PRODUCTION OF RIBOFLAVIN BY *ASHBYA GOSSYPII* FROM WHEY

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ABSTRACT

PRODUCTION OF RIBOFLAVIN BY *ASHBYA GOSSYPII* FROM WHEY

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Fermentation studies on the production of riboflavin were carried out with an industrial byproduct whey, in shake flask and laboratory fermentor using *Ashbya gossypii* NRRL Y-1056. Effect of supplements; yeast extract, sucrose, glycine, peptone, soybean oil, soybean flour, glycine+peptone and bran on the rate, and yield of riboflavin production in whey were studied in shake flask fermentation. In fermentor studies, whey, and whey supplemented with bran and glycine+peptone were used.

Riboflavin production started to increase more rapidly after 2 days of fermentation and continued to increase until 5 days. The quantities of riboflavin produced by *Ashbya gossypii* in whey after 8 days of shake flask fermentation with supplements; bran, soybean flour, and glycine+peptone were 389.5, 120.7 and 101.7 mg/L respectively. Lower yields were obtained using supplements; peptone and soybean oil (23.2 and 17.5 mg/L). The best yield was obtained by using bran supplement.

Yield of riboflavin produced in whey, and whey supplemented with bran and glycine+peptone by *Ashbya gossypii* after 8 days of fermentation in fermentor were found to be nearly four times higher than that of shake flask studies and were 113.4, 1227.7 and 400.0 mg/L respectively.

Key words: whey, riboflavin, *Ashbya gossypii*, fermentation.

PEYNİR ALTI SUYUNDAN *ASHBYA GOSSYPİİ* KULLANARAK RİBOFLAVİN ÜRETİMİ

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Sallamalı flask ve laboratuar fermentörü ile, *Ashbya gossypii* NRRL Y-1056 kullanılarak endüstriyel bir yan ürün olan peynir altı suyundan riboflavin üretimi üzerine fermentasyon çalışmaları yürütülmüştür. Peynir altı suyuna eklenen maya ekstraktı, sukroz, glisin, pepton, soya fasulyesi yağı, soya fasulyesi unu, glisin+pepton ve kepeğin riboflavin üretim hızı ve verimi üzerindeki etkisi sallamalı flask fermentasyonu ile çalışılmıştır. Fermentör çalışmalarında peynir altı suyu ve peynir altı suyuna eklenmiş kepek ve glisin+pepton kullanılmıştır.

Riboflavin üretimi fermentasyonun 2. günü sonunda daha hızlı artmaya başlamış ve 5. güne kadar artmaya devam etmiştir. Sallamalı flask fermentasyonuyla peynir altı suyunda 8 gün sonunda, peynir altı suyuna eklenen kepek, soya fasülyesi unu ve glisin+peptonla *Ashbya gossypii* ile üretilen riboflavin miktarları sırasıyla 389.5, 120.7 ve 101.7 mg/L olarak bulunmuştur. Peynir altı suyuna eklenen pepton ve soya fasülyesi yağı ile daha düşük verimler elde edilmiştir (23.2 ve 17.5 mg/L). En iyi verim kepek eklenmesiyle elde edilmiştir.

Peynir altı suyunda ve peynir altı suyuna eklenen kepek ve glisin +pepton ile, *Ashbya gossypii* tarafından fermentörde 8 gün sonrasında üretilen riboflavin miktarı, aynı ürünler için sallamalı flask çalışmalarına göre yaklaşık dört kat daha yüksek bulunmuştur ve riboflavin miktarları sırasıyla 113.4, 1227.4 ve 400.0 mg/L'dir.

Anahtar kelimeler: Peynir altı suyu, riboflavin, *Ashbya gossypii*, fermentasyon.

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CHAPTER I

INTRODUCTION

Utilization of agro-industrial wastes is one of the present challenges of biotechnology [1]. Whey is produced in very large quantities as a byproduct in the dairy industries. If it is not utilized or processed, it can cause environmental problems. Whey contains proteins, carbohydrates (mainly lactose), minerals and trace elements required for microbial growth [2]. Possible uses of whey include the production of lactose, ethanol, ammonium lactate, galactose, baker's yeast and single cell protein. Riboflavin is clearly of basic importance for nutrition and metabolism in the respiratory cycle. So the vitamin B_2 content of milk and cheese, muscle, liver, and kidney from warm blooded animals, of yeast and of certain vegetables is important in human nutrition. Deficiency of it causes some non-specific symptoms. Nowadays all pasta products such as macaroni and spaghetti, bakery products are supplemented with B vitamins to improve their nutritive values. So far, the B vitamins are imported and they are rather expensive in our country. Thus being able to utilize whey for B vitamins fermentation, might lower the cost of B vitamins and at the same time reducing environmental threat of disposing whey.

In this present work, the production of vitamin B_2 was carried both in shake flask and 1.5 liter laboratory fermentor studies, using cheese whey , which was obtained from local cheese factories, as a fermentation medium, by using the ascomycete *Ashbya gossypii* NRRL Y-1056. For shake flask studies whey was supplemented individually with 8 different ingredients at 28°C, pH 5.1, with a fermentation time of 8 days. For 1.5 liters laboratory fermentor, the best two based on shake flask studies, of the 8 supplements were tried under the same conditions,

but providing 0.1 NL/min aeration and 300 rpm agitation. The produced vitamins were analyzed by microbiological assay method according to AOAC [3] and Difco [4].

CHAPTER II
LITERATURE REVIEW

2.1 Whey

Whey is the aqueous phase or serum that is separated from the curd in conventional cheese making and casein manufacture. It comprises 80-90 % of the total volume of milk entering the process and contains about 50 % of the nutrients in the original milk: soluble protein, lactose, vitamins and minerals. Whey as a byproduct from the manufacture of hard, semihard or soft cheese and rennet casein is known as sweet whey and has a pH of 5.9-6.3. Manufacture of mineral acid precipitated casein yields an acid whey with a pH of 4.3-4.6. The approximate composition of whey from cheese and casein manufacture are given in Table 2.1 [5].

Ever since people started making cheese on a large scale it has been a great problem to dispose of the growing volumes of whey. At one time the whey was simply discharged into lakes and rivers without a thought to the environmental consequences. But gradual realization of those consequences has now led to discharge of untreated whey being banned in many countries. In many places, too, whey has been used as animal feed and fertilizer [5].

In recent years, utilization of whey has been felt to be an inexorable necessity in vies of the current requirements for alleviating environmental pollution as well as using available nutrients for feeding the malnourished segments of human population. Presence of several nutritionally important constituents having excellent functional characteristics enhances opportunities for a wide-range application of whey and whey constituents in the food industry. Technology is

Table 2.1. Approximate composition of whey, %

Constituents	Cheese whey	Casein whey
Total solids	6.350	6.500
Water	93.700	93.500
Fat	0.500	0.040
Protein	0.800	0.750
Lactose	4.850	4.900
Ash (minerals)	0.500	0.800
Lactic acid	0.005	0.400

being developed to utilize whey for the manufacture of a variety of new food products as well as for the replacement of cooperatively costly food ingredients [6].

A survey of production and utilization trends in the U.S.A. during 1974 indicated that approximately 13.8 billion kg of cheese whey or 0.9 billion kg of whey solids were produced. Of these available whey solids, only about 56 % were used in human and animal feeds and the rest was wasted. Whey solids for human nutrition are being produced in a variety of forms such as dried whey, condensed whey, partially delactosed whey, partially demineralized whey, etc. There has been a steady upward trend in the utilization of whey for human food during recent years, primarily due to a better understanding of the unique nutritional, biological, and functional characteristics of whey components. Many of the available methods of utilization, such as production of whey protein concentrates, aim at fractionating some of the constituents selectively rather than using whole whey, thus only partially solving the problem of utilization and disposal [7].

In our country cheese is one of the foodstuff being produced in large quantities. In one year period (1990), 229.000 tons different type of cheese were manufactured together with about 2.000.000 tons of whey [8].

2.1.1 Usefulness of Whey for Food Manufacturers

Whey can be incorporated advantageously into various food formulations. Lactose, which is the major component, acts as a carrier for flavor and color when added to many foods.

Whey solids tenderize and help retain moisture and freshness in foods in contrast to the firmness of products containing skim milk.

Whey-based coatings have been suitable for food applications. For space foods, whey-based coatings containing high-melting fats are used to reduce the rate at which these products disintegrate in the mouth and permit easy swallowing [9].

2.1.2 Cheese and Cheese Foods

Ricotta, a soft unripened variety of Italian cheese, is popular in the U.S.A. and ranked second in production to Mozzarella in this group of cheeses. Traditionally, Ricotta is produced by heating blends of about 3 to 10% whole milk with whey. However only about 50% protein is recovered by the traditional process. When whey is heated in the presence of 500 or 2000 ppm of Ca^{+2}, nearly 75% of the proteins may be recovered during the manufacture of Ricotta cheese.

Another large use of whey solids in the dairy industry is in the manufacture of the processed cheese and cheese spreads. Whey protein concentrates have been blended successfully with processed cheese foods [10].

2.1.3 Whey-Protein Concentrates (WPC)

The geometric increase in world population and arithmetic increase in food production have created the problem of protein-calorie malnutrition. Various vegetable protein sources are being tested for their efficacy in overcoming shortages of dietary protein. However, they lack one or more essential amino acids, which is deleterious to protein utilization in vivo. Whey protein concentrates on the other hand, have adequate essential amino acids, are easily digestible, and are considered to be highly nutritional and physiologically complete [11].

2.1.4 Infant Food Formulations

Infants are born with relatively underdeveloped organ functions, especially of the kidneys and intestines. This requires that certain special nutritional demands be met, especially during the first 3 months of life. Research work in this area has shown that feeding infants exclusively on cow's milk leads to the development of altered intestinal physiology. This has been attributed to not only gross compositional characteristics but also to the chemical makeup of various constituents of cow's milk which differ appreciably from that of human milk [6].

Modified whey solids may be added to bovine milk to give it characteristics of human milk. Studies in Japan have shown that infants fed on such humanized formulations having casein to whey ratio of 60:40 (as in human milk) showed normal physiological functions. Feeding of humanized formulations exerts considerably lower osmolar loads on the kidneys and increases nitrogen retention and utilization [12].

2.1.5 Bakery Products

Bakeries in the U.S.A. have been the largest single

user of whey solids. As a measure of reducing costs of manufacture, whey solids steadily have been replacing more costly nonfat dry milk.

However, usage of whey solids has certain limitations even when a high heat treated product is used. Use of whey solids increases dough mixing time, lowers water absorption, and depress bread volume. This problem can be overcome by modified whey products which are low in lactose and high in protein content [6].

2.1.6. Confectionery Products

Whey solids have been favored as an ingredient for formulation of confectionery products. Whey formulas make caramels with a fine flavor. It is possible to make a light caramel with little or no color when only sweetened condensed whey is used. Caramels produced by using sweetened condensed whey and soya proteins closely resemble those produced from skim milk or whole milk. Confectionery coatings prepared by using whey in the formulations have enhanced the texture, flavor, and color characteristics of the finished product and preserved freshness in year-round usage.

Various formulae for the manufacture of confections contain 2 to 7% soluble proteins to provide improved nutritional value. Use of demineralized whey in such formulations gives products of superior quality [9].

2.1.7 Ice Cream and Frozen Desserts

Probably the largest single use of whey in a dairy product is in ice cream. As a result, many million kg of whey solids are used each year in ice cream. There are strong economic reasons for using less expensive whey solids in place of nonfat milk. At present, both concentrated whey and dried whey are used in ice cream

formulations [9].

2.1.8 Culture Medium

Making a starter medium for cultured dairy products such as cheese, sour cream, yogurt, buttermilk etc., represents another potentially large use for whey. Usually skim milk fortified with nutrients is used industrially, but media made with whey as the base also can be used successfully to grow these starter cultures [6].

Several processes have been proposed for whey utilization largely based on fermentation by microorganisms that utilize lactose naturally. It would be convenient to utilize lactose as a carbon source for the production of ethanol, but yeast cells that utilize lactose are not able to carry on sustained alcoholic fermentation [13, 14], whereas the yeast *Saccharomyces cerevisiae* does not utilize lactose since it lacks both lactose permease and betagalactosidase activity but can produce ethanol [15].

Whey has been shown to be a useful substrate for the production of n-butanol by fermentation. Using whey supplemented with 0.5 % (w/v) yeast extract, yields of 1.5 % (w/v) n-butanol have been obtained [16].

Whey permeates can be transformed into a nitrogen-enriched wort, which in turn, can be used as culture medium for the fermentation process in the production of baker's yeast [6]. This nitrogen-enriched wort was prepared by fermentation of lactose into lactic acid with *Lactobacillus bulgaricus* or *Streptococcus thermophilus*, and then by transforming lactic acid into ammonium lactate through neutralization with ammonia. Parallel work on the screening of thermophilic lactobacilli revealed a strain of *Lactobacillus helveticus*, which consistently yielded highest concentrations of lactic acid in milk.

Furthermore *L. helveticus* is able to ferment galactose, a breakdown product in the fermentation of the lactose. Because of these advantageous properties of *L. helveticus*, it would be of interest to investigate the use of this microorganism for the production of lactic acid from whey permeate [17].

2.2 Riboflavin

2.2.1 Occurrence and Economic Significance

Riboflavin (also called lactoflavin or vitamin B_2)was first isolated from whey. Riboflavin is present in milk as free riboflavin, but is present in other foods (liver, heart, kidney or eggs) as part of flavoproteins which contain the prosthetic groups FMN (Flavin mononucleotide) or FAD (Flavin adenine dinucleotide) [18].

Riboflavin is produced industrially by several processes.

(a) Chemical synthesis, primarily for pharmaceutical use(20 % of world-wide production).

(b) Biotransformation of glucose to D-ribose by mutants of *Bacillus pumilus* and subsequent chemical conversion of ribose to riboflavin (about 50 % of world-wide production).

(c) Direct fermentation (about 30 % of world-wide production).

Total production of riboflavin on a world-wide basis is around 2000 tons per year [18].

In our country the riboflavin can not be produced yet, so it is imported from foreign countries. The amount that we are still paying is more than 1 million USD per year (Table 2.2) [19].

Table 2.2 The amount and prices of vitamin B_2 imported by Turkey between years 1990-1993.

Year	Amount(Kg)	Price(USD)
1990	19,183	1,019,122
1991	21,454	1,231,931
1992	25,978	1,660,676
1993	24,260	1,534,883

2.2.2 Deficiency

Ariboflavinosis is a disease in humans caused by riboflavin deficiency. Riboflavin deficiency manifests itself by a number of non-specific symptoms: general lethargy and disinclination to work, changes in the mucosa of the lips, mouth and tongue, dystrophy of the fingernails and finally changes in the skin of the whole body. Reduced rasistance to infection (typhus, pnitmonia) is often a sign of riboflavin deficiency. Due not to a lack of food, but to a badly balanced diet, the body may only receive 60 % of its actual riboflavin requirements. Mild deficiency in riboflavin may also result from insufficient absorption of the vitamin, due to disturbances in the digestive tract and to liver disease [20].

Appearing as a type of dermatitis, this disease can be counteracted by administering riboflavin at a daily dosage of 1 mg. The recommended daily dosages are as follows [20]:

- Men, of all ages 1.6 mg.
- Women, of all ages 1.3 mg.
- Growing boys and girls 0.4 to 1.7 mg depending on the age. During pregnancy it should be 1.6 mg and during lactation 1.8 mg [19].

2.2.3 Structure

Riboflavin is an alloxazine derivative which consists of a pteridine ring condensed to a benzene ring. The side chain consists of a C_5-polyhydroxy group, a derivative of ribitol. The structure of riboflavin(6,7-dimethyl-9-(D-1'-ribityl)-isoalloxazine) is given in Figure 2.1.

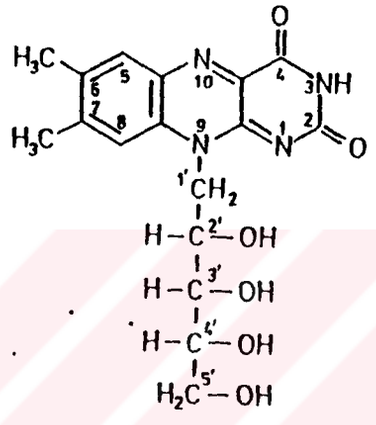


Figure 2.1 Structure of Riboflavin

Riboflavin is soluble in water and insoluble in acetone, chloroform, ether and benzene, it melts at about 553 K with decomposition. Generally, if foods are protected from light, increasing temperature does not increase its decomposition rate. Riboflavin in solution degrades rapidly when exposed to ultraviolet or visible radiation. For example, if milk is left under sunlight for 2 hours, more than 50 % of the riboflavin will be lost. Under acidic or neutral conditions, riboflavin looses the ribityl side chain to form lumichrome whereas in alkali solutions, riboflavin is photochemically converted to lumiflavin.

2.2.4 Biosynthesis

The most recent work on the biosynthesis of riboflavin has been carried out by Brown and Williamson [21]. The vitamin originates from a guanine derivative, guanosine triphosphate, whose purine ring, excepting the carbon 8, is incorporated intact into the isoalloxazine ring. The various stages of its biosynthesis are outlined in Figure 2.2. Under the action of an enzyme, GTP cyclohydrolase 11, guanosine triphosphate is converted into 2,5-diamino-oxy-4-(5'-phosphoribosylamino)-pyrimidine (PRP) which in turn undergoes deamination at C_2 and reduction of the ribosylamino group giving 5-amino-2,6-dioxy-4-(5'-phosphoribitylamino)pyrimidine (ADRAP-P). In the next stage the interaction of two molecules of the ADRAP-P gives one molecule of 6-methyl-7-(1',2'-dihydroxyethyl)-8-ribityl-lumazine (MERL) with release of one molecule of diaminouracil and of phosphate, one of the ADRAP-P molecules giving up a 5-carbon unit from its ribityl chain. The C_2 is than removed to give 6,7-dimethyl-8-ribityl-lumazine (DMRL). The conversion DMRL into riboflavin, catalyzed by riboflavin synthetase, requires two molecules of DMRL, one of which gives the other its newly acquired 4-carbon unit. One molecule of ADRAP is obtained as a side product of the reaction and this is recycled [21].

2.2.5. Microbial Process for Riboflavin Production

Microbiologically produced riboflavin has long been available in yeast and related preparations in association with many other vitamins of the B-complex. It is a unique vitamin in that it can be produced to a high concentration rather rapidly by certain microorganisms. Fermentation processes have been described in which the riboflavin content of the fermented medium amounted to more than 7

g/liter. The organisms involved in these processes are ascomycetes, namely, *Eremothecium ashbyii* and *Ashbya gossypii*. These ascomycetes are from the *Spermohtraceae* family, from the genus Eremothecium and Ashbya.

Although riboflavin is produced by many microorganisms, including bacteria, yeasts and molds, other microorganisms require riboflavin for growth. The organisms which have been found to produce sufficient riboflavin to be of interest from a commercial standpoint are listed in Table 2.3, where the yields are mentioned along with the sensitivity of the microorganism to iron [22].

Commercial fermentation processes for production of riboflavin or riboflavin concentrates are relatively recent, having been developed in the past 40 years. Aside from food yeasts, the first organisms employed primarily for riboflavin production was *Clostridium acetobutylicum* which, when grown in grain mashes or on whey low in iron, yielded residues containing 4-5 mg riboflavin per gram fermentation solids. These fermentations were succeeded in about 1940 by a process using *E. ashbyii* with yields of 2 mg/mL. In 1946 processes using the ascomycete *A. gossypii* were started.

Table 2.3 Microorganisms producing considerable amounts of riboflavin and the effects of iron on biosynthesis of the vitamin.

Microorganism	Riboflavin Yield (mg/L)	Optimum iron conc. (mg/L)
Clostridium acetobutylicum	97	1-3
Mycobacterium smegmatis	58	Not critical
Mycocandida riboflavina	200	Not critical
Candida flareri	567	0.04-0.06
Eremothecium ashbyii	2480	Not critical
Ashbya gossypii	6420	Not critical

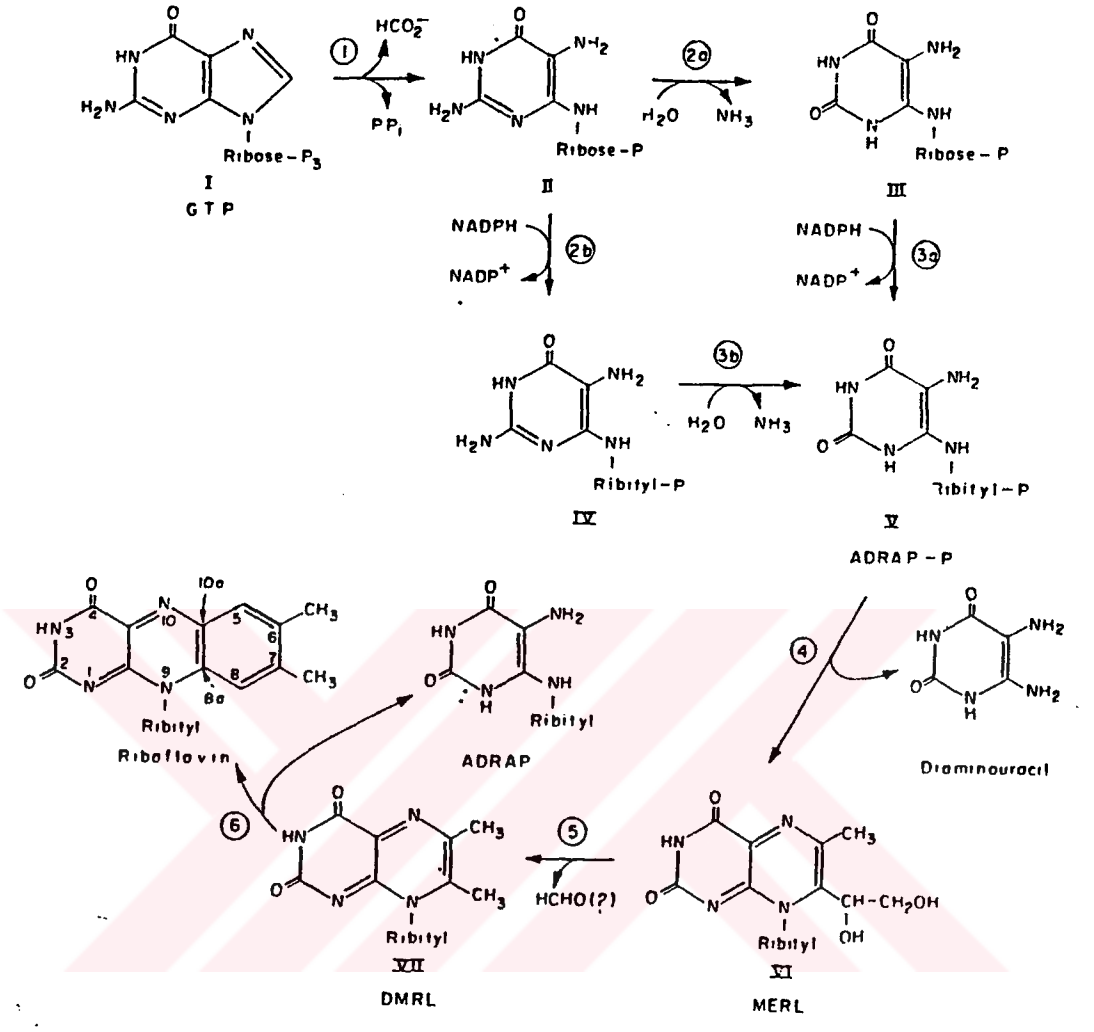


Figure 2.2 Probable pathway of riboflavin biosynthesis.

Development of the current processes used for riboflavin production by *A. gossypii* NRRL Y-1056 focused on three aspects:

1- Preparation of the culture medium.

2- Selection of mutant cultures and optimization of inoculum preparation.

3- Optimization of the fermentation conditions, e.g., incubation temperature, aeration level, and

fermentor design.

The most important increases in fermentation productivity have resulted from the changes in the culture media and the selection of high riboflavin-producing mutant cultures.

Initial studies showed that good growth of *A. gossypii* occurred in a medium containing glucose, corn steep liquor, and animal stick liquor, tankage or meat scraps [22]. It was also shown that a sterilization time of less than 30 minutes, a low concentration of young inoculum, e.g., 2 % (v/v), and efficient aeration were important.

Optimum conditions for the fermentation media have been summarized by Perlman [22]. Glucose, sucrose, and maltose are good sources of carbon but vegetable oils (soya or maize oil) are better. Peptones are recommended as nitrogen sources, especially those produced by enzymatic hydrolysis of collagen-like proteins (e.g., gelatin pancreatic hydrolysate) and corn-steep, yeast extract or distillers' solubles which also supply the vitamin factors (biotin, inositol, thiamine) required for growth. It has been shown that the addition of 1-3 g of glycine(the known precursor of guanine) per liter increases production by 10-30 %.

The optimal fermentation conditions include aeration at about one-third volume of air per volume of liquid per minute, and agitation with impellers at a rate of 1.0 hp/1000 liters of medium. When foaming is excessive, it might be controlled by initial addition of emulsified silicone antifoam, and later by addition of soybean oil, which also acted as a nutrient. Sterilization of the medium was accomplished by heating at 121°C for 3 hours (which improved productivity), and the incubation temperature was maintained at 28 °C for the 7-day

incubation period [22].

2.3 Turbidimetric Measurements

Turbidity, or optical density, is the cloudiness of a suspension. The more turbid a suspension, the less light will be transmitted through it. As bacterial cultures grow in broth, the clear liquid medium becomes turbid. Since the turbidity increases as the number of cells increases, this property can be as an indirect indicator of bacterial concentration. In the laboratory, turbidity is quantified with a spectrophotometer, an instrument that measures the amount of light transmitted directly through a sample. Some inaccuracy is unavoidable in turbidimetry since that cells and contaminating debris also block the direct passage of light.

Cultures grown overnight can be adjusted quickly by dilution to the turbidity that represents the desired concentration of cells. Turbidimetric measurements may be converted to values representing cell counts (number of cells per milliliter) by experimentally constructing a standard curve. Both turbidity and direct count of samples containing different cell concentrations are determined [23].

CHAPTER III
MATERIALS AND METHODS

3.1 Preparation of Cultures and Media

3.1.1 Cheese Whey Collection and Preparation

One lot (25 liters) of Turkish Feta cheese whey was obtained from a local cheese factory and autoclaved for 20 min at 121°C. After cooling, it was stored at 4°C until used in the experiments. Prior to being fed into fermenters, the cheese whey was removed from the refrigerator and allowed to equilibriate with room temperature. The supernatant parts of the whey was poured into flasks or fermentor.

3.1.2 Cultures

Ashbya gossypii NRRL Y-1056 known to produce riboflavin was kindly obtained from USDA, Agricultural Research service, Nat. Center for Agricultural Util. Research, University St. Peoria, USA. The culture was maintained in Malt extract agar (MEA; Difco laboratories, Detroit, MI) slants by periodic three monthly transfers and kept at -30°C. When needed the tubes were thawed and a loopful of this stock culture was streaked onto MEA slants. The tubes were then placed in an incubator at 28°C for 3 days. After visual growth appeared on the slants, they were removed from the incubator and stored in a refrigerator at 4°C until required for inoculum preparation.

The test culture, for determining the level of the riboflavin produced, in microbiological assay method was *Lactobacillus casei* ATCC 7469 was kindly obtained from USDA, Agricultural Research Service, Nat. Center for Agricultural Util. Research, University St. Peoria, USA.

The culture was maintained in Lactobacilli agar (Difco laboratories, Detroit, MI) slants. Transfers were made at twice monthly intervals and stored at -30°C. When needed, the culture was thawed, inoculated into 10 mL Lactobacilli broth and incubated for 24 hour at 35°C. All of the media were sterilized for 15 min at 121°C.

3.1.3 Fermentation media

In this study the production of riboflavin was carried out by using cheese whey as a fermentation medium with or without supplements. Supplements were added into cheese whey in order to increase the riboflavin yield in both shake flask and 1.5 liter jar fermentor MBF (Eyela, Tokyo Rikakikai Co., Ltd., Tokyo) studies. For shake flask studies the whey was used alone and also the following supplements were added to the whey:

(1) Soybean oil, as an antifoam and carbon source 1 % (v/v).

(2) Yeast extract, as a nutrient source 1 % (w/v).

(3) Sucrose, as a carbohydrate source 1 % (w/v).

(4) Glycine, known precursor of guanine, which is the first step in riboflavin synthesis 0.1 % (w/v).

(5) Peptone, as a nitrogen source 1 % (w/v).

(6) Glycine and peptone.

(7) Soybean flour, as a protein source 1 % (w/v).

(8) Bran, as a protein and mineral source 1 % (w/v).

For the 1.5 L fermentor studies, fermentations were conducted for each of the following substrate conditions:

(1) Whey alone.

(2) Whey + 0.1% (w/v) glycine + 1% (w/v) peptone.

(3) Whey + bran 1% (w/v).

The pH of fermentation media was adjusted to 5.1 by

using NaOH or tartaric acid. The fermentations were carried for 8 days at 28°C. The fermentation media were sterilized for 30 min at 121°C.

3.2 Fermentation

3.2.1 Activation of *Ashbya gossypii*

The culture was activated by inoculating it into 100 ml Malt extract broth and incubating for 72 hours at 28°C.

3.2.2 Shake Flask Studies

Two hundred ml of supernatant of cheese whey and required supplements were taken into 250 mL erlenmayer flasks and sterilized for 30 min at 121°C . They were inoculated with 2% (v/v) 3 days old *A. gossypii* and allowed to grow in dark, in a bench type water bath shaker ST-402 (NÜVE, Sanayi ve Malzemeleri Imalat ve Ticaret A.Ş., Istanbul) with 55 rpm wrist-shaking at 28°C for 8 days. Periodic samples were removed daily, under aseptic conditions and analyzed for riboflavin content.

3.2.3 Fermentor Studies

After shake flask studies, best riboflavin producing media were selected and studied in a 1.5 L laboratory fermentor. One liter of cheese whey supernatant and required supplements were added into fermentor, and sterilized as in shake flask studies. Then 2% (v/v) 3 days old *A. gossypii* was inoculated, and allowed to fermentationat 28 °C for 8 days. Aeration was provided by means of a gas sparger and maintained at 0.1 NL/min. The fermentation media was agitated by a stirrer with a speed of 300 rpm. The sampling was done by sterile pipettes under aseptic conditions. The foaming was prevented by using 1 % (v/v) soybean oil as an antifoaming agent.

3.2.4 Sampling

At every 24 hour interval, 5 mL aliquots of fermentation medium were taken into sterile test tubes by means of sterile pipettes under aseptic conditions. Riboflavin is present both in solution and bound to the mycelium in the fermentation broth. In order to release the bound vitamin, sample tubes were heated for 1 hour at 120°C [18]. After heating, the mycelium was separated by centrifugation (Hattich Roto Silenta II) for 15 minutes at 8000 rpm. The supernatant was then used to detect the level of riboflavin produced by *A. gossypii*.

3.3 Analyses

Riboflavin analyses were made according to the microbiological assay methods described in AOAC [3] and Difco [4].

The growth of microbes provides one of the most sensitive indicators the presence and concentrations of many chemical compounds. Because these assays use living organisms as indicators, they are known as bioassays. The selective test bacterium will grow only in the media that contain the vitamin. When the vitamin has been consumed, bacterial growth will cease. The amount of microbial growth will therefore be proportional to the concentration of the limiting vitamin as long as all other nutrients are supplied in excess. Thus a solution containing 0.2 microgram/mL of the vitamin will allow the production of twice as many bacteria as one containing 0.1 microgram/mL. A standard curve that relates vitamin concentration to total growth is experimentally constructed. Then by measuring total bacterial growth in a vitamin-free medium supplemented with a standard amount of the substance to be tested, the concentration of vitamin can be determined in any unknown materials, such as vitamin pills, cereal, or

other foods. The concentrations of many nutrients listed on food labels are often determined by bioassay [23].

3.3.1 Preparation of Tubes for Standard Curve

It is essential that a standard curve be constructed each time an assay is run, since conditions of autoclaving, temperature of incubation, etc., which influence the standard curve readings, can not be duplicated exactly from time to time (Appendix Figure A.1).

The standard curve was obtained by using riboflavin (Sigma) at levels of 0.0, 0.025, 0.05, 0.1, 0.15, 0.2, and 0.3 microgram riboflavin per assay tube.

The concentration of riboflavin adjusted for the preparation of the standard curve was prepared by dissolving 0.1 g of riboflavin in 1000 mL of distilled water by heating, giving a stock solution of 100 microgram per mL. The stock solution was diluted by adding 1 mL to 999 mL distilled water. From this diluted stock solution 0.0, 0.25, 0.5, 1, 1.5, 2, 3 mL aliquots per tube were taken and adjusted to the 10 mL final volume by adding 5 mL riboflavin assay medium and sufficient distilled water.

The tubes were sterilized by autoclaving for 5 min at 121°C. After sterilization the tubes were cooled quickly in cold water.

3.3.2 Preparation of Tubes for Test Sample Curve

The tubes were prepared in a similar manner to standard curve, except sample solution was added at appropriate volumes in place of standard riboflavin solution. If required, in order to maintain the turbidity readings in the standard curve readings range, the assay solution could be diluted into appropriate dilutions.

3.3.3 Inoculation of *Lactobacilli casei*

Inoculum for assay was prepared by subculturing from a stock culture of *L. casei* into 10 ml of Lactobacilli broth. Following incubation for 24 hours at 35°C, the cell suspension was diluted to 1:10 with sterile water. One drop of this diluted suspension was then used to inoculate each of the assay and standard curve tubes.

3.3.4 Incubation

The assay tubes and standard curve tubes which were inoculated with *L. casei* were then incubated at 35 °C for 24 hours.

3.3.5 Results

Light transmissions were measured using a spectrophotometer (Pharmacia LKB Biotech, NovaspecII, Cambridge, England) at 600 nm wavelength. The standard curve was constructed by plotting % light transmissions versus vitamin concentrations (mg/L). Then the % light transmissions of the assay tubes were read at the same wavelength. From standard curve, by using the % light transmissions of the assay tubes, the corresponding vitamin concentrations were obtained.

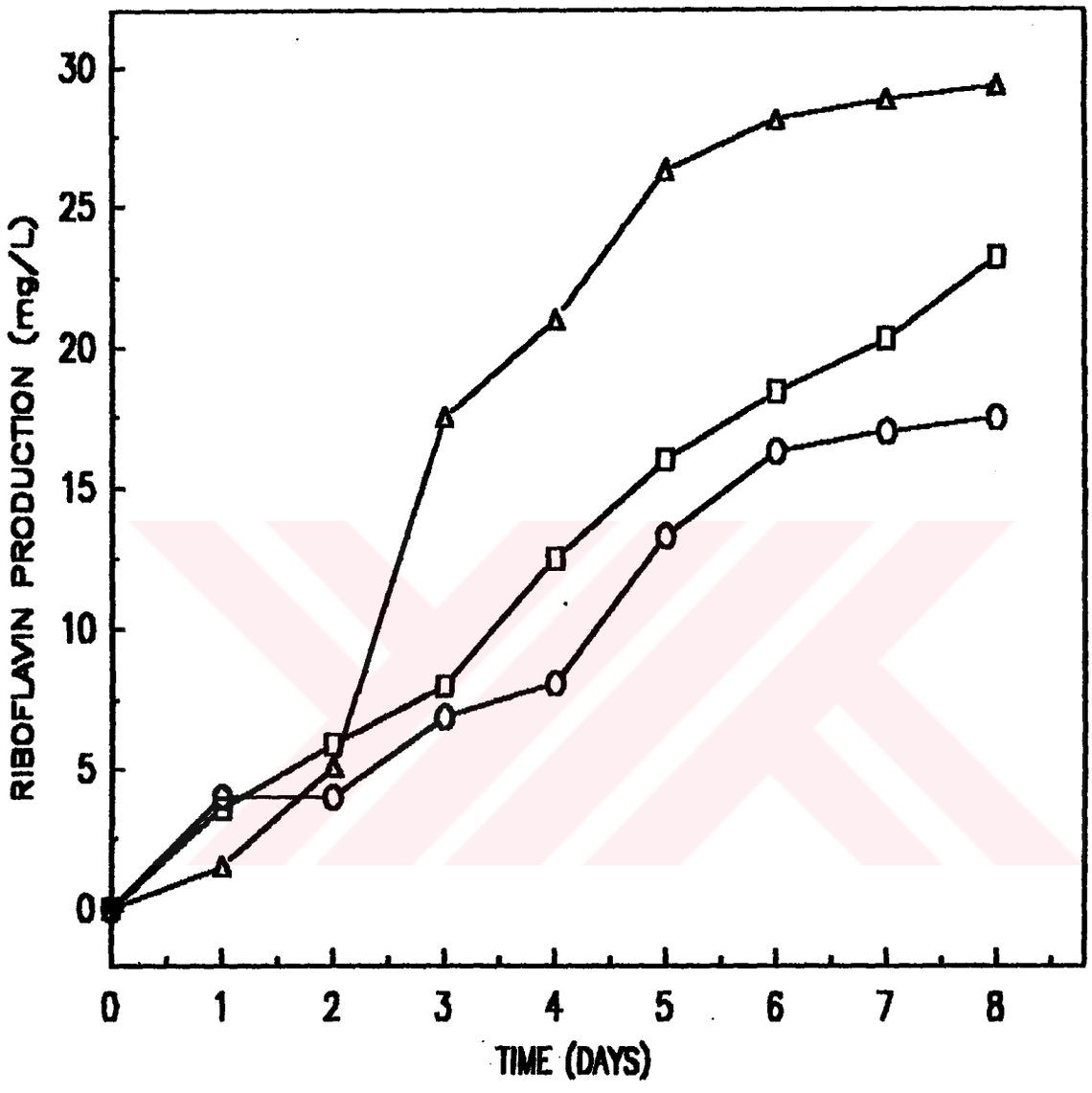
CHAPTER IV
RESULTS AND DISCUSSIONS

In this study experiments were carried out in shake flask and fermentor studies to produce riboflavin in only cheese whey and supplementation of cheese whey by using *A. gossypii*.

4.1 Effect of Time

Fermentation studies on riboflavin production during 8 days by *A. gossypii* NRRL Y-1056 in only whey and with different supplements in shake flask studies are given in Figures 4.1-3. By the use of linear curve fitting method, it was easily seen that the riboflavin production rate increased more rapidly after 2 days of fermentation and continued to increase until 5 days for all the fermentation media with a few exceptions (Table 4.1). But there was a decceleration in the rate between the days 5 to 8 which was considered to be stationary phase. For the first 2 days of fermentation there was a lag period in the riboflavin production and is consistent with literature [24]. Product formation started during the first 2 days, attained a maximum level after about 5 days and continued to increase slowly up to 8 days of fermentation (Appendix Table A-1).

In natural fermentation of corn meal, however, Murdock and Fields [25], found that during lactic acid fermentation of corn meal, riboflavin declined between days 3 and 4. This indicates the presence of microorganisms requiring vitamin for growth. Chung and Fields [26] found that, there was no decline in vitamin concentration in fermentation of corn meal by *Bacillus megaterium* ATCC 13639. The results obtained from



gure 4.1. Production of riboflavin by *Ashbya gossypii* from ey, and whey supplemented with 1% (v/v) soybean oil and 1% /v) peptone in shake flask studies. (O = whey + soybean oil; = whey + peptone; Δ = whey)

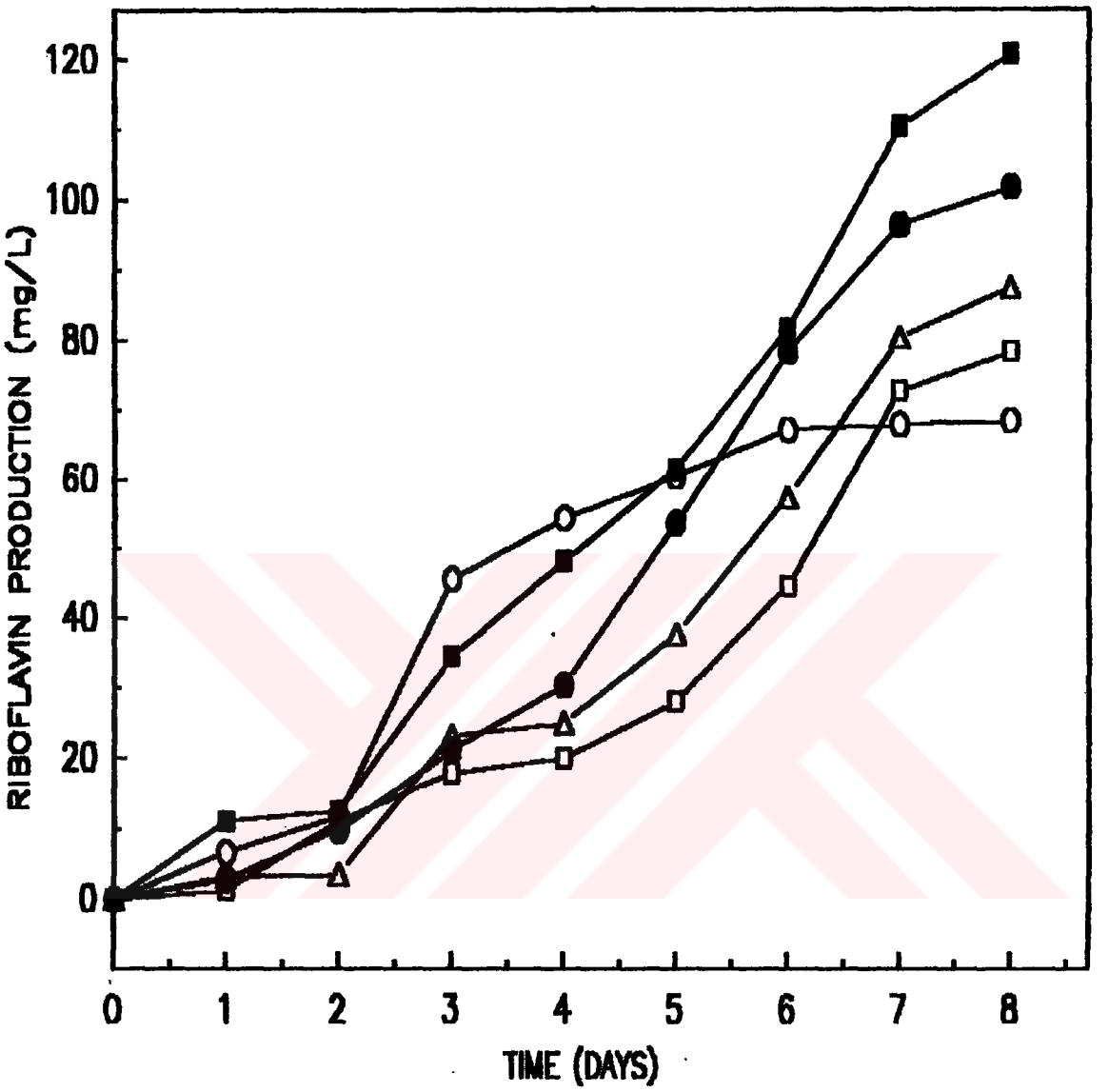


Figure 4.2. Production of riboflavin by *Ashbya gossypii* from whey supplemented with 1% (w/v) yeast extract, 0.1% (w/v) glycine, 1% (w/v) sucrose, 0.1% (w/v) glycine + 1% (w/v) peptone, and 1% (w/v) soybean flour in shake flask studies. (O = whey + yeast extract; □ = whey + glycine; ▲ = whey + sucrose; • = whey + glycine + peptone; ■ = whey + soybean flour)

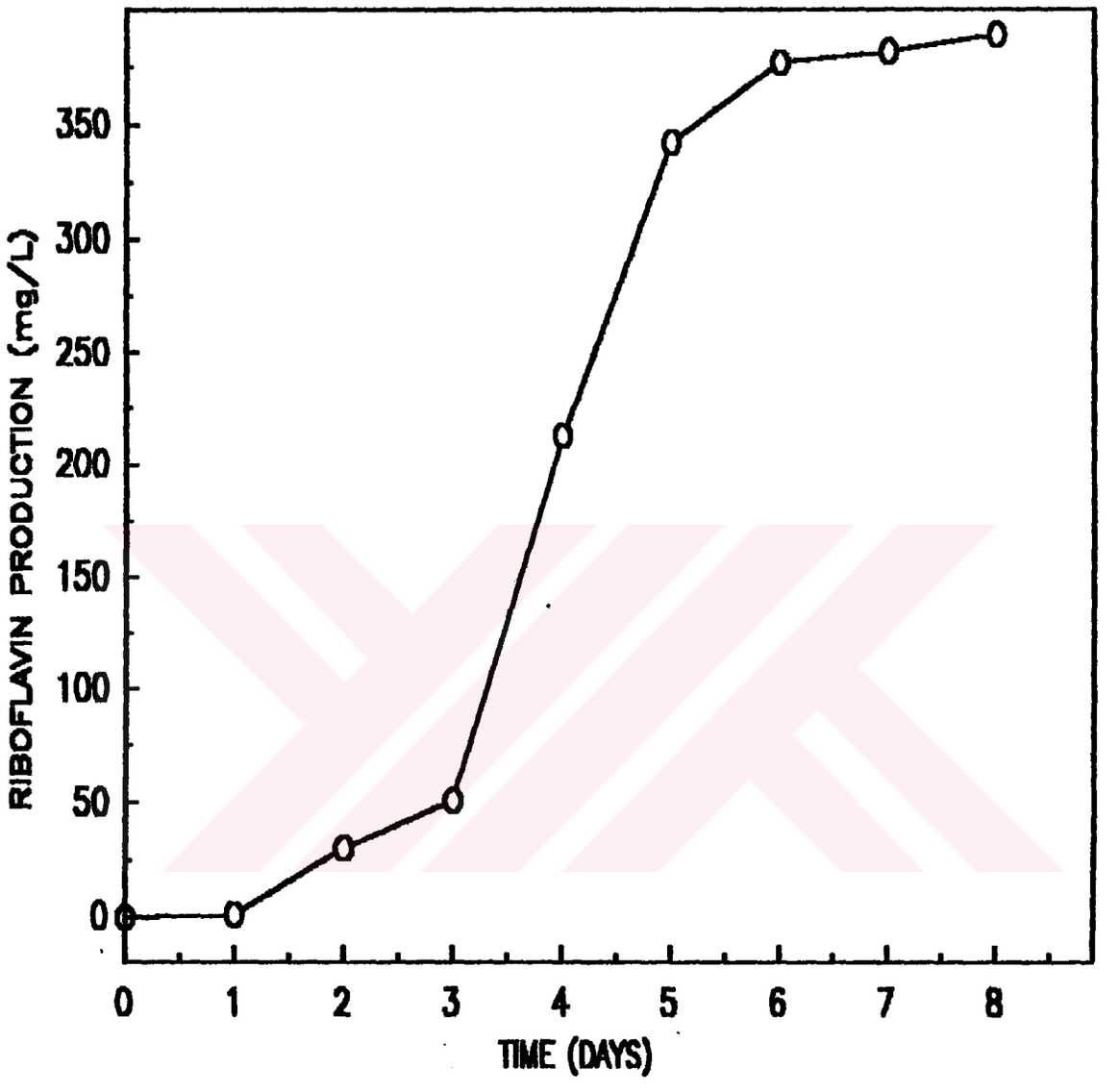


Figure 4.3. Production of riboflavin by *Ashbya gossypii* from whey supplemented with 1% (w/v) bran in shake flask studies.

Table 4.1. Production rates (mg/L.day) of riboflavin by *Ashbya gossypii* at various time intervals of fermentation process.

	0-2 Days		2-5 Days		5-8 Days	
	R^2	Slope	R^2	Slope	R^2	Slope
Whey[1]	0.95	2.55	0.92	6.71	0.91	0.97
Soybean Oil[1]	0.75	2.00	0.93	2.91	0.83	1.33
YeastExtract[1]	0.99	5.90	0.85	15.46	0.73	2.48
Glycine[1]	0.84	5.50	0.96	5.31	0.95	17.90
Sucrose[1]	0.83	1.80	0.91	10.38	0.96	17.26
Peptone[1]	0.98	2.95	0.98	3.48	0.99	2.35
Gly+Pep[1]	0.95	4.90	0.95	14.10	0.93	16.21
SoybeanFlour[1]	0.83	6.25	0.98	16.02	0.97	20.68
Bran[1]	0.78	15.00	0.93	109.83	0.81	14.64
Whey[1,2]	0.88	10.20	0.99	20.85	0.95	9.95
Gly+Pep[1,2]	0.83	21.20	0.93	56.40	0.91	60.90
Bran[1,2]	0.90	62.70	0.93	218.70	0.95	181.92

* 1 indicates whey+supplements
* 2 fermentor studies.

Table 4.2. Comparison of riboflavin production yield (mg/L) between shake flask and fermentor studies.

Days	Whey[1]	Gly+Pep[1]	Bran[1]	Whey[2]	Gly+Pep[2]	Bran[2]
0	0.0	0.0	0.0	0.0	0.0	0.0
1	1.5	2.9	1.2	3.7	4.3	27.3
2	5.1	9.8	30.0	20.4	42.4	125.4
3	17.5	21.1	51.3	47.6	89.3	253.2
4	21.0	30.4	213.0	63.8	118.4	648.3
5	26.3	53.7	342.2	84.5	220.7	722.7
6	28.1	78.3	377.2	97.3	316.3	845.2
7	28.8	96.4	382.0	110.1	387.4	1150.3
8	29.3	101.7	389.4	113.4	400.0	1227.4

* 1 indicates whey+supplements
* 2 fermentor studies.

riboflavin prodution during fermentation by *A. gossypii* on whey showed no declination.

4.2 Relative Yields

Yields do not account for total solids loss due to microbial activity; therefore, the values are not absolute but are relative yields of riboflavin.

The quantities of riboflavin produced by *A. gossypii* (at pH 5.1, 28°C, for 8 days) in whey with different supplements; bran, soybean flour, glycine+peptone, sucrose, glycine, yeast extract, peptone and soybean oil were 389.5, 120.7, 101.7, 87.5, 78.3, 68.4, 23.2 and 17.5 mg/L respectively. The quantities of riboflavin produced with only whey was 29.2 mg/L. Özbaş and Kutsal [27] studied the conversion of various substrates; glucose, glycerol, sunflower oil, whey and various combinations of these to riboflavin yield for whey at various pH values and temperatures by using *A.gossypii*. They found 170 mg/L riboflavin production at 28 °C and pH 6.5. This riboflavin production rate is about 6 times higher than the our results. Because in present work the riboflavin production was carried out at pH 5.1. Özbaş and Kutsal reported that *A.gossypii* has a optimum pH value of 6.5.

Riboflavin production in whey supplemented with bran was nearly four times higher than other supplements (Figures 4.4-6). This riboflavin quantity with bran supplementation was also four times higher than the vitamin produced in fermentation of molasses and lentils (84 mg/L) by *Eremothecium ashbyi* [24]. The wheat mill bran is rich in various mineral composition, pentosans, starch, total sugar, sucrose, and reducing sugar [28]. Increase in riboflavin production on bran supplemented whey might be explained by high mineral and sugar composition of bran.

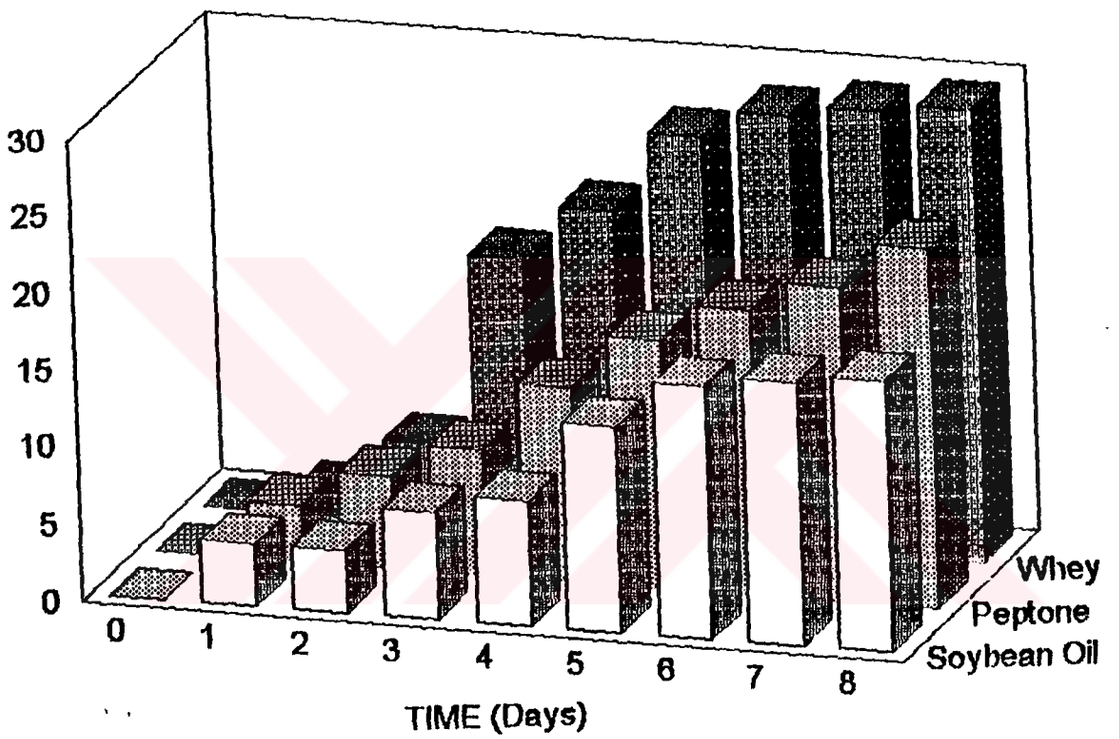
The maximum yield of 17.5 mg/L was obtained at the

end of fermentation in whey with 1 % soybean oil. But the yield was lower than the yield of only whey (Figure 4.4). Ghanem et al [29] found that riboflavin production was appreciably enhanced in the presence of the corn oil by *Candida guilliermondii*, (about 13 % increase), but use of cotton seed oil, olive oil and some fatty acids showed no stimulatory effect. Cruger and Cruger [18] reported that soybean oil is metabolized by *A. gossypii* during fermentation and with a fermentation medium of corn steep liquor 2.25 %, commercial peptone 3.5% and soybean oil 4.5 % with a yield of 10-15 g/L [18]. Özbaş and Kutsal [27] reported that the sunflower oil was increased the yield of riboflavin when it was added into fermentation media.

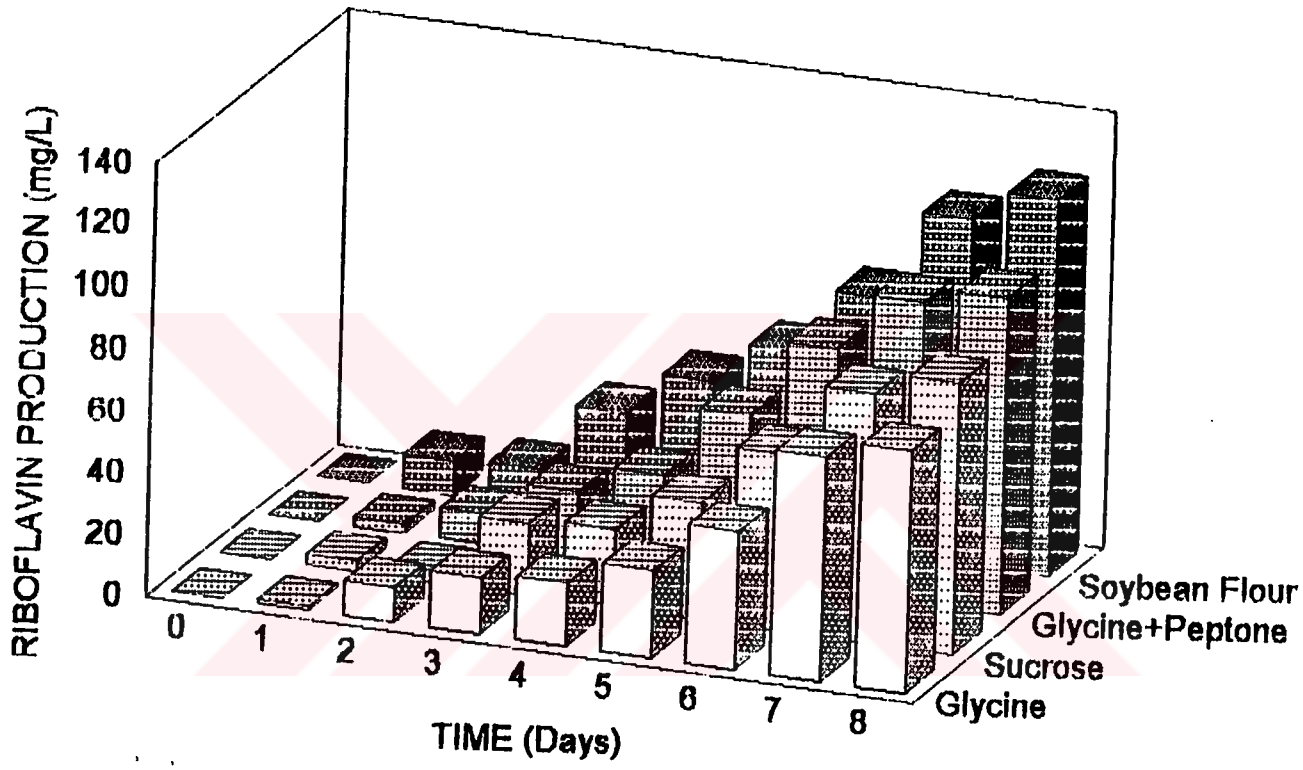
The effect of glycine+peptone on riboflavin production is given in Figure 4.5. The yield was greater than the yields when glycine (Figure 4.5.) and peptone (Figure 4.4.) added individually. Typical data on stimulation of riboflavin production as a result of including various peptones and glycine in the media are summarized by Perlman [22] who reported 3620 and 4200 mg/L riboflavin yield for A. gossypii NRLL Y-1056 from fermentation media supplemented with peptone and peptone+glycine respectively.

Riboflavin production in the laboratory fermentor (at pH 5.1, 28 °C, with a stirrer speed of 300 rpm, with an aeration rate of 0.1 NL/min, for 8 days) with only whey and supplements; bran and glycine+peptone were 113.2, 1227.4, 407.4 mg/L respectively and given in Figures 4.7-9. Riboflavin produced by *A. gossypii* with only whey and different supplements after 8 days of fermentation in a 1.5 liters laboratory fermentor were found to be four times higher than that of found in shake flask studies (Table 2).

The increase in the yield of riboflavin in the



gure 4.4. Comparison of riboflavin production from whey and whey pplemented with 1% (w/v) peptone and 1% (v/v) soybean oil by *Ashbya ssypii* in shake flask studies.



igure 4.5. Comparisoh of riboflavin production from whey supplemented ith 1% (w/v) soybean flour, 0.1% (w/v) glycine+1% (w/v) peptone, 1% w/v) sucrose and 0.1% (w/v) glycine by *Ashbya gossypii* in shake flask tudies.

fermentor studies with 300 rpm can be explained by the fact that agitation gives rise to greater oxygen transfer rates and better distribution of oxygen in the system. Prabhakar et al. [24] has found that riboflavin production was reached to higher levels with 300 rpm stirrer speeds.

Not only the detailed growth rate including the death phase but also the effect of parameters like temperature and pH on the fermentation needs to further experimentation. According to the results *A. gossypii* NRRL Y-1056 has potential for the production of riboflavin in only whey and with natural supplements; bran and soybean flour.

4.3 Economic Significance

As it was discussed before, Turkey is importing about 25.000 kg riboflavin from foreign countries [19]. Whey is produced in our country in large amounts (about 2.000.000 tons/year) and only a few percent of it is being used in whey powder production. Remaining part of it is discarded. In this study, 29.3 and 113.4 mg/L of riboflavin were produced from whey in shake flask and fermentor studies by using *A.gossypii* respectively. If all of the whey is used for riboflavin production, about 60.000 and 220.000 kg of riboflavin can be produced respectively. This will be 2 and 4 times higher than riboflavin requirements of our country. This means that about 1.500.000 USD will be annually saying from importation expenses.

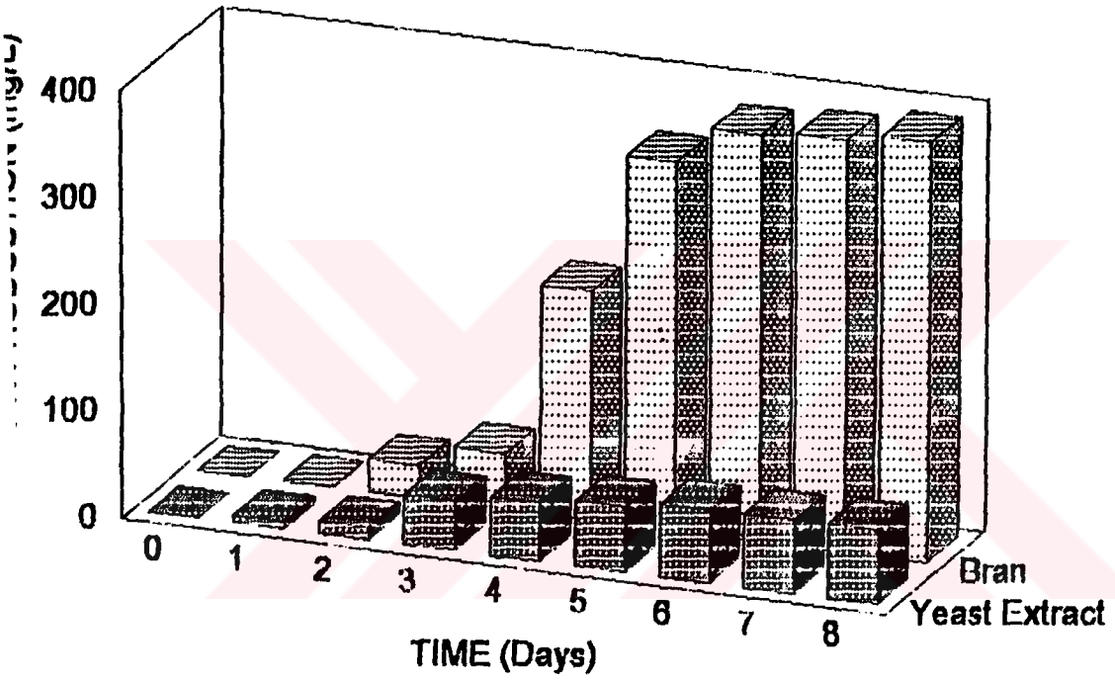


Figure 4.6. Comparison of riboflavin production from whey supplemented with 1% (w/v) bran and 1% (w/v) yeast extract by *Ashbya gossypii* in shake flask studies.

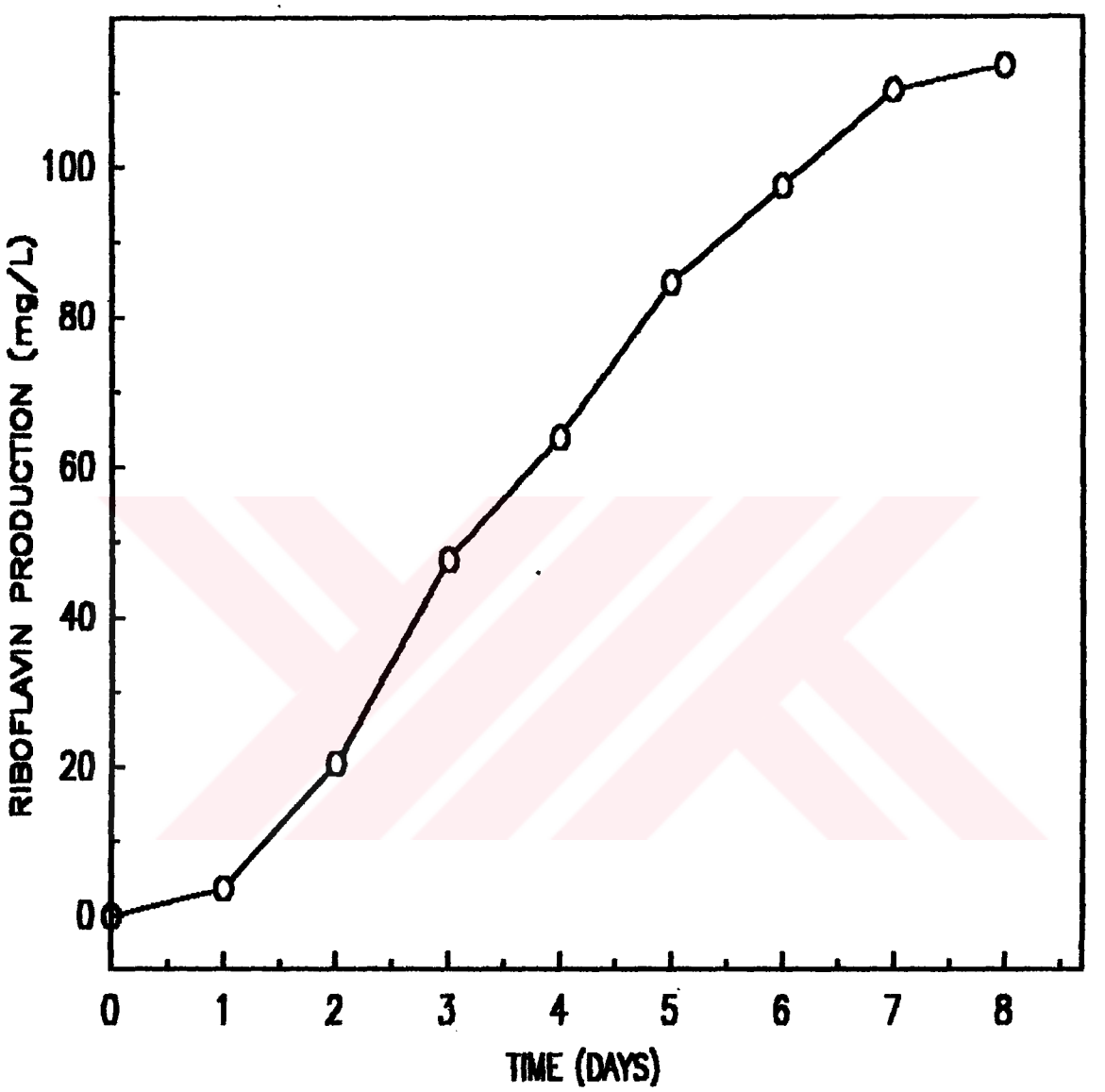


Figure 4.7. Production of riboflavin by *Ashbya gossypii* from whey in fermentor studies.

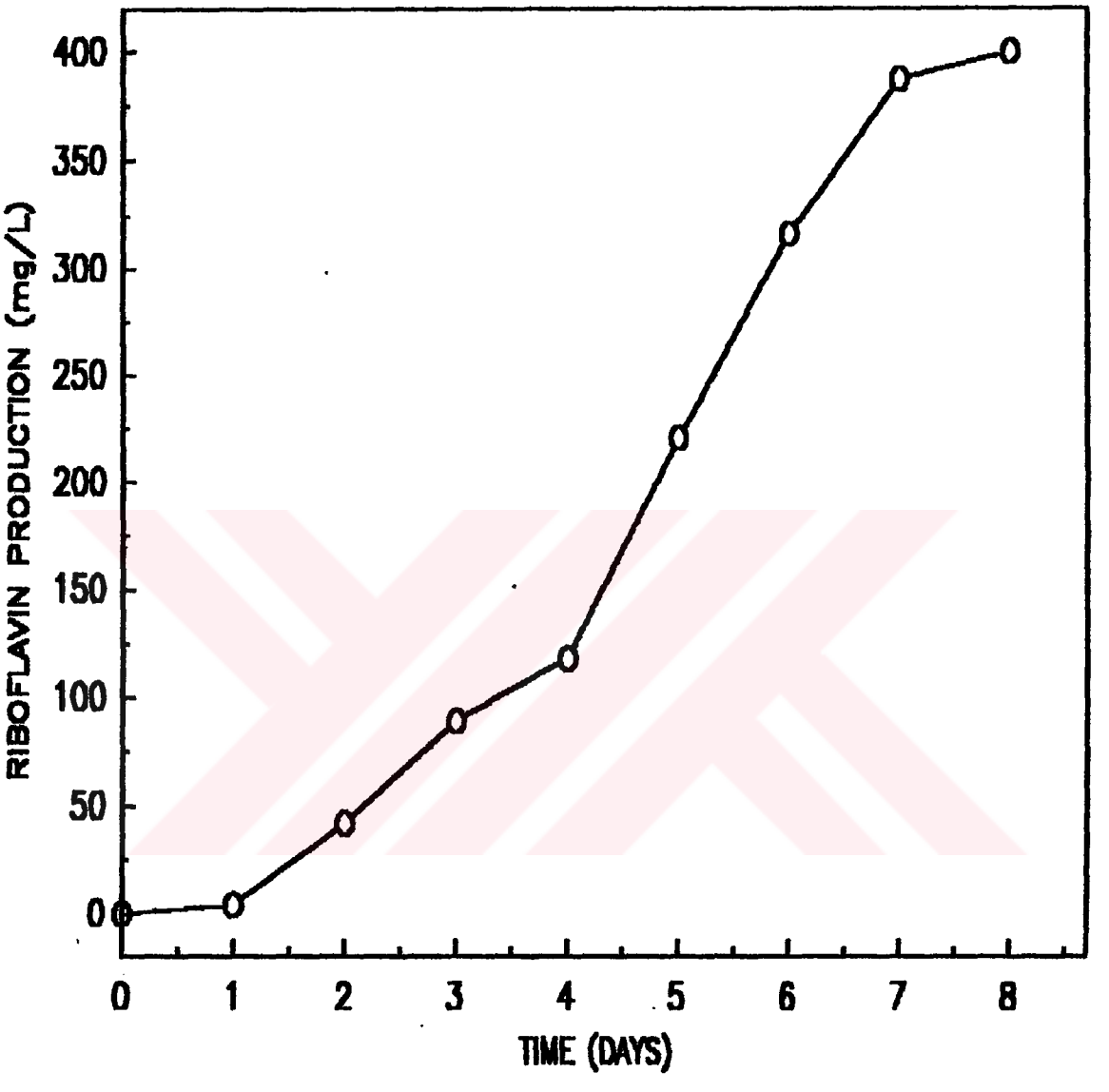


Figure 4.8. Production of riboflavin by *Ashbya gossypii* from whey supplemented with 0.1% (w/v) glycine+1% (w/v) peptone in fermentor studies.

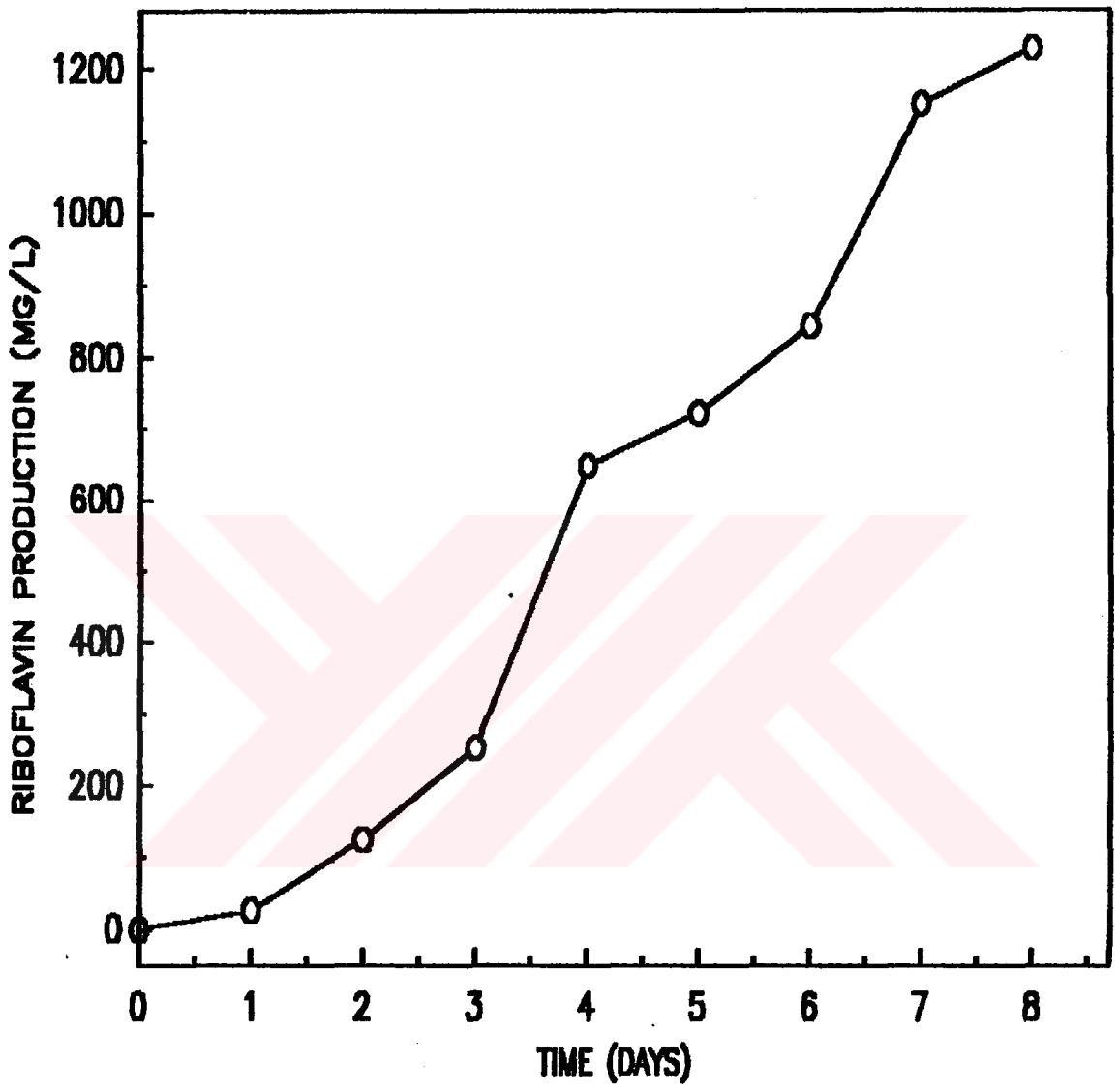


Figure 4.9. Production of riboflavin by *Ashbya gossypii* from whey supplemented with 1% (w/v) bran in fermentor studies.

CONCLUSION

Eventhough about 6 chemical routes are available for the synthesis of riboflavin, a substantial amount (30%) of vitamin is being produced by fermentation [18].
Previously, considerable research has been carried out to identify the nutritional requirements of *A. gossypii* and almost all of them used pure chemicals-glucose, mannose, sucrose, as the carbon source and peptone, yeast extract, casein as the nitrogen source. As these synthetic media are expensive, there is a need to look for an alternative, natural media for the synthesis of the riboflavin.

1. The quantities of riboflavin produced by *A.gossypii* on whey after 8 days of fermentation in shake flask and fermentor studies were 29.2 and 113.2 mg/L respectively.

2. Riboflavin production by *A. gossypii* with only whey and different supplements in fermentor studies were found to be four times higher than the shake flask studies.

3. The best yield was obtained by using of 1 % bran which is another agroindustrial byproduct has a yield of riboflavin nearly four times higher than only whey and other supplements.

4. By using two industrial byproducts, whey and bran, the production costs of riboflavin may be lowered to considerable amounts.

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APPENDICES

Table A-1. Results of riboflavin productions in whey and various whey supplemented with various ingredients in both shake flask and fermentor studies.

Days	Whey[1]	Soy Bean Oil[1]	Yeast Ext.[1]	Glycine[1]	Sucrose[1]	Peptone[1]
0	0	0	0	0	0	0
1	1.5	4.0	6.7	1.3	3.2	3.6
2	5.1	4.0	11.8	11.0	3.6	5.9
3	17.5	6.9	45.6	17.9	23.2	8.0
4	21.0	8.1	54.4	20.0	25.0	12.5
5	26.3	13.3	60.4	28.0	37.6	16.0
6	28.1	16.3	67.1	44.6	57.4	18.4
7	28.8	17.0	67.9	72.7	80.3	20.3
8	29.3	17.5	68.4	78.3	87.5	23.2

Days	Gly+Pep[1]	Soy Bean Flour[1]	Bran[1]	Whey[2]	Gly+Pep[1,2]	Bran[1,2]
0	0	0	0	0	0	0
1	2.9	11.1	1.2	3.7	4.3	27.3
2	9.8	12.5	30.0	20.4	42.4	125.4
3	21.1	34.4	51.3	47.6	89.3	253.2
4	30.4	48.2	213.0	63.8	118.4	648.3
5	53.7	61.3	342.2	84.5	220.7	722.7
6	78.3	81.7	377.2	97.3	316.3	845.2
7	96.4	110.3	382.0	110.1	387.4	1150.3
8	101.7	120.7	389.4	113.4	400.0	1227.4

* 1 indicates whey+supplements
* 2 fermentor studies.

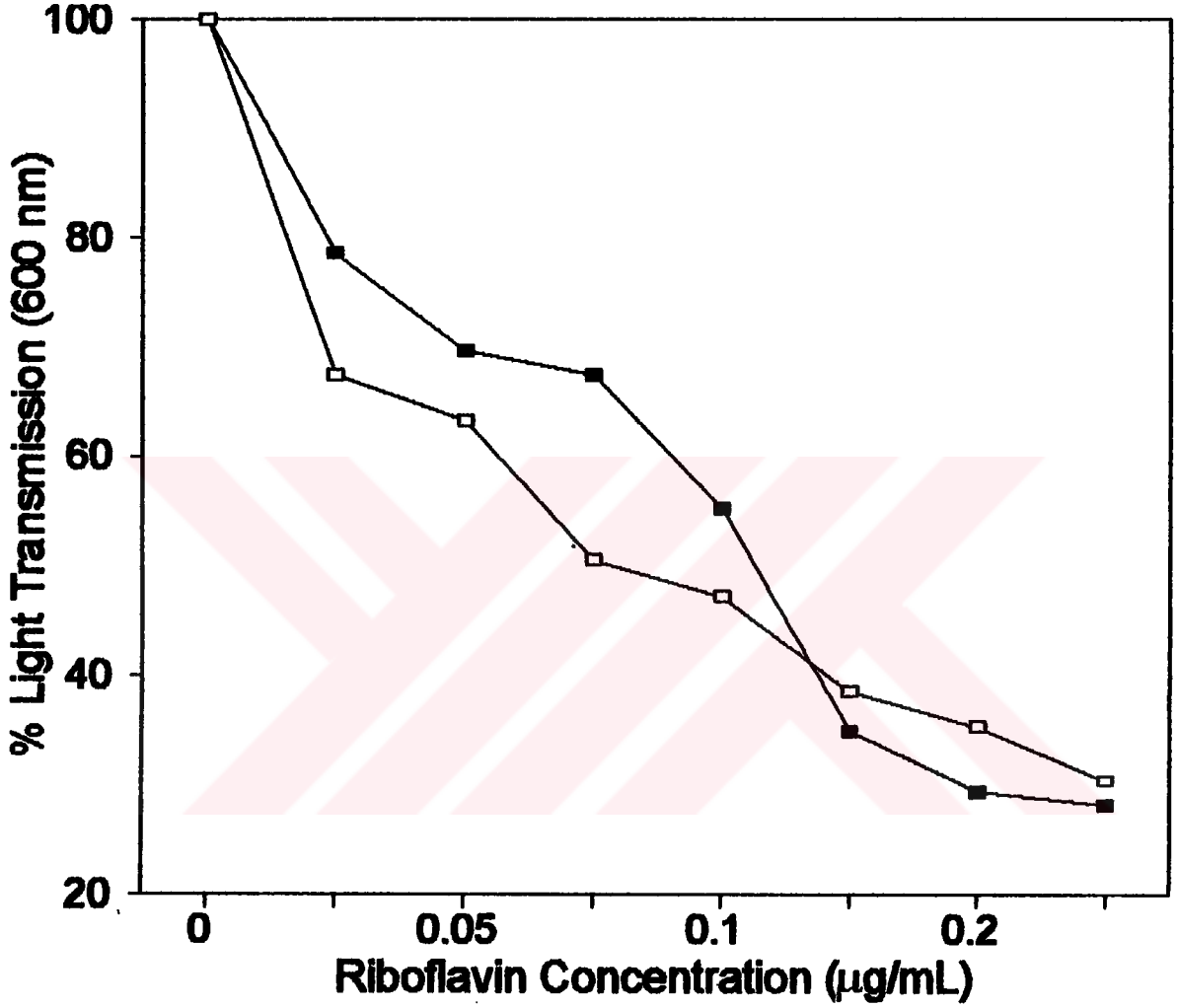

Figure A-1. Examples of two standard curves prepared at two different sampling time in order to determine the riboflavin content in the assay samples.