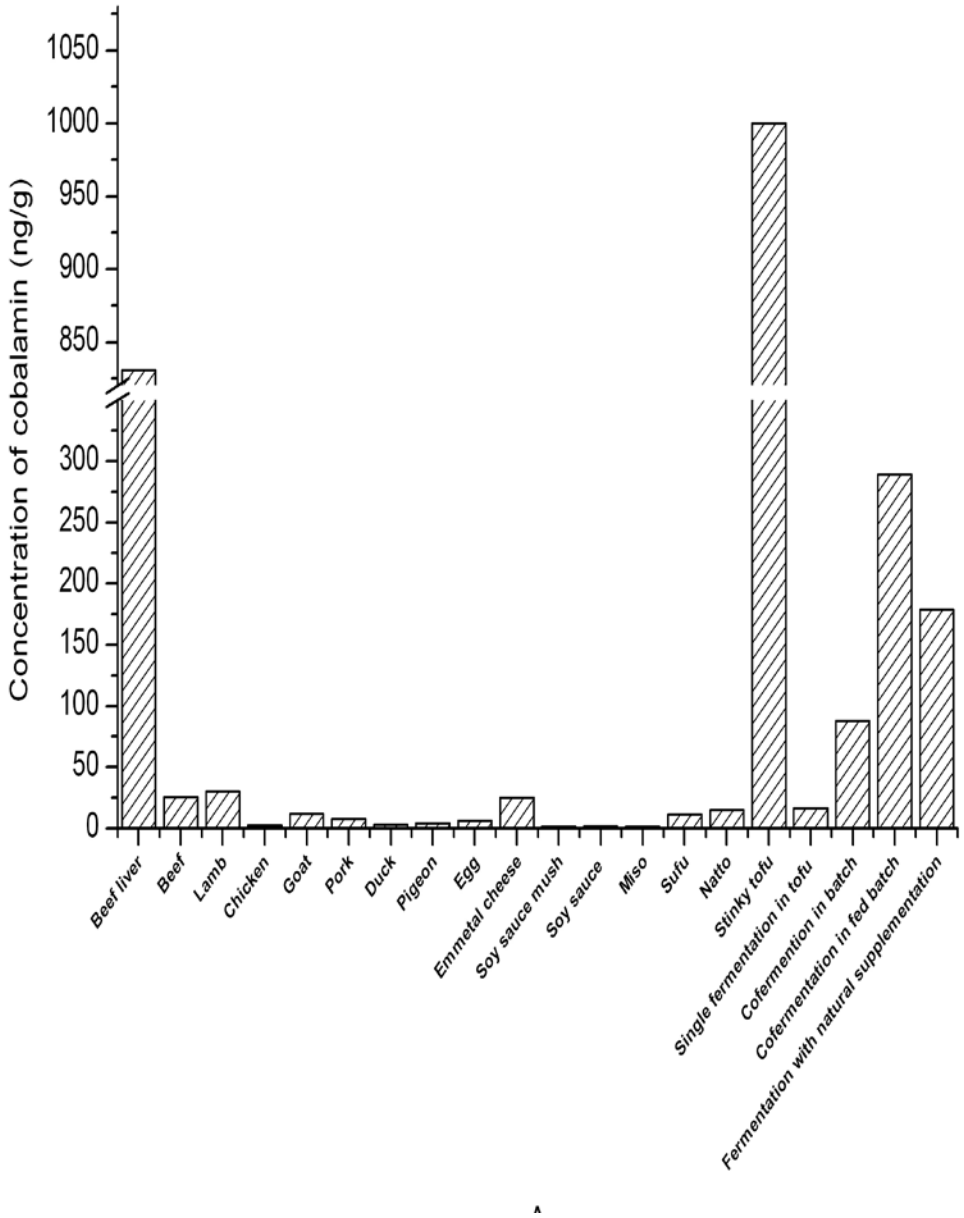


Fig 5-1 Concentrations of cobalamin in different foods and our products (Li et al. 2004; USDA 2007)

However, fermentations with *Lactobacillus reuteri* on tofu merely produced 16.3 ng/g of cobalamin. That was lower than in Emmetal cheese and stinky tofu (Fig. 5-1) even after optimization. The low cobalamin productive ability of this species was demonstrated by Taranto and coworkers (2003) and may be the reason for the low concentration of cobalamin in our

experiment. Another possible reason could be the inhibition by low pH caused by a fast accumulation of lactate and acetate. Miyano et al. (2000) had reported an inhibiton by own metabolites which can reduce and retard the growth of microorganisms and the accumulation of cobalamin. The pseudovitamin B_{12} formed under anaerobic fermentation by *L. reuteri* detected and confirmed by researchers (Santos et al. 2007) could be another plausible reason for the low productivity as it binds with a riboswitch in some mRNA to form inhibitory structure (Biedendieck et al. 2010).

A co-fermentation with *P. freudenreichii* and *L. reuteri* stimulated by procedures of Emmetal cheese and Miyano's commensalistic interaction research was employed to solve the problem of low cobalamin concentrations generated in tofu. *P. freudenreichii* with an ability of high cobalamin production prefers to consume lactate as the main energy and carbon source (Lee et al. 1974; Piveteau 1999). This can reduce the lactate stress on *L. reuteri* and retard the decrease of pH. Moreover, *Propionibacterium* sp. has a 100-fold stronger activity of hydrolyzing triglycerides of fat, compared with lactic acid bacteria (Dupuis et al. 1993). Hence production of glycerol inducing vitamin B_{12} dependent enzyme in *L. reuteri* would be increased during the growth of *P. freudenreichii*. In the mean time, *L. reuteri* can decompose proteins from tofu relying on full proteolytic system to meet the nitrogen requirement of itself and *P. freudenreichii* with a low ability of protease. Another hypothetical assumption is about the synthesis of Dmbi. 5,6-dimethylbenzimidazole, an important precursor of cobalamin, can only be formed in the presence of oxygen by *Propionibacterium freudenreichii* and *Propionibacterium shermanii* (Hoellriegl et al. 1982). Because of this, after several days of fermentation under anaerobic conditions with *Propionibacterium freudenreichii* or *Propionibacterium shermanii*, the fermentation should be switched to aerobic conditions. However, Santos *et al.* (2008) found that the gene of cobT of *Lactobacillus reuteri* is 59% similar with *Salmonella typhimurium*, which could mean that *L. reuteri* has the ability to form Dmbi without oxygen. Furthermore, some analogues can improve production of cobalamin by protecting an inhibitory riboswitch (Thirupathaiah et al. 2012).

Hugenschmidt et al. (2011) reported that a co-fermentation of whey by *Lactobacillus plantarum* SM39 and *Propionibacterium freudenreichii* has achieved to obtain 8 µg/mL of cobalamin. A synergistic effect in the co-fermentation of *Lactobacillus acidophilus* and *P. shermanii* was described by Liu and Moon (1982). They reported tthere was no lactate accumulation in the medium. Acetic acid production rates per generation were lower in mixed cultures and growth rate was faster than before. We agreed with the results from Liu and Moon (1982) and partly confirmed them. In our work, the robust growth of mixed cultures was indicated and observed by fast propionic acid and ethanol production, and also OD values. Meanwhile an accumulation of lactate was also observed, particularly for cofermentations at 37 °C (Fig. 4-16a and b). The surplus supplemented glucose and rapid decreasing of pH may be an explanation for this contradictory result in our work.

Moreover, Liu and Moon (1982) also stated that the maximum cell numbers of *L. acidophilus* and *P. shermanii* were higher than in sigle culture fermentation. Our present work also illustrated these phenomena (Table 4-20). The average values of interaction coefficient of a (-1.7621) and b (5.5934) from our work represented a positive effect from *P. freudenreichii* on *L. reuteri* and a negative effect from *L. reuteri* on *P. freudenreichii*, confirming the previous reported work.

Some reseachers demonstrated that spent media used previously to grow LAB strains to improve the production of cobalamin by *P. shermanii* led to a low cell concentration (Gardner and Champagne 2005). Nevertheless mixed cultures in our work produced 1.6 to 2.4 fold more cobalamin than single fermentation. For the further work, a doubtless conclusion can be made that a cofermentation with *P. freudenreichhii* and *L. reuteri* leads to a good cobalamin production in tofu. The best results (289 ng/g cobalamin in tofu) got in our tests still have a great gap from value of cobalamin in stinky tofu fermented by *Bacillus* sp. (Friberg and Hui 2005; Li et al. 2004). This genus may also be employed into a cofermentation to develop the production of cobalamin after the problem of a big amount of ammonia generation is solved.

5.3. Carbohydrates

Glycerol and fructose have an enhancing effect on the production of cobalamin (Table 4-3 and Table 4-4). *L. reuteri* is a heterofermentative LAB. But Arskold et al. (2008) reported that the main flux was through the Phosphoketolase pathway (PKP) in *L. reuteri* ATCC 55730, while the Embden-Meyerhof pathway (EMP) was used as a mere shunt. All genes to code the enzymes of EMP pathway were found in *L. reuteri* DSM 20016 (Copeland et al. 2007) we used in our work by NCBI gene bank database (NC_009513.1). That means this strain has a ability to use EMP. In contrast with the fact that the EMP is recognized as naturally optimized ATP synthesis system (Melendez-Hevia et al. 1997), the energy yield of the PKP usually used by LAB to ferment pentoses is poor. However, the PKP can also work as efficient as the EMP, when more acetate instead of ethanol is generated from acetyl phosphate accompanied with generating an additional ATP. Unluckily, a disaster problem of redox balance will appear. Arskold et al. (2008) demonstrated that without external electron acceptor, a severely restricted growth of *L. reuteri* is happening. This phenomenon is accompanied by a relatively low growth rate, a low biomass yield, and a low ATP level in the presence of non-limiting concentrations of glucose, which was also observed by this work. Lower comsuption of glucose, lower production of mannitol, lactate, acetate, 1,3 propanediol, and cells, but higher production of ethanol in combination 1 supplemented with only a high amount of glucose were observed in our work (Fig. 4-14), compared with other combinations. Even tofu in fact contains a few oxidizing agents such as fructose, glycerol, and other hydrogen acceptors, but the production of acetate and lactate were low. Instead of the pathway of acetate and lactate which can generate ATP, *L. reuteri* in this case tried to generate more NAD^+ by producing more ethanol, but bacteria still could not grow well due to the imbalance of redox reaction. The low production of cobalamin could be a plausible result from the limitation of growth owing to imbalance of redox.

The growth limitation was alleviated in a presence of fructose, reported by Arskold et al. (2008). The strains of *Lactobacillus pontis, L. reuteri, L. amylovorus, and L. fermentum* were also reported to use fructose as an electron acceptor when maltose was available in excess (Stolz et al. 1995). Fructose can be used as a carbon source instead of an electron acceptor in the fructose and

glucose cofermentation with *L. reuteri*, which can be confirmed by our carbon selection experiments depicted in Fig. 4-18-1 and -2. The additional production of lactate (Fig. 4-14) meant anyhow some fructose was treated as a carbon source instead of an acceptor through the PKP pathway to increase the production of lactate. However glucose was normally selected as a primary carbon source in the presence of fructose through the PKP which is as energetically efficient as the EMP. The additional production of acetate would be a persuasive evidence of this hypothesis. All other combinations (Fig. 4-14) produced less ethanol, more acetate and mannitol than combination 1 supplemented only with glucose.

L. reuteri could produce ATP through the EMP and PKP with the end products acetate and lactate depicted in Fig. 5-2 to boost the growth of cells. But the excess NADH blocking both pathways can be got rid of by generation of ethanol and mannitol. The pathway to ethanol depicted in Fig. 5-2 is the only way to balance the redox intracellularly without the help of other oxidants. A similar phenomenon of the low production of acetoin and diacetyl was reported when hexoses are the only carbon sources (Kandler 1983). Regarding the decrease of cobalamin production with the increasing supplementations of fructose, two tentative interpretations can be cited. Firstly, fructose was used as an electron acceptor to regulate PocR (Bobik et al. 1992); sencondly, as the B_{12} sythensis cluster inducer by mediating the ratio of NAD^+/NADH. At the beginning a high amout of fructose supplementations enhanced and accelerated the growth of cells. Then the rest of fructose can still oxidize NADH to NAD^+, which may inhibit the generation of vitamin B_{12} dependent glycerol dehydratase (Ailion et al. 1993). On the other hand low concentrations of fructose only improve the growth of cells at the beginning. Furthermore, the vitamin B_{12} synthesis inhibition can retard or block a series of vitamin B_{12} dependent reactions such as methionine synthesis, DNA synthesis, and deoxyribonucleotide synthesis. This can explain the reason why concentration of cells was low with a high supplementation of fructose.

Glycerol is a vital interesting topic not only for vitamin B_{12} production but also for the growth of cells. Glycerol works as an inducer of vitamin B_{12} synthesis cluster. These genes are involved in the conversion from glycerol to 3-HPA in order to regenerate NAD^+ with more ATP generation. Some researchers demonstrated that glycerol serves only as an external hydrogen acceptor in the

glycerol fermentation of *L. reuteri*, and does not work as a carbon source (Radler and Schütz 1984; Talarico et al. 1988; Talarico and Dobrogosz 1989). Our work of glycerol used as an external hydrogen acceptor (Fig 4-17) is in agreement with the above results. An obvious shift of end products from ethanol to acetate was observed in our results (Fig. 4-14). The notably reduced production of 1,3-propanediol (1,3-PD) was found in supplementations of glycerol. Similar results were found in the work of Luthi-Peng and coworkers. They (Luthi-Peng et al. 2002a) stated that a sufficient supplement of glucose compared to glycerol can improve the generation of 1,3-propanediol and acetate and reduce the accumulation of 3-HPA and lactate. Another interesting sudden descent of acetate concentration between 20 to 30 hours occurred (Fig. 4-14), which also was reported by Gerez (2008). The appreciated explaination of those phenomenon would be that acetate was used to generate acetyl-CoA to join into other pathways. No matter what happend, cobalamin production (Table 4-3 and Table 4-4) was indeed improved with the supplementation of glycerol since glucose went through a more efficient pathway to generate APT and was not involved in the redox balance. But high concentrations of glycerol supplementations definitely inhibited the growth of cells (Fig. 4-14). It may attribute to the glycerol dehydratase activity inhibited by a qorum sensing effect from high concentrations of reuterin (Bauer et al. 2010b).

The accumulated 3- HPA can be reversibly dehydrated to acrolein (Fig. 1-5), hydrated to HPA hydrate and also dimerized to HPA cyclic dimer. Acrolein is pulmonary toxicant and an irritant of mucous membranes (Esterbauer et al. 1991) and develops bitterness in wine (Noble 1994). In this case 3-HPA will be focused in the prevention of terrible taste and safety problem in our fermented tofu.

Production of 3-HPA was not only dependent on the microorganisms and specialized enzymatic systems, but also on other conditions such as acidification, cell concentrations and fructose supplementations. Bauer (2010b) described that *L. reuteri* DSMZ 20016 produced and accumulated 3-HPA when cell concentrations were increased up to the threshold concentration that may affect viability of cells. A sudden and severe drop in 3-HPA content was seen at high cell concentrations. All these phenomena indicated that a quorum sensing system is involved in

regulating 3-HPA. An influence of pH was also revealed by the fact that pH 6.0 was the favorite pH to produce 3-HPA, and at lower pH the yield of 3-HPA would be dramatically reduced or inhibited (Wall et al. 2007). Normally glycerol can enhance the production of 3-HPA up to 300 mM, at the concentration of which the glycerol dehydratase activity seems to be inhibited (Bauer et al. 2010b). The NAD^+/NADH ratio was increased and 3-HPA was obviously accumulated while fructose was added as an oxidant (Bauer et al. 2010b). More 1,3-PD converted from glycerol was accumulated when the ratio of glucose to glycerol in fermentations with *L. reuteri* was over 1.6 (Bauer et al. 2010b). The accumulation of 3-HPA was increased at a ratio of glucose to glycerol less than 0.33 (Bauer et al. 2010b). The varied ratios of glucose and glycerol disturb the redox balance to affect the reduction of 3-HPA. According to what we mentioned above, excess glucose compared to glycerol and fructose was used to prevent the accumulation of 3-HPA in tofu.

Propionibacteria can use glucose, glycerol, fructose, and lactate as carbon sources. But all these carbon sources have different priorities. This genus prefers lactate as the primary carbon source in a mixture with glucose, even though less ATP was generated by lactate than glucose (Lee et al. 1974). A NAD^+ dependent lactate dehydrogenase is involved in oxidation of lactate to pyruvate. Pyruvate also can be degraded from other sugars through the EMP pathway depicted in Fig. 5-3. In the presence of no sufficient hydrogen acceptors more glucose is consumed by *P. freudenreichii* to take part in the propionic acid synthesis pathway to balance the redox. A conenzyme B_{12} is required for activity and catalyses the rearrangement of succinyl-CoA to methylmalonyl-CoA (Kellermeyer et al. 1964). Fig. 5-3 depictes that 2 mol NAD^+ and 1 mol ATP are generated by the conversion from oxaloacetate to succinate, whereas 1 mol of acetate is converted from pyruvate with 1 mol of NADH and 1 mol of ATP generation. Some researchers (Himmi et al. 2000; Liu et al. 2011) reported that considerably more propionic acid was produced under a co-fermentation of glycerol with other sugars, since this metabolic pathway in conversion of glycerol to propionic acid is redox-neutral and energetic. They (Himmi et al. 2000) also stated that a considerable lower biomass yield was accompanied with a high consumption of glycerol.

Cofermentations by two microorganisms with glycerol (Fig. 4-17) did not improve the cobalamin production, whereas a low amount of glucose supplementation led to the best result of cobalamin production. This may be explained by the results provided in Table 4-11 and Fig. 4-17. The rapid drop of pH inhibits the growth of cells and also the cobalamin synthesis (Fig. 4-18-1 and -2). The supplementations of glycerol produced more propionic acid, but less acetate and lactate (Fig. 4-18-1 and -2). The production of cell mass, propionic, and acetic acids behaved similarly as reported by Liu and Moon (1982), but the results of lactate were different. That may result from scarcity of NAD^+ to support the conversion of lactate.

Fructose in cofermentations was still an attractive topic for cobalamin production. The pathway of fructose is depicted schematically in Fig. 5-3. Besides the conversion to mannitol, fructose can be transformed to fructose-6-phosphate with the help of ATP. Further on fructose-1,6-bisphosphate (FBP), which plays an inhibiting role in the conversion of dihydroxyacetone phosphate to glyceraldehyde-3-phosphate, is generated with another ATP (Fraenkel and Vinopal 1973). A hypothesis that the consumption of fructose in tofu blocks the pathway of glycerol may interpret the low cobalamin production after glycerol supplementations in the paragraph above. A definite higher value of cobalamin production with a supplementation with 0.1 g/kg of fructose was observed (Fig. 4-20-1b and). The benefit of fructose to *L.reuteri* has been presented in the paragraph above, whereas for *P. freudenreichii* a low concentration of fructose can generate NAD^+ with the generation of mannitol, but not inhibit the consumption of glycerol which boosts the production of propionic acid. The lower accumulations of lactate and ethanol and higher accumulations of propionic acid and acetate in the combination 7 (Fig. 4-20-2b) were the best evidence to prove our hypothesis of redox balance. This situation can lead to a high production of cobalamin. The interaction coefficient b (Table 4-20) suggests that the pressure from *L. reuteri* on *P. freudenreichii* increased significantly during the increase of fructose. The interaction coefficient a showed the same trend albeit very light. The slightest interactions between both bacteria can explain the reason why more cobalamin was produced with 0.1 g/kg of fructose in another aspect.

5.4. Amino acids

The results of nitrogen source supplementations of *L. reuteri* shown in Fig. 4-9 indicated that *L. reuteri* has a substantially efficient proteolytic system, which was also reported by other researchers (Rollan and Font de Valdez 2001; Tobiassen et al. 1997). The reverse truth for *P. freudenreichii* has been illustrated from the results of less growth and no cobalamin production in tofu substrate. Marwaha et al. (1983) reported that some amino acids including L-aspartic acid, L-lysine and glycine in *Propionibacterium* sp. have a negative effect on cobalamin production, but L-glutamic acid has a positive effect on cobalamin production. Limpinsathian (2005) has reported that *P. freudenreichii* could only survive on the chemical modified media without amino acids by supplementing sera out of curd cheeses treated by strains of *Streptococcus thermophilus* or *Lactobacillus helveticus*. Results of Limpisathian (2008) insprited us to use a cofermentation to imitate the symbiosis in cheese making. Initial fermentations with *L. reuteri* can offer amino acids to *P. freudenreichii* (Fig. 4-28e). The earlier *L. reuteri* was added, the sooner amino acids were set free and consumed. After concentrations of amino acids decreased down to ca. 3 mg/kg, the concentrations went up again. The inoculation titers and time of incubation of *L. reuteri* influenced growth and cobalamin production of *P. freudenreichii* (Fig. 4-28b, d). Low productivity of cobalamin in some cofermentation may result from the aging of *L. reuteri* that could not preform a vigorous commensalistic interaction with *P. freudenreichii*.

Furthermore, an interesting research about the omission of several single amino acids that led to great increases in cobalamin production by *L. reuteri* has been published (Santos et al. 2009). Omission of aspartate, glycine, alanine, lysine and cysteine by leading to an increase of 500% had an undoubtedly positive effect on cobalamin production. Tofu (100 g) normally contains 11.70 g of aspartic acid, 4.00 g of threonine, 19.26 g of glutamic acid, 4.14 g of glycine, 1.43 g of methionine and no cysteine (Wang and Cavins 1989). Unlike the omission of other amino acids, the growth rate of *L. reuteri* would drastically drop by omission of glycine and cysteine due to the fact that an increase demand cannot be satisfied by self-synthesis. Contradictory results were obtained from our present work. A slight increase of glycine concentrations can notably enhance the cobalamin production (Fig. 4-20-1a). At the same time the growth of both microorganisms

was improved, but no significant difference in metabolites was found. The inhibition of microorganism growth with abundant glycine supplementations was observed in our work. Iida and Kajiwara (2007) reported that glycine acted as a methyl carbon donator to corrin ring through S-adenosyl-methionine derived from catabolically generated L-methionine and a precursor of ALA. The interaction coefficient b (Table 4-20) suggested that with an increase of glycine, the pressure from *L. reuteri* on *P. freudenreichii* was dropping down significantly. At 1 g/kg of glycine a very strong pressure from *L. reuteri* on *P. freudenreichii* (b = 57.8) was seen. The slightest interaction between both microorganisms can explain the question of why more cobalamin was produced with 5 g/kg of glycine in another aspect.

The result of L-methionine supplementation (Fig. 4-19) showed a negative effect on cobalamin production, which was not in agreement with Iida's results (Iida and Kajiwara 2007). This may result from a block of conversion of homocysteine to methionine which was catalyzed by vitamin B_{12} dependent coenzyme.

Other amino acids such as threonine and glutamate which are the precursor of 1-amino-2-propanol and ALA were also supplemented into fermentations. Unfortunately, all of these reduced the cobalamin production unlike what was expected. Ford and Friedmann (1976) have reported the relation of L-threonine and vitamin B_{12}. But they also found L-threonin functioned not only as the source of cobalamin constituent but passed through extensive metabolic changes unrelated to cobalamin. The possible reason for the failure of experiment has not been explained and studied on in present work.

5.5. Dmbi and riboflavin

There are two different pathways of cobalamin synthesis found in microorganisms. The present work used two anaerobic pathway bacteria. But oxygen still plays a critical role in Dmbi generation for *P. freudenreichii*. Although the synthetic pathway was still unknown, Renz (1970) had yet proposed and proven an assumptive pathway of *Propionibacterium* that Dmbi may originate from riboflavin in the presence of oxygen. However Dmbi can also be synthesized by *Salmonella* spp. without oxygen (Johnson and Escalante-Semerena 1992), as the single CobT

protein may catalyze the complete Dmbi. A gene of cobT in *Lactobacillus reuteri* is 59% similar with *Salmonella typhimurium* (Santos et al. 2008). Unfortunately, tofu contains only 0.1 mg per kg riboflavin, and it was reported to rapidly loose 78% riboflavin on storage in water even at 4 °C (Fernando and Murphy 1990). Regarding the importance of Dmbi and low concentration of riboflavin, Dmbi or riboflavin were considered to be supplied. It was shown that both bacteria can be used to counter riboflavin deficiency in rats (Burgess et al. 2009) as they have a powerful capability of riboflavin productivity (Murdock and Fields 1984). Research has been done on Dmbi supplementations to improve the production of cobalamin (Bullerman and Berry 1966; Hugenschmidt et al. 2011; Miyano et al. 2000). Dmbi in Table 4-7 shows that a T-value of 2.102 that means Dmbi is not as important as we imaged before. A prudent explanation would be that riboflavin was converted to Dmbi to meet the need of cobalamin production. A further effect of riboflavin and Dmbi on cobalamin production depicted in Fig. 4-21 shows that riboflavin can take the place of Dmbi. No cobalamin production was found in anaerobic fermentations with riboflavin supplementations for less than 4 days. This may tentatively be explained by the fact that oxygen can inhibit the growth of both microorganisms and cobalamin production, which was also confirmed by Miyano et al. (Miyano et al. 2000). The results in Fig. 4-21 led to the conclusion that 5-day anaerobic and 2-day aerobic fermentation can produce the highest concentration of cobalamin, as suitable and adequate oxygen promotes generation of Dmbi from riboflavin to synthesize cobalamin.

Santos et al. (2008) demonstrated a presence of a regulatory feedback mechanism that inhibited the biosynthesis of cobalamin when it was available from the environment. During the late exponential phase in the presence of cobalamin, cobalamin biosynthesis genes were also less expressed. A riboswitch regulation of cobalamin was also reported by Vitreschak et al. (Vitreschak et al. 2003). All these reports influence the decision when to add riboflavin or Dmbi. The results concering riboflavin supplementations at different times suggest that abundant cobalamin can be produced when riboflavin is supplemented after 2 days of fermentations. This could be caused by the rapid synthesis of cobalamin that can activate the regulation to inhibit the

cobalamin synthesis. On the other hand, cobinamide was accumulated at scarcity of Dmbi, further to influence normal metabolisms of microorganisms such as more riboflavin synthesis.

5.6. Cobalt

Cobalt as a central atom of cobalamin is required by cobalamin synthesis. To some extent, the bioavailability of cobalt is a bottleneck to limit the increase of cobalamin production. This has been confirmed by Santos et al (2008). Tiffany and coworkers (2006) reported that the supplementation concentrations of cobalt affected production of cobalamin directly. Our results (Fig. 4-22) depict that high concentrations of cobalt have a negative effect on cobalamin production and lowered the pH. Tiffany's work also found the descending values of pH during the increase of cobalt supplementations (Tiffany et al. 2006). Furthermore excess cobalt can produce polycythemia, cardiomegaly or diffuse interstitial pulmonary fibrosis in animals and men (Payne 1977). The suitable supplementation of cobalt concentration should be prudently considered and checked. In a following industrial production work, a natural material derived from normal food should replace pure chemical substances in order to prevent harmful effects from cobalt. A result of FFD experiment (Table 4-15) suggests that 2500 µg/kg of cobalt would be recognized as a reference to further work as the change of cobalt supplementation concentrations from 2500 µg/kg to 7500 µg/kg did not have influence on cobalamin production.

5.7. Trimethylglycine

Trimethyglycine also called betaine is found in high contents in sugar beet molasses. Betaine was recognized as a beneficial supplementation to improve the production of cobalamin, even though it showed an inhibition to cell growth (Li et al. 2008). Two reasons may be used as explanation. The first explanation for this improvement is an increased need for the cobalamin-dependent betaine-homocysteine methyltransferase, which converts homocysteine with a methyl from betaine to methionine (White et al. 1973). The other possible explanation is that betaine is thought to be of a regulatory nature and is applied in general porphyrin synthesis as well (White and Demain 1971). An alluring and fascinating, almost 2 folds, increment was obtained in our results (Fig. 4-19). But no significant difference has been found between a supplementation of 0.1 and 1

g/kg of betain. A further optimization results done by an FFD design (Table 4-15) showed no significantly different effect of betaine between 250 mg/kg to 750 mg/kg on cobalamin production in tofu.

5.8. Heme

A heme dependent catalase is removing the stress from oxygen and hydrogen peroxides (Wolf et al. 1991). Heme and cobalamin share the same pathway from ALA to urophyrinogen III (Roth et al. 1996). The expression of the gene hemA operon coding a glutamyl-tRNA reducatse is known to be strictly regulated in microorganisms including *E. coli*, *S. typhimurium*, *Pseudomonas aeruginosa*, *L. reuteri*, and *P. freudenreichii* (Darie and Gunsalus 1994; Moser et al. 2002; Piao et al. 2004; Santos et al. 2008; Schobert and Jahn 2002). The expression level of hemA is inhibited by a feedback from heme, whereas the degradation of HemA is catalyzed by a heme-dependent proteolytic enzyme (Schobert and Jahn 2002). Considering these facts, experiments were done using microorganisms surviving in a medium in the presence of heme for 20 generations to attenuate expressions of hemE or hemZ under heme stress. After 20 generations in the presence of heme, more cobalamin was found in bacetia cultured in heme medium than cultured in normal medium (Fig. 4-34 and 35). To avoid effects from heme, the medium with heme is used as a control. The strains cultured in the normal medium produced more cobalamin in the medium with heme, due to a catalytic activity of heme. However strains cultered in heme medium for 20 generations generated less cobalamin in the medium with heme, but the values were still higher than normal strains in both media. For further work, it would be interesting to check the influence of growth of cells of *P. freudenreichii* and *L. reuteri* in a concentration of heme of 10 mg/L in the media on the transcription of hemeA and hemeE to avoid the feedback inhibition of heme on Uro III, as the intracellular production of heme would be regulated down under these circumstances.

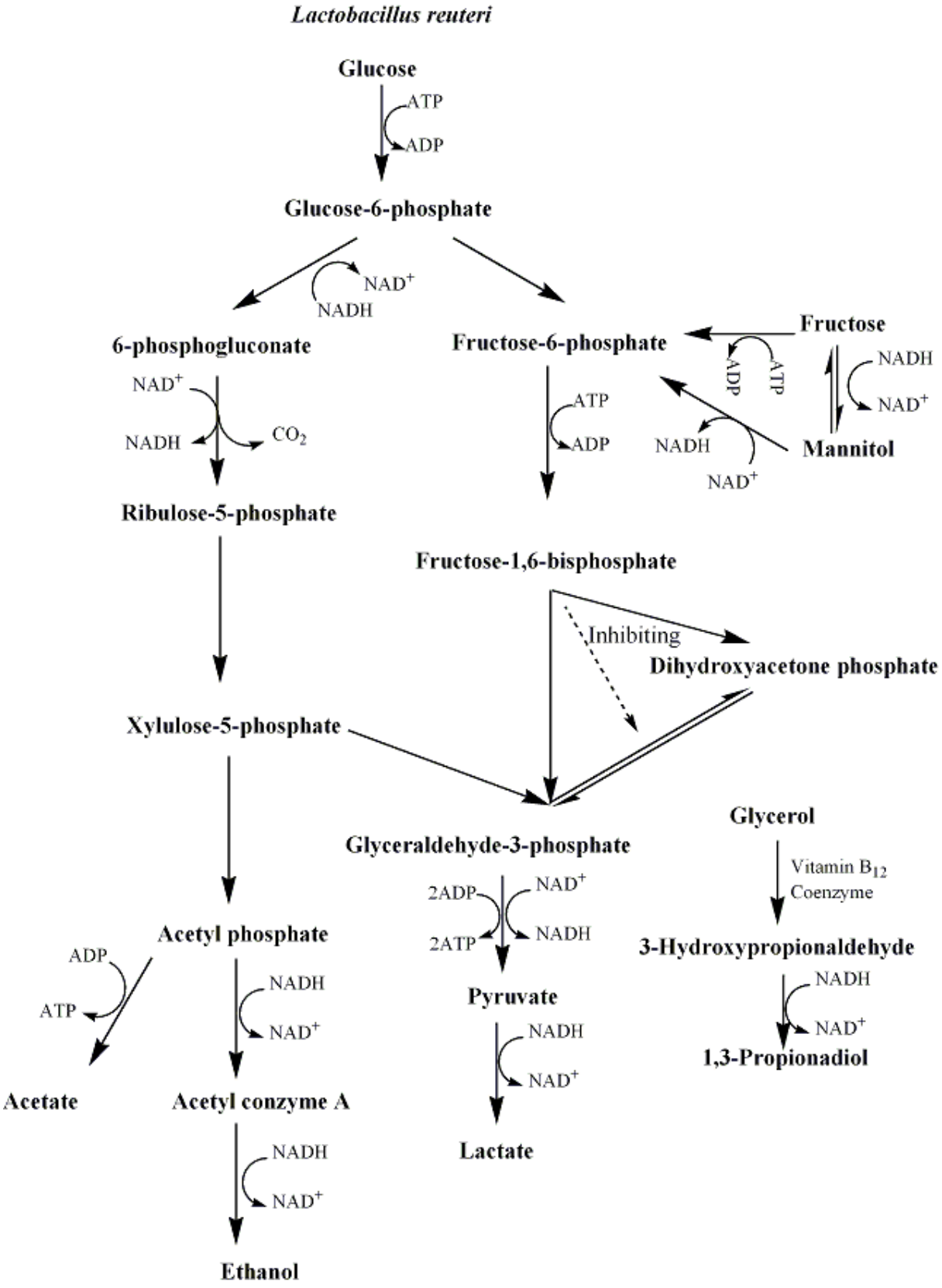


Fig. 5-2 Schematical pathway of carbohydtate metobalisms of *Lactobacillus reuteri*

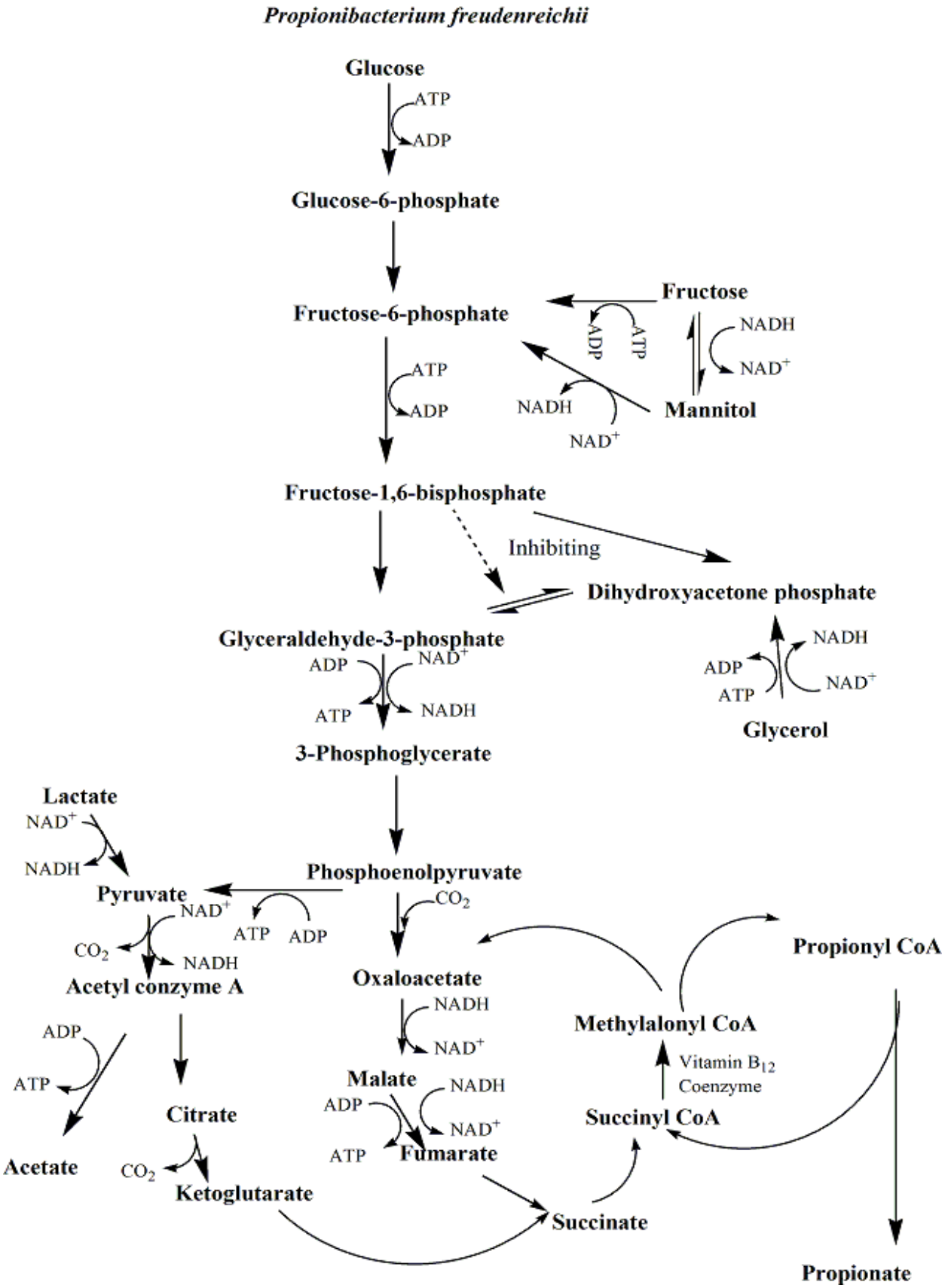


Fig. 5-3 Schematical pathway of carbohydrate metabolism of *Propionibaceterium freudenreichii*

5.9. **Temperatures**

There is no report about the relationship between temperature and cobalamin production. But some researchers (Doleyres et al. 2005; Liu and Moon 1982; Luthi-Peng et al. 2002b; Piveteau 1999) reported a correlation between temperature and 3-HPA production or propionic acid, in which a cobalamin dependent enzyme was involved. Doleyres et al. (2005) had stated no significant difference in 3-HPA production at temperatures between 15 and 37 °C. Another contradictory result mentions that 3-HPA production at 37 °C was significantly higher than at other temperatures in any kind of media (Luthi-Peng et al. 2002b). On the other hand, the optimum growth temperature for *Propionibacterium* sp. is almost 30 °C (Liu and Moon 1982). In our work (Fig. 4-27b), 30 °C is an optimum temperature for co-fermentation to produce cobalamin. An unexpected drop in cell numbers for *L. reuteri* from 28 to 37 °C was observed. The decreasing of final concentrations of ethanol and increasing of final concentrations of propionic acid from 28 to 37 °C can be recognized as a reason of low cobalamin production and low cell densities of *L. reuteri,* which is confirmed by increasing values of interaction coefficient a (Table 4-20). With the purpose of improving the cobalamin yield, the optimum temperature of 30 °C was used in further work.

5.10. Initial pH

The pH value is an important factor to influence metabolites and cobalamin production. Both microorganisms have their own optimum pH and adapted ranges. The optimum pH for 3-HPA production is at 6.0 (Luthi-Peng et al. 2002b), whereas the best pH for propionic acid is between 7.0 and 7.2 (Piveteau 1999). The question of what is the optimum initial pH value for cobalamin production in co-fermentation was solved by our work (Fig. 4-26b). At pH 7.0 and pH 6.5 the highest values of cobalamin were reached. The changed pH values from pH 6.0 to pH 8.0 did not have strong effects on final pH and cell numbers. But a very strong influence on production of ethanol, propionic acid, and acetate was observed (Fig. 4-26c). A possible interpretation of high cobalamin production was that more cobalamin was needed to meet a higher production of propionic acid under acid condition (pH 6.5), which was also reported (Hsu and Yang 1991).

Moreover another explanation contributes to this phenomenon was the low activity of 3-HPA generation above pH 7.0 (Luthi-Peng et al. 2002b), whereas more ethanol was generated to balance redox reaction in *L. reuteri*.

5.11. Oxygen supplementation

Oxygen, as we mentioned before, is involved into the Dmbi generation in *P. freudenreichii*. In the presence of oxygen, growth is slower due to inhibition of propionic acid, acetate, and succinate formation, but pyruvate is accumulated (Schwartz et al. 1976). However propionic acid which is an inhibition factor to both microorganisms can be decomposed in the presence of oxygen. Some researchers (Miyano et al. 2000; Ye et al. 1996) have conducted an oxygen cycle to improve cobalamin production by mediating catabolism of glucose to propionic acid and acetate in the presence and absence of oxygen (Piveteau 1999). On the other hand oxygen also has effects on *L. reuteri* to synthesize more heme against toxic forms of oxygen (Wolf et al. 1991). In our work (Fig. 4-25), 2-day aerobic fermentation in standing culture and 2-day aerobic fermentation in rotating cultures showed a higher productivity of cobalamin than 1-day or 0-day aerobic fermentation. Some researchers (Ye et al. 1996; Ye et al. 1999) demonstrated that low dissolved oxygen was found to be advantageous for the cell growth, for decomposition of propionate, and for lowering the production of acetate by *P. freudenreichii*, which was also confirmed by our work (Fig. 4-25a, c). The dissolved oxygen obviously inhibited growth of *L. reuteri* and led to a reduction of final ethanol concentrations (Fig. 4-25a, c). Regarding the submerged solid fermentation technology we used, oxygen cannot be distributed as homogeneously as we designed. But the standing fermentation without rotating incubations also met the need of oxygen to improve cobalamin production.

5.12. Inoculation

Culture age and ratio of inoculation volumes had an influence on cobalamin production and other metabolites. Inoculum titers of $5*10^7$ of both microorganisms led to the highest cobalamin production, but no significant difference in cell numbers or pH were found. After t-test (data not shown), differences of cobalamin production between various inoculation cell concentrations

were not significant. No matter what inoculation titers of *L. reuteri* were inoculated earlier than *P. freudenreichii,* all these experiments showed low production of cobalamin and also a bad growth of *P. freudenreichii*, even though the pH value was adjusted back to 6.5. That can possibly be explained by inhibitors such as reuterin. Lower inoculum concentrations of *L. reuteri* led to a drop of cobalamin production. That indicated an important role of *L. reuteri* in the cooperation of cobalamin production.

5.13. Batch and fed fermentations with natural substrates

According to above paragraphs, glucose is the main factor to improve cobalamin production besides pH, temperature and other supplementations. But a catabolite repression would appear when a rapidly metabolizable carbon source such as glucose was used. A slower metabolization of this energy source was caused by an increase of the intracellular concentration of ATP that leads to the repression of enzyme biosynthesis (Jeude et al. 2006; Lin et al. 2004; Smets et al. 2002). In this case, batch fermentations with various glucose concentrations were conducted. We found that the highest cobalamin production can be attained with 5 g/kg of glucose (Fig. 4-29I). The fast drop of pH, high production of acetate, propionic acid, and ethanol in the fermentation supplemented with 7 g/kg of glucose (Fig. 4-29) was the reason for low cobalamin production and low cell concentrations of *P. freudenreichii*. The nutrient limit could reasonably answer the poor cobalamin production and low cell concentrations of *L. reuteri* at 1 g/kg. Compared with the other two experiments, 5 g/kg of glucose supplementations could produce more cobalamin due to the appropriate cell numbers of both microorganisms and metabolites that did not inhibit growth of any microorganisms.

To develop cobalamin production, a fed batch fermentation method was applied. A tremendous progress was made so that cobalamin production of all fed batch fermentations was beyond 200 ng/g (Fig. 4-30-1). This phenomenon was attributed to alleviation of catabolite repression and no nutritional limitation. The low cell concentrations can be interpreted by effects from inhibition of metabolites and high amount of oxygen because fermentation flasks were opened to supplement glucose. We also found (Table 4-20) that supplementations of glucose were

enhancing antagonistic effects of *L. reuteri* to *P. freudenreichii*, which can explain the low cell concentrations. Higher amounts of metabolite production of fermentations were resulting from higher glucose supplementations (Fig. 4-30-2). In brief, a fed batch submerged solid fermentation succeeded to produce 289 ng/g of cobalamin which is much higher than in other foods (Fig. 5-1).

Supplementations of pure chemical substrates such as cobalt chloride, glucose, riboflavin, glycine, and fructose are not only expensive but cobalt chloride may also cause a safety problem. Several plant-based foods were chosen to do fermentations with natural substrates. According to USDA National Nutrient Database (http://ndb.nal.usda.gov/), bananas are containing 4.98 g of glucose, 4.85 g of fructose, 2.39 g of sucrose, and even 5.38 g of starch per 100 gram and may take the place of glucose as a carbon source. Spinach with 0.189 mg of riboflavin, 6.5 mg of cobalt and 102.6 mg of betaine per 100 g can be a good supplementation source for cobalt and betaine. Kelp seaweed including 0.15 mg of riboflavin and 0.1 g of glycine can be treated as a glycine source. Some authors reported seaweed as a good vitamin B_{12} source (Schneider and Stroinski 1987). Analysis showed no biological active vitamin B_{12} in our samples. By orthogonal experiments, all supplementations had positive effects on cobalamin production. But the order is banana > spinach > kelp seaweed. That is corresponding to our pure chemical optimization results. Cobalamin productions were ranging rom 92.4 to 179. 3 ng/g. The low cell concentrations (Fig. 4-41 and 42) may be due to the same reason as the above mentioned effect of oxygen and inhibitors. Except of combination 2, pH of other combinations decreased slowly (Fig. 4-40). That means seaweed and spinach did not only function as nutritional sources but also as pH buffering substances. Based on hints from Fig. 4-39 and 38, production of propionic acid was corresponding to seaweed and production of lactate was corresponding to banana. The latter is easy to understand. The former can be explained by generation of propionic acid accompanied with synthesis of methionine, which was promoted by glycine (Iida and Kajiwara 2007) from seaweed. However some problems still existed in our work to scale up fermentations. In order to execute fermentation in an industrial size, natural materials should be more homogenously distributed into tofu and dissolved oxygen should be controlled.

6. Summary

The present work is concerning the vitamin B_{12} enrichment in tofu by submerged solid substrate fermentations with *L. reuteri* and *P. freudenreichii*.

After screening, eight strains of bacteria were confirmed to be able to produce vitamin B_{12} in tofu. *L. reuteri* was considered the best strain to carry out further experiments. The optimization of nitrogen source, moisture, time of fermentation, and carbon source had been performed. An appropriate co-fermentation with glucose, fructose, and glycerol can improve cobalamin production by balancing the redox reaction. After further optimizations by a fractional factorial design, a steeptest ascent design and a central composite design, 16.33 ± 0.58 ng/g (wet weight) of cobalamin were produced by fermentation in 100 gram of tofu supplemented with 16.41 g/kg of glucose, 0.7 g/kg of glycerol, 1.5 g/kg of fructose, 2.5 mg/kg of $CoCl_2$, 3.5 mg/kg of Dmbi, and 100 mL DDW at 37 °C for 3 days.

A cofermentation with *L. reuteri* and *P. freudenreichii* was applied because the cooperation of *L. reuteri* and *P. freudenreichii* can improve vitamin B_{12} production in tofu. A series of fermentation supplementations and conditions were screened and optimized. Riboflavin, betaine, Dmbi, glycine, glucose, fructose, and glycerol supplementations have been proven to have significantly positive effects on vitamin B_{12} production. Riboflavin can replace Dmbi to produce vitamin B_{12} under at least 4-day anaerobic fementations for *L. reuteri*. Supplementations of riboflavin after 2-day fermentations have been proven to improve vitamin B_{12} production as it can avoid an inhibitory riboswitch feedback of vitamin B_{12}. Conditions of initial pH values, fermentation temperature, oxygen supply, and inoculum titers have also been optimized. Optimum initial pH values were between 6.5 and 7.0. 30°C was the optimum fermentation temperature. The last 2-day aerobic fermentation can afford enough oxygen to convert riboflavin to Dmbi for *P. freudenreichii*. Unlike Emmental cheeses, high vitamin B_{12} production can be found only if $5*10^7$ of both bacteria were inoculated simultaneously. After a series of single factor experiments and FFD experiments, 90 ng/g of vitamin B_{12} (wet weight) were produced in 100 gram of tofu

fermentations supplemented with 0.5 g/kg glucose, 0.1 g/kg fructose, 80 µg/kg riboflavin, 1 mg/kg cobalt chloride, and 0.5 g/kg betaine.

Batch fermentations of 1 kg tofu were successfully conducted based on above conditions. Supplementations of 5 g/kg glucose cannot only produce more cobalamin, but also alleviate the catabolic repression. Fed batch fermentations obviously improved vitamin B_{12} production. In particular, a fed batch supplemented with 1 gram glucose every day produced almost 289 ng/g of vitamin B_{12}. Fermentations with natural substances have also been performed to produce 179 ng/g of vitamin B_{12}. After breeding experiments were perfomed by inoculating bacteria in vitamin B_{12} test broths supplemented with heme for 20 generations, Strains can produce 2 fold more vitamin B_{12} than before. Optimizations of tofu whey as preculture media for both bacteria were successfully perfomed.

As the vitamin B_{12} content in fermented tofu was almost the same as in meat, fermented tofu can be recognized as a potential alternative diet for vegetarians.

Zusammenfassung

In der vorliegenden Arbeit wurde die Anreicherung von Vitamin B_{12} in Tofu durch eine Fermentation von Tofu mit *Lactobacillus reuteri* und *Propionibacterium freudenreichii* untersucht.

Nach einem „Screening“ konnte gezeigt werden, dass 8 Bakterienstämme die Fähigkeit besitzen in Tofu Vitamin B_{12} zu bilden. Da sich *L. reuteri* als der am besten geeignete Stamm herausstellte, wurden die weiteren Untersuchungen mit diesem Stamm durchgeführt. Es wurden Optimierungsversuche für die Parameter: Fermentationsdauer, Kohlenstoffquelle, Stickstoffquelle, Feuchtigkeitsgehalt (Wassergehalt) durchgeführt. Es konnte gezeigt werden, das eine Co-Fermentation aus *L. reuteri* und *P. freudenreichii* nach einer Optimierung der Zusätze an Glucose, Fructose und Glycerin durch eine Balance des Redox-Gleichgewichtes geeignet ist die Vitamin B_{12}-Produktion in Tofu zu steigern. Nach weiteren Optimierungen durch ein „fractional fractorial design“, ein „steepest ascent design“ und ein „central composite design“ konnten 16,33 ± 0,58 ng/g (Naßgewicht) Cobalamin erreicht werden. Dazu wurden 100g Tofu mit 16,41 g/kg

Glucose, 1,5 g/kg Fructose, 0,7 g/kg Glycerin, 2,5 mg/kg $CoCl_2$, 3,5 mg/kg Dimethylbenzimidazol und 100mL entmineralisiertem Wasser für 3 Tage bei 37°C fermentiert.

Es wurde eine Co-Fermentation mit *L. reuteri* und *P. freudenreichii* durchgeführt, da die Kooperation der beiden Stämme die Vitamin-B_{12}-Produktion in Tofu verbessert. Nach der eingehenden Prüfung unterschiedlicher Fermentationssupplementierungen und unterschiedlicher Fermentationsbedingungen konnte gezeigt werden, dass Riboflavin, Betain, Dimethylbenzimidazol, Glycin, Glycerin, Glucose und Fructose Supplementierungen eindeutig positive Effekte auf die Vitamin-B_{12}-Produktion in Tofu haben. Unter den Bedingungen einer mindestens viertägigen anaeroben Fermentation für *Lactobacillus reuteri* kann Riboflavin Dimethylbenzimidazol als Vorstufe für die Vitamin-B_{12}-Produktion ersetzen. Es konnte gezeigt werden, dass Riboflavin-Supplementierungen, die nach einer zweitägigen Fermentationszeit erfolgten, die Vitamin-B_{12}-Produktion verbesserten, da so ein hemmender „Riboswitch“ durch eine Vitamin-B_{12}-Rückkopplungshemmung (feedback inhibiton) vermieden werden konnte. Des Weiteren wurden die Parameter Ausgangs-pH-Wert, Fermentationstemperatur, Sauerstoffversorgung und Inokulumstiter optimiert. Der optimale Ausgangs-pH-Wert betrug zwischen 6,5 bis 7,0. Die optimale Fermentationstemperatur betrug 30°C. Eine anschließende zweitägige aerobe Fermentation kann eine ausreichende Sauerstoffversorgung für die Umwandlung von Riboflavin zu Dimethylbenzimidazol durch *P. freudenreichii* gewährleisten. Im Gegensatz zu Emmentaler Käse kann eine hohe Vitamin-B_{12}-Produktion nur stattfinden, wenn beide Bakterienstämme gleichzeitig in einer Zellzahl von 5×10^7 Zellen pro mL zugegeben werden. Nach einer Reihe von Einzelfaktor-Experimenten und „fractional factorial design“ Experimenten konnten in 100g Tofu, die mit 0,5 g/kg an Glucose, 0,1 g/kg an Fructose, 80 µg/kg an Riboflavin, 1 mg/kg Cobaltchlorid und 0,5 g/kg an Betain supplementiert worden waren, 90 ng/g Vitamin B_{12} (Naßgewicht) produziert werden.

Unter den beschriebenen Bedingungen wurden Batch-Fermentationen mit 1 kg Tofu erfolgreich durchgeführt. Supplementierungen von 5 g/kg Glucose führten nicht nur zu einer besseren Cobalamin-Produktion, sondern führten auch zu einer Abmilderung der Katabolitrepression. Fed-Batch-Fermentationen konnten die Vitamin-B_{12}-Produktion deutlich steigern. Insbesondere führte eine Fed-Batch-Fermentation, bei der jeden Tag 1 g Glucose zugefüttert wurden, zu einer

Vitamin-B_{12}-Konzentration von 289 ng/g. Fermentationen, die mit natürlichen Substraten durchgeführt wurden, führten zu Vitamin-B_{12}-Konzentrationen von 179 ng/g Tofu. Durch Kultivierungsexperimente, bei denen die für die Inokulation verwendeten Bakterienstämme über 20 Generationen in Vitamin-B_{12}-Testbouillon angezogen worden waren, die mit Häm supplementiert worden war, konnte die Vitamin-B_{12}-Produktion um das Doppelte gesteigert werden. Optimierungsversuche, die auf einem Tofu-Molke enthaltenden Anzuchtmedium beruhten, konnten für beide Bakterienstämme erfolgreich durchgeführt werden.

Da der Vitamin-B_{12}-Gehalt in fermentiertem Tofu in der gleichen Größenordnung liegt, wie der Vitamin-B_{12}-Gehalt in Fleisch, kann fermentierter Tofu als potentieller Fleischersatz für Vegetarier angesehen werden.

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8. Hazardous chemicals

8.2.1. List of hazardous chemicals

Chemicals	Hazard symbol	Risk phrase	Safety phrase
1,2-Propanediol			S24 S25
Acetate		R10, R35	(S1/2), S23, S26, S45
Betaine			
Cobalt(II) chloride	T, Carc. Cat. 2, N	R49, R60, R22, R42/43, R68, R50/53	S53, S45, S60, S61
Potassium cyanide	T+, N	R26/27/28, R32, R50/53	(S1/2), S7, S28, S29, S45, S60, S61
Cyanocobalmin			S24/25
Dmbi			S22 S24/25
Ethanol	F, Xn	R11, R61	S2, S7, S16
Formic acid	C	R10, R35	(S1/2), S23, S26, S45
Hexane		R11, R38, R48/20, R62, R65, R67, R51/53	S2, S9, S16, S29, S33, S36/37, S61, S62
Hydrogen chloride	T, C	R23, R35	(S1/2), S9, S26, S36/37/39, S45
Methanol	F, T	R11, R23/24/25, R39/23/24/25	(S1/2), S7, S16, S36/37, S45
Ninhydrin		R22, R36, R37, R38	S26, S28, S36
Sodium hydroxide	C	R35	(S1/2), S26, S37/39, S45

8.2.2. Abbreviation and description of hazard

Abbreviation	Desription of hazard
F	Highly flammable
T+	Very toxic
T	Toxic
Xn	Harmful
C	Corrosive
N	Dangerous for enviroment
Carc. Cat. 2	

8.2.3. Risk phase and desription of risk

R10: Flammable
R11: Highly flammable
R22: Harmful if swallowed
R22: Harmful if swallowed
R23/24/25: Toxic by inhalation, in contact with skin and if swallowed
R23: Toxic by inhalation
R26/27/28: Very toxic by inhalation, in contact with skin and if swallowed
R32: Contact with acids liberates very toxic gas
R35: Causes severe burns
R36: Irritating to eyes
R37: Irritating to respiratory system
R38: Irritating to skin
R39/26/27/28: Very Toxic: danger of very serious irreversible effects through inhalation, in contact with skin and if swallowed
R42/43: May cause sensitization by inhalation and skin contact
R48/20: Harmful: danger of serious damage to health by prolonged exposure through inhalation
R49: May cause cancer by inhalation
R50/53: Very toxic to aquatic organisms, may cause long-term adverse effects in the aquatic environment
R51/53: Toxic to aquatic organisms, may cause long-term adverse effects in the aquatic environment
R60: May impair fertility
R61: May cause harm to the unborn child
R62: Possible risk of impaired fertility
R65: Harmful: may cause lung damage if swallowed
R67: Vapours may cause drowsiness and dizziness
R68: Possible risk of irreversible effects

8.2.3. Safety phase and desription of safety

(S1/2): Keep locked up and out of the reach of children
S2: Keep out of the reach of children
S16: Keep away from sources of ignition - No smoking
S22: Do not breathe dust
S23: Do not breathe gas/fumes/vapour/spray (appropriate wording to be specified by the manufacturer)
S24/25: Avoid contact with skin and eyes
S24: Avoid contact with skin
S25: Avoid contact with eyes
S26: In case of contact with eyes, rinse immediately with plenty of water and seek medical advice
S28: After contact with skin, wash immediately with plenty of ... (to be specified by the manufacturer)
S29: Do not empty into drains
S33: Take precautionary measures against static discharges
S36: Wear suitable protective clothing
S36/37: Wear suitable protective clothing and gloves
S37/39: Wear suitable gloves and eye/face protection
S45: In case of accident or if you feel unwell seek medical advice immediately (show the label where possible)
S53: Avoid exposure - obtain special instructions before use
S60: This material and its container must be disposed of as hazardous waste
S61: Avoid release to the environment. Refer to special instructions/safety data sheet
S62: If swallowed, do not induce vomiting: seek medical advice immediately and show this container or label where possible
S7: Keep container tightly closed
S9: Keep container in a well-ventilated place

9.1. List of figures

9.2. List of tables

10. Abbreviations

%	Percent
°C	Celsius degree
1,3-PD	1,3-propanidiol
3-HPA	3-hydroxypropionaldehyde
Ado	Adenosyl
Ado-Cbi	Adenosylcobinamide
Ado-cobalamin	Adenosyl-cobalamin
ALA	Delta-aminolevulinic acid
ANOVA	Aanalysis of variance
AOAC	Association of official analytical chemists
APD	Animal protein factor
APF	Animal protein factor
ATP	Adenosine triphosphate
BC	Before Christ
CCD	Central composite designs
CFU	Colony-forming unit
CN-	Cyanide
CoA	Coenzyme A
CobA	Cobalamin adenosyltransferase
CobG	Precorrin 3 biosynthesis protein
CobT	Nicotinate-nucleotide-dimethylbenzimidazole phosphoribosyltransferase
DAD	Diode array detector
DDW	Double distilled water
Dmbi	Dimethylbenzimidazole
DmbMN	Ribofuranosyl dimethylbenzimidazole phosphate
DSMZ	Deutsche Sammlung von Mikroorganismen und zellkulturen
DNA	Deoxyribonucleic acid
dTMP	Deoxythyminemonophosphates
DRI	Dietary reference intakes
dUMP	Deoxyuracilmonophosphates
DW	Dry weight
HPLC	High performance liquid chromatography
EMP	Embden Meyerhof-Parnas
FAD	Flavin adenine dinucleotide
FMN	Flavin mononucleotide
FFD	Fractional factorial design
g	gram(s)
GDL	Glucono-delta-lactone
GRAS	Generally recognized as safe

GysG	Sirohaem synthase
IF	Intrinsic factor
L	Liter
LAB	Lactic acid bacteria
mL	Millilitre
ng	Nanogram
NCBI	National Center for Biotechnology Information
PBA	*Propionibacterium*
PKP	Phosphoketolase pathway
PocR	Transcriptional regulator
RSD	Relative standard deviation
RSM	Response surface methodology
SAS	Statistical analysis system
SPE	Solid phase extraction
spp.	Species
ssp.	Subspecies
k	Kilo
µ	Micro

11. Curriculum vitae

Personal Data

Name: Xuan Zhu

Place, date of birth Jiaxing, P. R. China, 03,03,1982

Eduction experience

2009 to 2013 Doctor Candidate in Lebensmittelchemie

Abteilung Lebensmittelmikrobiologie/Hygiene, Department of Lebensmittelchemie, University Hamburg, Hamburg, Germany

Research on Vitamin B_{12} Production during Tofu Fermentation by *Lactobacillus reuteri* and *Propionibacterium freudenreichii* (supported by Deutscher Akademischer Austausch Dienst and by Federal Ministry of Education and Research (BMBF, Bonn-Bad Godesberg) grant 0315825)

2005 - 2007 Master of Food Science

School of Biosystems Engineering and Food Science, Zhejiang University, Hangzhou, China

Resarch on Enzyme link Immunoassay for Group-Specific Determination of Chloramphenicol and Clenbuterol Hydrochloride

2001 - 2005 Bachelor of Biological Science and Engineering

College of food science and biotechnology, Zhejiang Gongshang University, Hangzhou, China

Research on thesis project of Transformation and Cloning of an Endochitinase Gene from *Trichoderma viride*

Career experience

Feb. 2007 - Jan. 2008 Eurofins China

Acted as a section manager of microbiology lab and consultant of customers.

Mainly focusing on the lab setting up and being responsible for design, purchase and SOP (Standard Operating Procedure) preparation.