YIELD AND KINETICS OF RIBOFLAVIN PRODUCTION BY *Ashbya gossypii*

A MASTER'S THESIS

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by

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Approval of the Graduate School of Natural and Applied Sciences.

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ABSTRACT

YIELD AND KINETICS OF RIBOFLAVIN PRODUCTION BY *Ashbya gossypii*

GÖK, İLKAY

M.Sc in Food Engineering

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In this research, effects of pH, different substrates and concentrations on riboflavin production by *Ashbya gossypii* NRRL Y-1056 in shake flasks and fermentor were studied. During fermentation; pH, riboflavin, sugar and biomass concentration were determined daily. Four pretreatment methods to release bound riboflavin were compared for riboflavin determination and no significant differences were observed. Modeling of growth, and riboflavin production were done and from obtained models, specific growth rate (μ), maximum specific growth rate (μ_{max}), product formation kinetic parameters, and carbon yields were calculated.

Glucose concentration of 20 (g/L) was found to be optimum for riboflavin production. When lactose, galactose and glucose used as substrate in shake flasks, 0.0172, 0.0096 and 0,00973 (g/L) riboflavin concentration were obtained respectively. Riboflavin production was 5 times higher in fermentor than shake flask studies when glucose was used as substrate.

Approximately 10-20% of the substrate carbon was used for growth and riboflavin production. Boltzman model was found to be a suitable model to fit growth and product formation data of *Ashbya gossypii.* Maximum specific growth rates were found to be 0.0193 and 0.0197 (hr^{-1}) on 20 (g/L) lactose and glucose in shake flasks respectively. However, maximum specific growth rate increased to 0.0461(hr^{-1}) on 20 (g/L) glucose in fermentor studies. Based on growth, riboflavin production, sugar

consumption and calculated product formation kinetic data, riboflavin production with *Ashbya gossypii* was found to be mainly growth associated.

Key words: *Ashbya gossypii* , riboflavin production, modeling, yield, kinetic parameters

ÖZET

Ashbya gossypii İLE RİBOFLAVİN ÜRETİMİNİN VERİMİ VE KİNETİĞİ

GÖK, İlkay

Yüksek Lisans Tezi , Gıda Mühendisliği Anabilim Dalı

Tez Yöneticisi: Prof. Dr. Mehmet D. Öner

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Bu çalışmada sallamalı flask ve fermentörde *Ashbya gossypii* NRRL Y-1056 ile riboflavin üretiminde pHnın, farklı substrat ve konsantrasyonun etkileri incelendi. Fermentasyon esnasında pH, riboflavin, şeker ve biyokütle konsantrasyonu belirlendi. Riboflavin analizinde bağlı vitamini serbest bırakmak için dört değişik önişlem karşılaştırıldı ve aralarında önemli bir fark gözlenmedi. Mikroorganizmanın üreme ve riboflavin üretim modellemesi yapıldı ve bu modellerden özgül üreme hızı (μ), maksimum özgül üreme hızı (μ_{max}), ürün oluşumu kinetik parametreleri ve karbon verimleri hesaplandı.

Riboflavin üretimi için optimum glukoz konsantrasyonun 20 (g/L) olduğu bulundu. Sallamalı flasklarda laktoz, galaktoz ve glukoz substrat olarak kullanıldığında sırasıyla 0.0172, 0.0096 ve 0.0973 (g/L) riboflavin konsantrasyonu elde edildi. Glukozun substrat olarak kullanıldığı fermentör çalışmalarında riboflavin üretimi sallamalı flasklardan 5 kat daha yüksek bulundu.

Substrat karbonunun yaklaşık %10-20 si üreme ve riboflavin üretimi için kullanıldı. *Ashbya gossypii* nin üreme ve ürün oluşum verilerine Boltzman modelinin uygun olduğu bulundu. Laktozun ve glukozun 20 (g/L) substrat olarak kullanıldığı sallamalı flask ortamlarında maksimum özgül üreme hızının sırasıyla 0.0193 ve 0.0197 (sa^{-1}) olarak bulundu. Bununla birlikte, 20 (g/L) glukozun substrat olarak kullanıldığı fermentör çalışmalarında maximum özgül üreme hızı 0.0466 (sa^{-1}) ya yükseldi. Üreme, riboflavin üretim ve şeker tüketim verileri ve hesaplanan ürün oluşum kinetik

parametrelerine dayanarak *Ashbya gossypii* ile riboflavin üretiminin temel olarak üremeye bağlı olduğu bulundu.

Anahtar Kelimeler: *Ashbya gossypii*, riboflavin üretimi, modelleme, verim, kinetik parametreler

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ABBREVIATIONS

SF.	:	Shake flask run
F.	:	Fermentor run
P	:	Product concentration (g/L)
P_C	:	Product carbon concentration (g/L)
S	:	Substrate concentration (g/L)
S_{C0}	:	Initial substrate carbon concentration (g/L)
S_C	:	Substrate carbon concentration (g/L)
X	:	Biomass concentration (g/L)
X_C	:	Biomass carbon concentration (g/L)
μ	:	Specific growth rate (hr^{-1})
μ_{max}	:	Maximum specific growth rate (g/L)

CHAPTER 1
INTRODUCTION

Riboflavin has the chemical name 7,8-dimethyl-10-D-ribitylisoalloxazine, $C_{17}H_{20}N_4O_6$, mol. wt. 376.37. It has a bitter taste. Aqueous solutions of riboflavin are greenish-yellow in color and exhibit an intense yellow green fluorescence with a maximum at pH 6.7-6.8 which is destroyed by acids and bases. In neutral aqueous solutions it is relatively heat-stable if protected from light. Its stability is pH dependent, being more stable under acidic conditions. Riboflavin is amphoteric and forms salts with acids and bases. No appreciable destruction occurs during the cooking of food, but exposure of milk in bottles to sunlight leads to destruction of more than half of the riboflavin in two hours. Its photochemical cleavage under alkaline conditions results in the formation of the highly reactive compound lumaflavin, which mediates the destruction of other vitamins.

Riboflavin is widely distributed in plant and animal tissues. Milk, liver, kidney, heart, egg white, and leafy green vegetables are excellent sources. Riboflavin is one of the components required for the enrichment of flour. This enriched flour, enriched bromated flour and enriched self raising flour and farina must contain at least 1.2 mg and not more than 1.5 mg of riboflavin per pound. It is also used for enrichment of bread, rolls, and other bakery products which should contain per pound not less than 0.7 mg and not more than 1.6 mg. Enriched cereals and bread contribute importantly to the riboflavin of the diet.

Riboflavin is essential for growth and reproduction of human beings and animals. It has assumed large importance as a component of feeds for poultry, swine, calves, sheep, dogs, horses, fox, mink, and other animals. Ariboflavinasis is a desease in humans caused by riboflavin deficiency.

Riboflavin is produced industrially by several process. Direct fermentation is one of the these process (about 30% of world-wide production) used to produce riboflavin by microorganisms.

Riboflavin is a unique vitamin in that it can be totally synthesized to very high concentrations rather rapidly by certain microorganisms including yeast, yeast like microbes, and bacteria. Commercial fermentation process for production riboflavin or riboflavin concentrates are relatively recent, having been developed in the past 40 years.

Thus the aim of this research is to synthesize riboflavin by using *Ashbya gossypii*, calculate the yield and kinetic parameters of riboflavin production, determine the effects of pH, substrate and concentrations on product formation yield.

Modeling of growth, riboflavin production were done and model equations were determined to describe behavior of microorganisms.

CHAPTER 2

LITERATURE REVIEW

2.1. Riboflavin

2.1.1. Early studies on riboflavin

Riboflavin (Vitamin B_2, vitamin G, lactoflavin, ovoflavin, lyochrome, hepatoflavin, uroflavin) has the chemical name 7,8-dimethyl-10-D-ribitylisoalloxazine, $C_{17}H_{20}N_4O_6$, mol. wt. 376.37 [1].

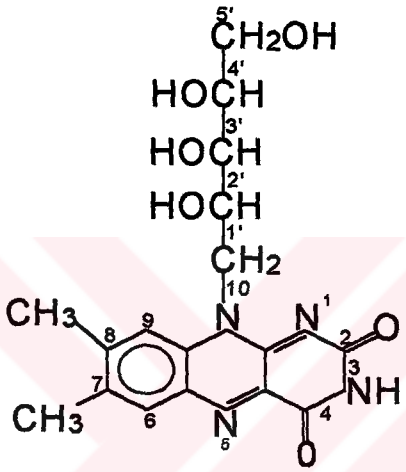

Figure 1. Riboflavin [1].

Early in the twentieth century it was found that certain unidentified dietary factors were necessary for the growth of rats. In 1916, McCollum and Kennedy suggested the designation of water-soluble B for one of these factors. At that time, it was not known whether the water-soluble growth substance for rats was identical with the antiberiberi vitamin. In 1920, Emmett and Luros showed that the two factors were not equally susceptible to destruction by heat and consequently should be considered as different substances. Subsequently, numerous investigators showed that vitamin B was not a single entity.

A deficiency syndrome was produced in rats which resembled pellagra in man. This syndrome was thought to be due to lack of the pellagra-preventive substance but was later shown to be largely the result of riboflavin deficiency. This new vitamin was designated vitamin G in America and vitamin B_2 in England and Germany. At one time, the terms vitamin G and B_2 were applied to the entire heat-stable fraction of the vitamin B complex, resulting in confusion in terminology which persisted for years.

A method for the determination of vitamin G with the rat was proposed as the experimental animal. This method of bioassay was used for a number of years, and the amount of the vitamin present was expressed as Bourquin-Sherman units. Studies clarified the picture of vitamin G (B_2) deficiency in rats and assisted in separating it from vitamin B_6 (pyridoxine) deficiency.

Chemical research on substance which subsequently became known as riboflavin began in 1879 when it was demonstrated in whey a yellow pigment with green fluorescence. This pigment was called as lactochrome. Concentrates of this pigment was obtained in 1925 and some of the its properties were described, but its chemical nature remained obscure [2].

Riboflavin from eggs was first isolated in a pure crystalline state, named it ovoflavin, and its function was determined as a vitamin. At the same time impure crystalline preparations of riboflavin were isolated from whey and named lyochrome and, later, lactoflavin. Soon there after, riboflavin from a wide variety of animal organs and vegetable sources was isolated and named it hepatoflavin. Ovaflavin from eggs, lactoflavin from milk, and hepatoflavin from liver were all subsequently identified as riboflavin. The discovery of the yellow enzyme in 1932 and their description of lumaflavin, a photochemical degradation product of riboflavin, was of great use for elucidation of the chemical structure of riboflavin. The structure was confirmed in 1935 by the synthesis. For therapeutic use, riboflavin is produced by chemical synthesis, whereas concentrates for poultry and livestock feeds are produced by fermentation using microorganisms such as

Ashbya gossypii and *Eremothecium ashbyii*, which have large capacities to synthesize large quantities of riboflavin [1].

Riboflavin is an essential growth factor for many species of animals and for some types of bacteria. It was found to be essential in man [2].

2.2. Chemistry and physiology of riboflavin

2.2.1. Structure and properties

Riboflavin, vitamin B_2, lactoflavin is a solid crystallizing in fine orange-yellow needles which melt at 282 °C with some decomposition [3]. The solubility of riboflavin in water is 10-13 mg/100 ml at 25-27.5 °C and in absolute ethanol 4.5 mg/100 ml at 27.5 °C; it is slightly soluble in amyl alcohol, cyclohexanol, benzyl alcohol, amyl acetate, and phenol, but insoluble in ether, chloroform, acetone and benzene.It is very soluble in dilute alkali, but these solutions are unstable. Various polymorphic crystalline forms of riboflavin exhibit variations in physical properties. In aqueous nicotinamide solution at pH 5, solubility increases from 0.1 to 2.5% as the nicotineamide concentration increases from 5 to 50% [1]. Aqueous solutions of riboflavin are greenish-yellow in color and exhibit an intense yellow green fluorescence with a maximum at pH 6.7-6.8 which is destroyed by acids and bases [3]. The isoalloxazine nucleus gives riboflavin certain chemical properties of a substituted benzine, an azine dye, and a pyrimidine; the ribityl side chain relates it to the pentose sugars [3]. In aqueous solution, riboflavin has absorption maxima at 220-225, 266, 371, 444, and 475 nm. Neutral aqueous solutions of riboflavin have a greenish-yellow color and an intense yellowish-green fluorescence with a maximum at 530 nm and a quantum yield of Φ_f = 0.25 at pH 2.6. Fluorescence disappears upon the addition of acid or alkali. The fluorescence used in the quantitative determinations. The optical activity of riboflavin in neutral and acid solutions is $[\alpha]_D^{20}$ = +56.5-59.5° (0.5%, dil. HCl). In an alkaline solution, it depends upon the concentration, e.g., $[\alpha]_D^{25}$ = -112-122° (50 mg in 2 ml 0.1 N

alcoholic NaOH diluted to 10 ml with water). Borate-containing solutions are strongly dextrorotatory, because borate complexes with the ribityl side chain of riboflavin; $[\alpha]_D^{20} = +340°$ (pH 12) [1].

In neutral aqueous solutions it is relatively heat-stable if protected from light. Its stability is pH dependent, being more stable under acidic conditions [4, 5]. Riboflavin is also stable against to heat and common oxidizing agents such as bromine nicotinic acid (except chromic acid, $KMnO_4$ and potassium persulfate). It is irreversibly decomposed on irradiation with ultraviolet rays or visible light. Its photochemical cleavage under alkaline conditions results in the formation of the highly reactive compound lumaflavin, which mediates the destruction of other vitamins. Under neutral and acidic conditions, this vitamin loses the ribityl side chain, forming lumichrome (Figure 2) [1,4]. Both lumichrome and lumaflavin have no biological activity. Moreover, the photolysis reaction is irreversible. Researches with pasta products indicated that lumichrome, a photolysate of riboflavin, was not the only, or final, degradation product. In fact, for these type of products, 60% of the losses were accounted for by the presence of lumichrome, and varied according to the process conditions [4].

2.2.2. Biological function

Riboflavin is found in tissues chiefly in the form of flavinadenine dinucleotide (FAD), with a smaller amount of riboflavin-5-phosphate or flavin mononucleotide (FMN) [4]. However, the lens, retina and the cornea of the eye, milk, whey, urine and plasma with small amount contain free riboflavin [1, 2, 6].

Principally, riboflavin fulfills its metabolic function in a complex form. In general, riboflavin is converted in to flavinmononucleotide (FMN,riboflavin5'-phosphate) and flavin-adenine dinucleotide (FAD)

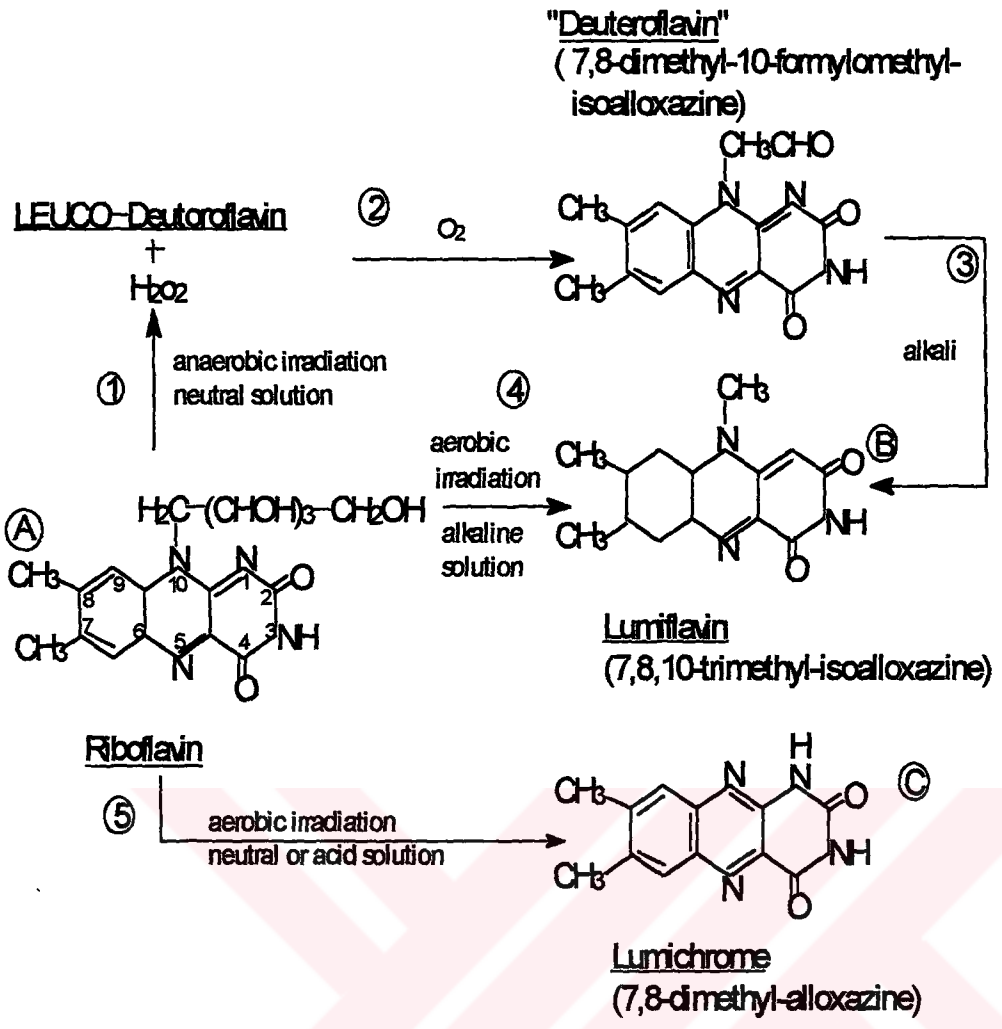

Figure 2. Degradation pathways of riboflavin [1, 4].

(Figure3), which serve as the prosthetic groups (coenzymes) ie, they combine with specific proteins (apoenzymes) to form flavoenzymes, in a series of oxidation-reduction catalyst widely distributed in nature [1].

Vitamin B_2, in the form of its phosphate ester (FMN) or of its dinucleotide (FAD), act as a cofactor for flavin-containing enzymes concerned with hydrogen transfer. As such, they are important cofactors in the citric acid cycle and play an especially important role in the generation of ATP in the respiratory chain [6]

2.2.3. Diatery sources of riboflavin

Riboflavin is widely distributed in plant and animal tissues. Milk, liver, kidney, heart, egg white, and leafy green vegetables are excellent sources of riboflavin seen in Table 1. Lean meat, beef, veal, pork, poultry, cheese, apricots and tomatoes furnish valuable amounts. Cereals are low in riboflavin, but the content is increased during germination [2].

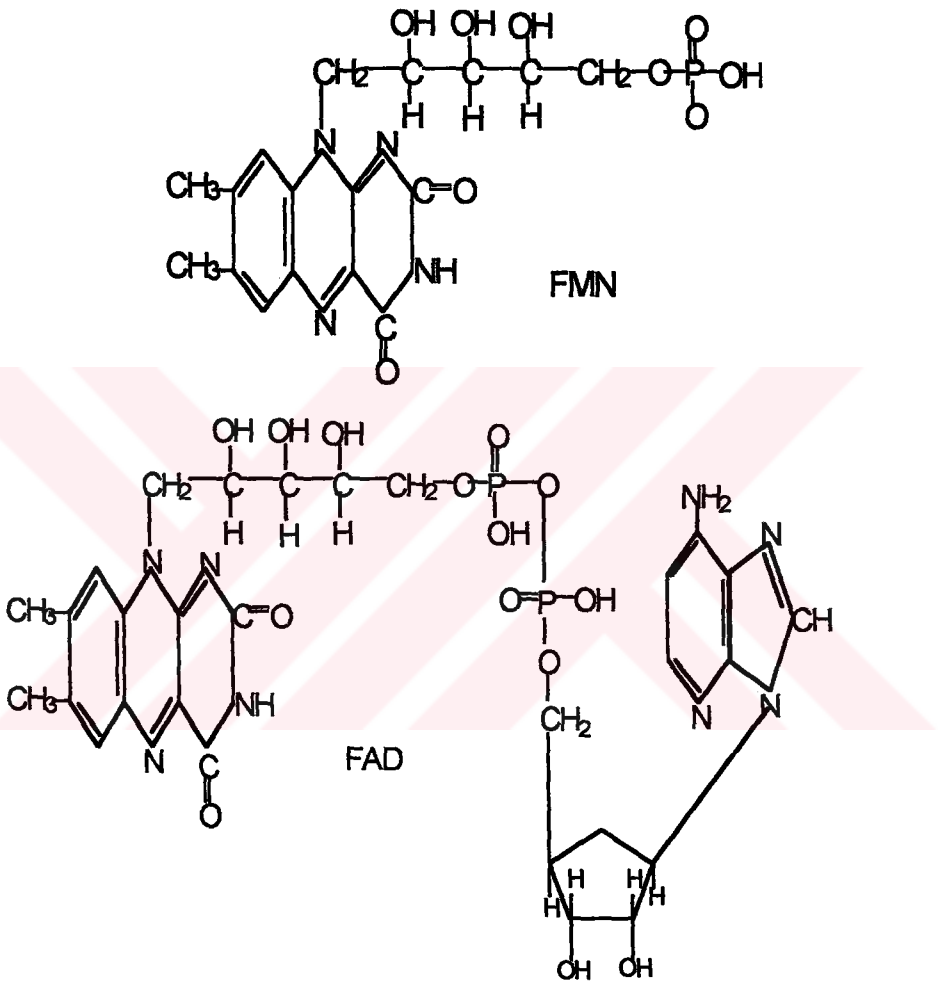

Figure 3. Structural formula of oxidized flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD) [1].

Enriched cereals and bread contribute importantly to the riboflavin of the diet [2]. Riboflavin is used with iron, thiamine, and niacin to enrich flour and bread because these four compounds are destroyed during the processing of wheat into flour [7].

Table 1. Riboflavin Content of Various Foods[a] [1].

Food	mg/100g	Food	mg/ 100g
fruit		fish	
apple, raw	0.01	cod, haddock, raw	0.17
banana, raw	0.04	salmon, canned	0.12
citrus, grapefruit,orange	0.03-0.04	tuna, canned	0.13
strawberry	0.03	whitefish,herring,halibut	0.17-0.29
vegetables		grain	
broccoli, raw	0.27	wheat, entire	0.10
cabbage, raw	0.05	wheat, germ	0.6-0.8
fresh green peas	0.14	rice, entire	0.06
mushroom	0.57	rye, entire	0.20
parsley	0.24	cereal products	
potato, raw	0.03	refined	
sweet corn	0.14	bread	0.07-0.10
sweet potato, raw	0.05	cereal	0.10
tomato, raw	0.03	soda cracker	0.1
meat		whole grain and enriched	
beef muscle	0.16-0.32	bread	0.12
pork muscle	0.19-0.33	cereal	0.20
chicken muscle	0.10-0.28	dairy products	
liver, beef, pork	3.00-3.60	cheese, cheddar	0.54
		eggs	0.48
		milk	0.15-0.17

[a] Averages from several sources, often of a wide range of analytical results; should be regarded as working estimates which vary with geography, season, and preparative method.

The heat stability of riboflavin protects it from destruction with ordinary cooking procedures. There is some loss due to extraction by water, and exposure to light during cooking in uncovered dishes may lead to substantial loss. Pasteurization or drying of milk does not lower riboflavin content very much, but exposure to sunlight destroys large amounts of the vitamin (up to 85%) [2].

2.2.4. Deficiency of riboflavin and human requirement

Early symptoms of riboflavin deficiency may be general in nature, or may be related to oral or ocular lesions. Soreness and burning of the lips, mouth and tongue are common complaints and this is usually accompanied

by discomfort in eating and swallowing. Ocular symptoms include photophobia, lachrymation, burning and itching of the eyes, visual fatique, blepharospasm and loss of visual acuity which cannot be accounted for by refractive error. A sensarion of "gritness" under the lids is often present.

Lesions of the lips begin with pallor and maceration at the angles of the mouth or with dryness, redness and denudation along the life of closure. In severe deficiency ulceration may occur, and at the transverse fissures appear which may extend outward for a centimeter or more. The lesions of the lips have been designed 'cheilosis' and those at the angles of the mouth 'angular stomatitis'. There is still some dispute about the tongue signs particularly in relation to distinguishing between the glossitis of riboflavin and nicotinic acid deficiency [8].

A lack in the human diet causes well-defined syndromes, such as seborrhoeic follicular keratosis of the nasolobial folds, nose, and forehead; dermotisis of the anogenital region (scrotum and valvua). Requirements are increased in pregnancy and lactation [1]. Riboflavin requirements in humans vary from 1 to 3 mg per day and are related to body weight, age and living conditions [6]. Daily requirement of riboflavin are shown in Table 2 [8].

Riboflavin is not stored in organism, any surplus taken in food or supplements is excreted (like Vitamin C) . Also riboflavin deficiency manifest itself by a number of non-specific symptoms: general lethargy and disinclination to work, dystrophy (breaking of the finger nails) and finally changes in the skin of the whole body. Reduced resistance to infection (typhus, pneumonia) is often sign of vitamin B_2 deficiency [5]. Aribоflavinasis is a disease in humans caused by riboflavin deficiency [7, 9].

2.3. Synthesis of riboflavin

Riboflavin is produced industrially by several processes:

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

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

Total production of riboflavin on a world-wide basis is around 2000 tons per year [9] .

Table 2. Daily requirements for riboflavin in humans [8].

		Riboflavin (mg/day)
Man	Sedentary	1.8
	Moderately active	1.8
	Very active	1.8
Woman	Sedentary	
	Moderately active	1.5
	Very active	1.5
	Pregnancy first half	2.0
	second half	2.0
	Lactation	2.5
Children		
Both sexes	Under 1 year	0.6
	1-3 years	0.9
	4-6 years	1.2
	7-9 years	1.5
	10-12 years	2.0
Boys	13-15 years	2.0
	16-20 years	2.5
Girls	13-15 years	2.0
	16-20 years	1.8

2.3.1. Chemical synthesis of riboflavin

It was proved that riboflavin was 7,8-dimethyl-10-D-ribitylisoalloxazine by total synthesis (Figure 4).

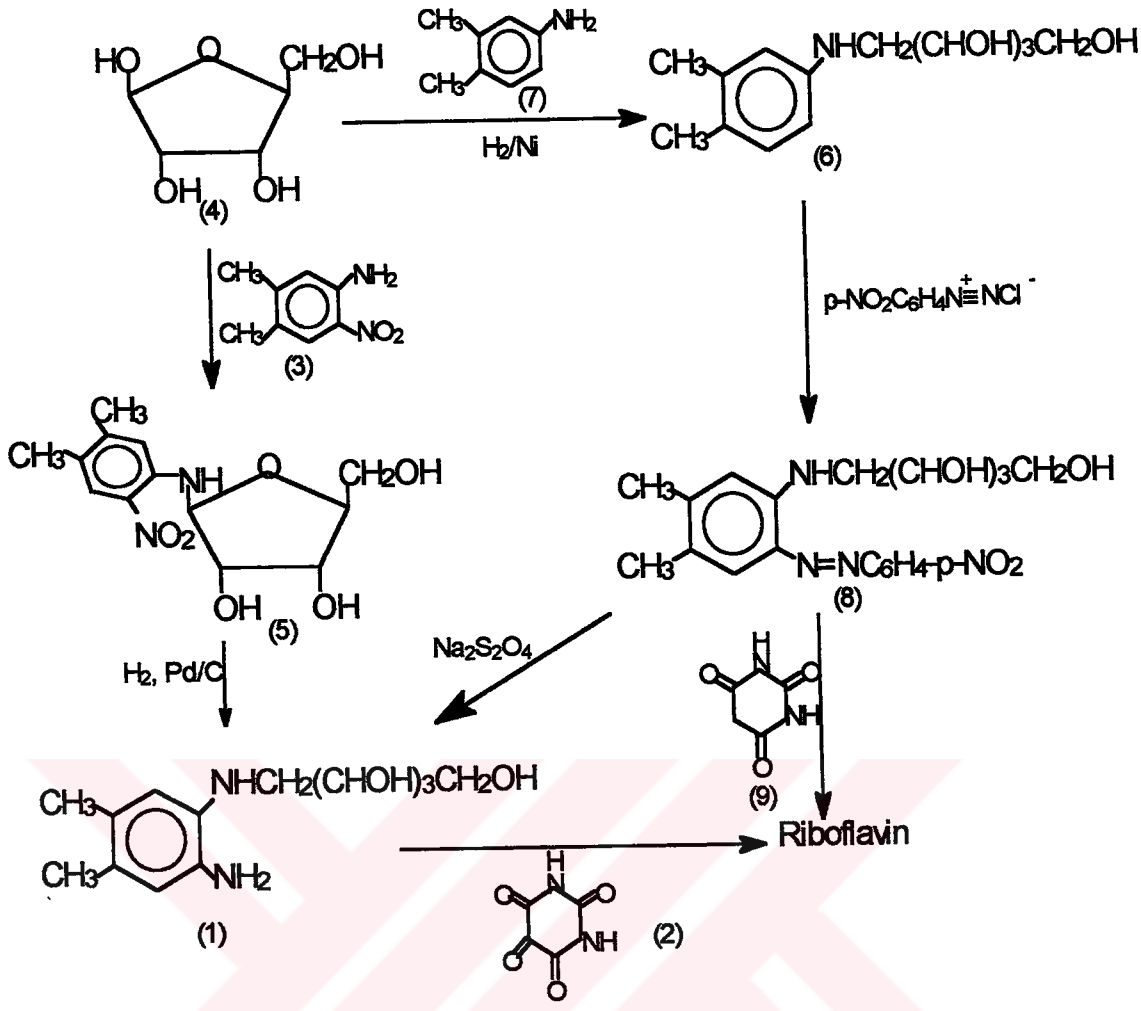

Figure 4. Synthesis of riboflavin [1].

These synthesis are essentially the same and involve a condensation of 6-D-ribitylamino-3,4-xylidine (1) with alloxan (2) in acid solution. Boric acid as a catalyst increases the yield considerably . The intermediate (1) was prepared by a condensation of 6-nitro-3,4-xylidine (3) with D-ribose (4), followed by catalytic reduction of riboside (5). The yield based on D-ribose was increased by using N-D-ribityl-3,4-xylidine (6), which was prepared by the condensation of 3,4-xylidine (7) with ribose (4), and followed by catalytic reduction, the reduced product was coupled with p-nitrophenyldiazonium salt to give 1-D-ribitylamino-2-p-nitrophenylazo-4,5-dimethylbenzene (8), which is reduced to (1) and treated with alloxan to give riboflavin. Replacement of

alloxan by 5,5-dibromobarbituric, or 5-bromobarbituric acid in the above syntheses yields riboflavin.

More conveniently, compound (8) was directly condensed with barbutyric acid (9) in acetic acid. The same azo dye intermediate (8) and alloxantin give riboflavin in the percentage of palladium on charcoal in the alcoholic hydrochloric acid under nitrogen. This reaction may involve the reduction of the azo group to the o-phenyl-enediamine by the alloxantin which is dehydrogenated to alloxan [1].

2.3.2. Microbial synthesis

Some microorganisms are capable of excreting large quantities of certain vitamins in to the medium in which they grow [6]. The industrially important microorganisms have at least five outstanding abilities:

- To grow rapidly in suitable organic substrates
- To be cultivated easily in large quantities
- To produce the necessary enzymes readily and profusely, in order to bring about the desired chemical changes
- To carry out the transformations under comparatively simple and workable modifications of environment conditions
- To maintain physiological constancy under these conditions [10].

A great number of microorganisms synthesize water soluble vitamins in amounts which appear to be somewhat in excess of their needs [11]. Microbiologically produced riboflavin, or lactoflavin as it is also known, has long been available in yeast and in related preparations in association with many other vitamins, particularly those of the B-complex [12]. The mechanism of riboflavin biosynthesis shown in Figure 5 has been deduced from data derived from several experiments involving a variety of organisms. Include are the conversion of a purine such as guanosine triphospate (GTP)

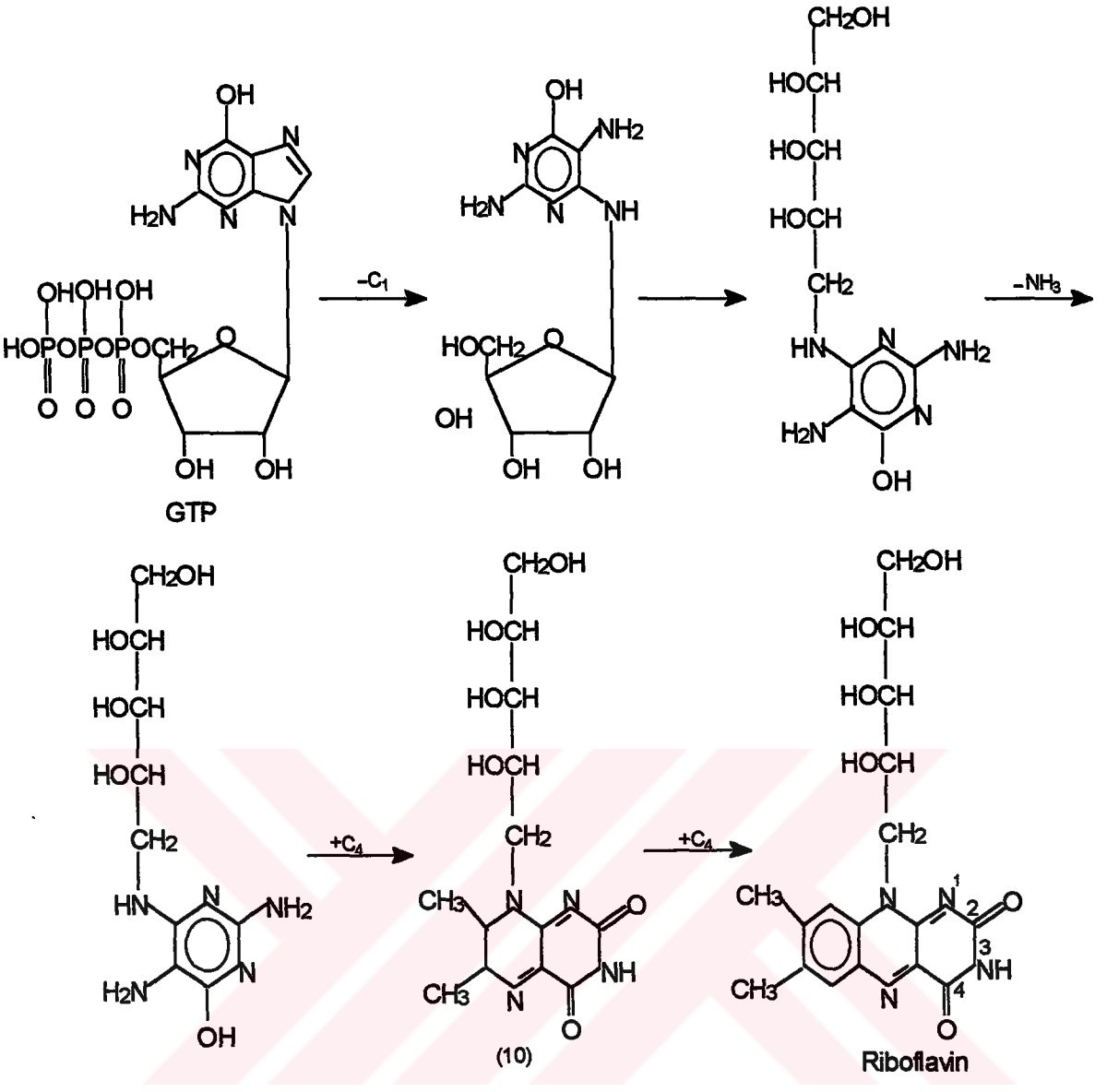

Figure 5. Biosynthesis pathway to riboflavin [12].

to 6,7-dimethyl-8-D-ribityllumazine (10), and the conversion of (10) to riboflavin.

Riboflavin is a unique vitamin in that it can be totally synthesized to very high concentrations rather rapidly by certain microorganisms [12], including yeast, yeast like microbes, and bacteria. Table 3 lists some of the microbes which are capable of synthesizing this vitamin [14]. Although riboflavin is produced by many microorganisms, other microorganisms require riboflavin for growth [13] and may therefore be used for assays [11].

The organism which have been found to produce sufficient riboflavin to be, or to have been, of interest from a commercial standpoint are relatively few. Those of prime importance for riboflavin formation rather than for B-complex are noted in Table 4 [12].

Table 3. Some microorganisms capable of synthesizing riboflavin [14].

Yeast-like	Yeasts	Bacteria
Ashbya gossypii	*Anascosporpgenus:*	*Clostridium*
*Butyricium**		
Eremothecium ashbyii	*Candida arborea*+	*Cl. acetobutylicum**
	C. flareri+	*Cl. felsineum*
	C. guilliermondia+	*Cl. propylbutylicum*
	C. utilis	*Cl. roseum*
	Rhodotorula sp.	*Aerobacter aerogenes*
	Torulopsis sp.	*A. cloacae*
	Ascosporogenous:	*A. oxytocum*
	Hansenula suaveolens	*Azetobacter agile*
	Sacharomyces sp.	*Az. chrococcum*
		Az. vinelandi

* Employed industrially
+ Employed on a pilot-plant scale

Commercial fermentation process for production riboflavin or riboflavin concentrates are relatively recent, having been developed in the past 40 years. Aside from food yeast, 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 [12, 15]. These fermentations were succeeded in about 1940 by a process using *E. ashbyii* with yields of about 2 mg/ml. In 1946 processes using the ascomycete *A. gossypii* were started. In 1978 the only manufacturer using the microbiological process is Merck & Co., Inc. (United States). Companies manufacturing riboflavin by chemical synthesis include Hoffmann-La Roche Inc. (United States and Switzerland), and E. Merck (West Germany) [13].

Table 4. Microorganisms producing considerable riboflavin and the effect of iron on the biosynthesis [12].

Organism	Riboflavin in culture fluid [μg/ml]	Optimum iron concentration, [μg/ml]
Mycobacterium smegmatis	57.5	Not critical
Clostridium acetobutylicum	97.0	1 to 3
Mycocandida riboflavina	20.0	Not critical
Candida flareri	567.0	0.04 to 0.06
Ashbya gossypii	1760.0	Not critical
Eremothecium ashbyii	2480.0	Not critical

Riboflavin production is not increased significantly by its addition to the medium. Comparatively large yields may be obtained by careful selection of the yeast and medium [14].

Success of the fermentation process on the industrial scale depends on a number of factors, notably:

- The ability of the selected organism to give consistently high yield of the desired product in a reasonable time from a cheap, available raw substrate;
- The easy recovery of the product in pure form; and
- The manufacture of a unique product which is demanded, but difficult to obtain by other methods [10].

2.4. Synthesis of riboflavin by *Ashbya gossypii* (Ashby and Novel)

2.4.1. Morphology

This ascomycete has also been described as *Nematospora gossypii* (Ashby and Novel) and *Ashbya gossypii* (Ciferri and Fragoso). This yeast-like microorganism has a dual "personality" [7]. On one hand it is a plant pathogen, first isolated from stained cotton bolls, cause disease in cotton plants, parasitizes many other economically important crops such as coffee, citrus fruit, okra, and tomatoes, infect beans [7, 11, 12]. On the other hand,

under different conditions they can be useful because they produce riboflavin [7]. Because of its pathogenicity, it is essential to sterilize all fermentation residues and cultures before they are discarded [14].

Morphologically, *Ashbya gossypii* is very similar to *Eremothecium ashbyii*, but differences exist in the arrangement and shape of their spores and in the fact that *Ashbya gossypii* is homotallic [11], this is in contrast with *Eremothecium ashbyii* which is a heterothallic Ascomycete, but only one sexual form has been described as yet [12]. The life cycle of *Ashbya gossypii* have been described and shown in Figure (6) [11].

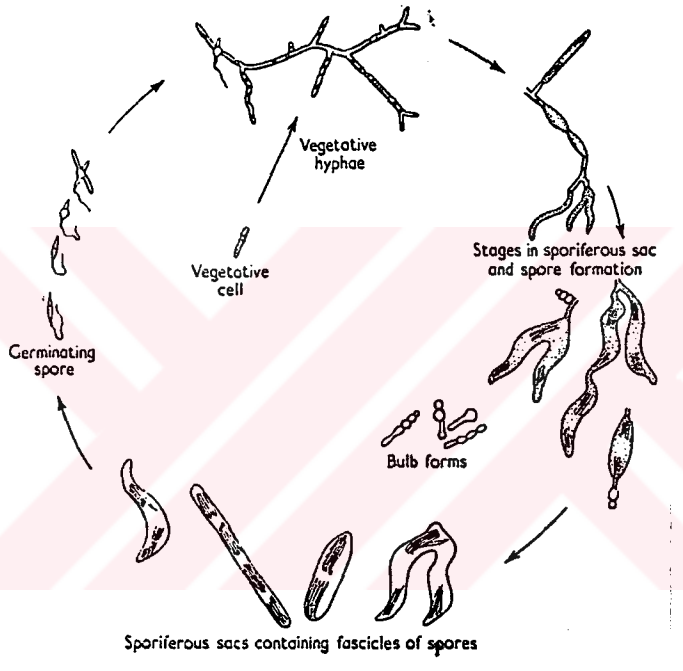

Figure 6. Life cycle of *Ashbya gossypii* [11].

2.4.2. Nutrients required

Ashbya gossypii requires certain nutrients for growth and maximum yield of riboflavin [14]. Carbohydrates other than sucrose will also support good growth, but many are not utilized (Table 5) [11]. It was reported that commercial glucose appeared to be the best source, but that sucrose and maltose could be substituted for it. Starch molasses (cane-sugar, beet-

sugar, and corn), and the common pentoses were apparently not utilized by *Ashbya gossypii* for synthesis of riboflavin [14].

Table 5. The ability of Various Carbohydrates to Support Growth of *Ashbya gossypii* [11].

Growth		
Good	Medium	Slight or nil
Glucose Fructose	Maltose Cellobiose	Rhamnose Arabinose Xylose Galactose Lactose Inulin Cellulose Starch

Washed suspensions of *Ashbya gossypii* completely metabolize glucose to carbon dioxide and water, probably via the conventional glycolytic and tricarboxylic acid pathways[11] . *Ashbya gossypii* requires some growth factors for growth. Inositol, thiamine, and biotin are essential for the growth of this organism. It was found that *Ashbya gossypii* grew rapidly in a synthetic-type medium composed of sucrose, aspargine, biotin, thiamine, inositol, and several salts [11, 12,14,15, 16]. A significant amount of riboflavin did not appear to form [15]. More complex media, containing peptone, yeast extract, and sugar, resulted in much better riboflavin formation. Lipids may be of importance for high riboflavin production by *Ashbya gossypii* [12], and riboflavin synthesis in that organism is unaffected by the iron level of the medium employed [7].

2.4.3. Cultural conditions

According to literature, satisfactory yields of riboflavin may be obtained when fermentation variables are carefully controlled [14]. Riboflavin

synthesis in *Ashbya gossypii* is a strongly aerobic process and for high yields it is necessary to aerate the cultures either by shaking or by bubbling air through the medium. However excessive shaking, which interferes with mycelial development, reduces riboflavin synthesis [16]. The air flow in liters/minute, and agitation rate should be at lest 0.25 times the volume of the medium and 250-300 rpm [11, 14, 19].

The object of the aeration and agitation is to provide;

- Oxygen to growing organism,
- Maintaining oxygen to a grown organism which is producing extra-cellular products,
- Complete mixing within the fermentor [19].

Prolonged autoclaving (over 30 min.) of the medium results in reduced yields of riboflavin (Table 6) and flash sterilization at 280°-290° for 3 min. is recommended [11].

Table 6. Effect of Sterilization Time on Flavinogenesis by *Ashbya gossypii*[+] [11].

Time of sterilization of medium	Riboflavin µg/ml
0*	678
15	648
30	680
45	494
60	308
90	248

[+] Sterilization by autoclaving at 121°C; 8-day cultures.
* Medium sterilized by filtration.

The age and proportion of inoculum influenced the riboflavin yield considerably. A 24-hour was found to be much preferable to 72-, 96-, or 120-hour inoculate. The preferred quantity of inoculum was 0.5 to 1.0% by volume. Higher levels were less satisfactory and a 10% by volume inoculum resulted in a much lower yield [12,18].

Özbaş and Kutsal [20, 21] described the studies on optimum temperature and initial pH on the specific growth and riboflavin production rates for *Ashbya gossypii*. The optimum initial pH value found to be 6.5 and the optimum temperature was 30°C in all medium compositions. They also found the optimum initial glucose for growth of *Ashbya gossypii* as 20 (g/L).

2.4.4. Constitution of the medium

Although riboflavin biosynthetic pathway is well understood, information on the physiology of overproduction and influence of culture parameters on yields is not so complete . There have been many studies on the effect of medium composition on riboflavin yields in this organisms, but in these pH was not controlled at a constant value during fermentation, and therefore its importance or influence is not clear. It was found that during biomass production in *E. ashbyii*, the pH dropped, but than rose during the phase of riboflavin formation. This decrease appeared to be the result of initial accumulation of pyruvate in the culture medium, which was then utilized. Özbaş and Kutsal [20] confirmed these observations with a different medium. They also demonstrated that highest riboflavin productivity in *Ashbya gossypii* occurred at an initial pH of 6.5, although they did not report subsequent changes in the medium pH over the fermentation period [22] .

Almost all the glucose of the medium is utilized during the first 24 hr of the fermentation; this is accompanied by a fall in pH from its initial value of 6.3 to 4.5 and no significant amount of riboflavin are produced. Thereafter up to 115 hr after inoculation, riboflavin is rapidly synthesized and the pH value gradually rises [11, 12, 17].

2.4.5. Recovery of riboflavin from fermented products

Fermentation liquors containing riboflavin may be recovered for use as animal or human food supplements simply by drying and powdering. Drying is generally accomplished where practicable by drum or spray-drying procedures [12].

A pilot plant for producing riboflavin by *Ashbya gossypii* fermentation was described. The flow sheet is outlined in Figure 7. The fermentor can be of glass, aluminum, copper, or stainless steel. The medium (2 per cent glucose, 2 per cent cornsteep liquor, 1 per cent animal stick liquor, 0.1-0.2 per cent soybean oil as antifoam, pH 4.5) was sterilized at 275°F by a continuous procedure, inoculated with 0.5-1.0 per cent volume of mother culture, aerated and gently shaken for 4-5 days at 28°-29°C. The resulting product was then dried on a drum drier, yielding a concentrate containing 2.5 per cent riboflavin [11,16,19].

In a more recent process, the optimum requirement are air an air flow rate of 0.33 vvm and agitation with three impellers at a power input of 1.0 HP per 100 liters of medium. Excessive foaming is controlled by initial addition of emulsified silicone antifoam followed by soya bean oil, which also act as nutrient. Sterilization of the medium is achieved by heating at 121°C for three hours. The optimum incubation temperature is 28°C over the seven-day incubation period. Using suitable mutants, yields of riboflavin are in the region of 4 g/l [16, 23].

When the riboflavin produced is to be used as animal feed supplement, the pH is adjusted to 4.5 and broth is concentrated to about 30 per cent solids and then dried in double dryers. When a crystalline product is required, the riboflavin content of the cells is solubilized by heating the broth for one hour at 121°C, with the subsequent removal of the insoluble matter by centrifugation. The riboflavin in solution is then converted to a less soluble reducing agent and a finely divided diatomaceous earth are used. Alternatively, the riboflavin is adsorbed by on to Fuller's earth, silica gel or other adsorbents, and then eluted with an aldehyde, ketone or alcoholic solution of an organic base. Microbial methods involve the riboflavin being converted to a less soluble form by the action of reducing bacteria, e.g. *Streptococcus faecalis*. The precipitated vitamin treated by the chemical or microbiological methods is dissolved in water, polar solvents or an alkaline

solution, oxidized by aeration and recovered by crystallization (the alkaline solution are acidified to crystallize riboflavin) [15, 19].

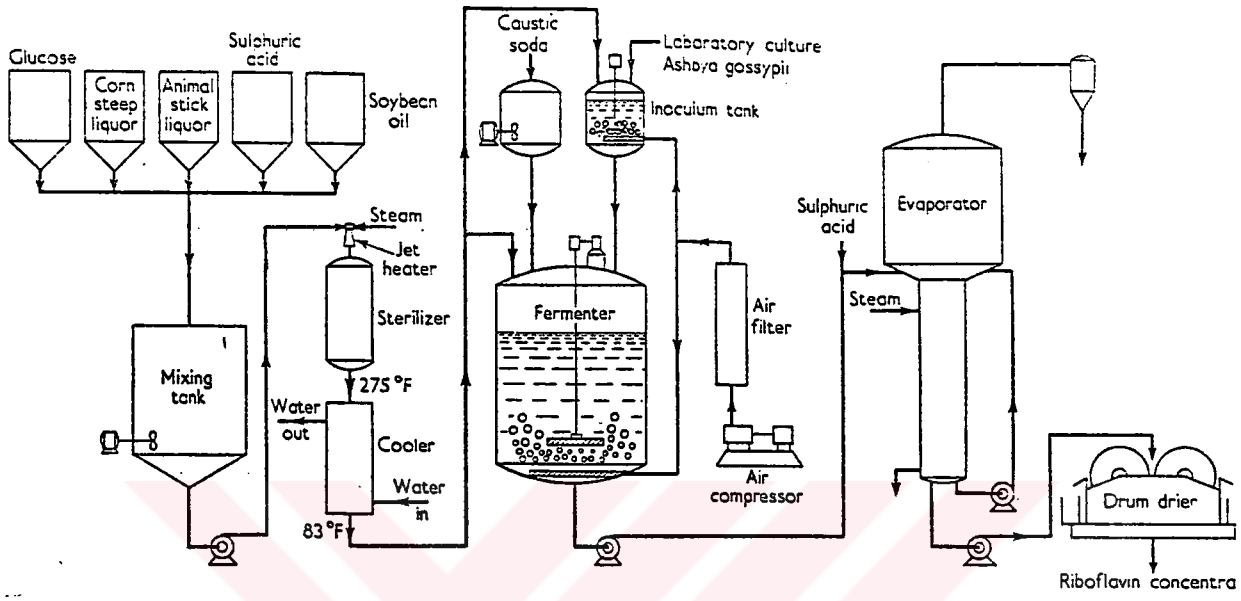

Figure 7. Flow sheet for riboflavin production by fermentation [19].

CHAPTER 3
MATERIALS AND METHODS

3.1. Preparation of cultures and media

3.1.1. Organism and other materials

Ashbya gossypii NRLL Y-1056 known to produce riboflavin was kindly obtained from USDA, Agricultural research service, Natural Center for Agricultural Utilization Research, University ST. Peoria, USA.

Galactose and 3,5-dinitrosaliciylic acid from Sigma Chemical Company; lactose, malt extract and sodium hydroxide from Merck; glucose, sodium potassium tartarate from Carlo Erba; peptone and malt extract from Oxoid; phenol, K_2HPO_4, $MgSO_4.7H_2O$ and sodium meta-bisülfite from Riedel-de Haen were used.

3.1.2. Inoculum preparation

Cultures of *Ashbya gossypii* were maintained in solid agar media in slant tubes. The composition of the culture medium was as follows (g/L) glucose, 20.0; peptone, 5.0; yeast extract, 5.0; malt extract, 5.0; $MgSO_4.7H_2O$, 0.2; K_2HPO_4, 0.2 [18, 19]. The pH of the medium was adjusted to 6.5 with 0.1N NaOH or 10% tartaric acid. Both liquid and solid media were sterilized at 121°C in an autoclave before inoculation.

The cultures was maintained in slant and deep agars by periodic three monthly transfers and kept at -30°C. When needed the tubes were thawed and a loopful of this stock culture was placed in to culture media broth and growth was provided at 30°C.

3.1.3. Fermentation media

The composition of the fermentation media was similar to the composition of the broth used for the inoculation except carbon sources.

Following substrates with different concentrations were used as a carbon source.

1. Glucose (20 g/L)

2. Glucose (40 g/L)

3. Glucose (60 g/L)

4. Lactose (20 g/L)

5. Galactose (20 g/L)

Experiments were carried out in shake flasks and an aerated stirred tank fermentor at 30°C [18, 19] for 8 to 12 days.

Solutions were prepared by using distilled water. Fermentation media for riboflavin production were prepared by dissolving the constituents of the medium in distillate water. After filtering the solution to eliminate suspended particles, pH of the media was adjusted to 6.5 [18, 19] by using 0.1N NaOH or 10% tartaric acid.

3.2. Fermentation

3.2.1. Shake flask studies

Prepared culture media with different carbon sources were placed into flasks and sterilized at 121°C for 20 minute. They were inoculated with 2% (v/v) *Ashbya gossypii* which was grown at 30°C. Inoculated flasks were covered with aluminum foil to prevent light degradation of riboflavin produced and placed in to a bench type water bath shaker ST-402 (NÜVE; Sanayi ve Malzemeleri İmalat ve Ticaret A.Ş., İSTANBUL) with 55 rpm wrist shaking at 30°C for 12 days. Samples were collected daily, under aseptic conditions, and analyzed for riboflavin, pH, sugar and biomass.

100 ml or 500 ml working volume were used for fermentation. 500 ml culture media were used when substrate, riboflavin and pH were determined. Samples were drawn from the same fermentation media at each sampling time. 12 flasks with 100 ml working volume of culture were used to determine pH, substrate, riboflavin and biomass. One of the 12 flasks were used at each sampling time during fermentation.

3.2.2. Fermentor studies

Fermentor studies were performed by using 5 L cell culture system (with interactive 4-gas DO/pH instrumentation type, New Brunswic Scientific CO. INC., U.S.A) fermentor. Prepared 3.7 L fermentation medium was placed into fermentor, and sterilized at 121°C for 30 min. Then 0.2% (v/v) 1 days old *Ashbya gossypii* grown in shake flask [11, 14] was inoculated and allowed to grow at 30°C for 8 days. Aeration were done by an air sparger at 0.25 vvm rate [16, 18]. Sterilization of the air was provided by using 0.2μm Millex-FG Hydrophobic Filter. The fermentation medium was agitated by a stirrer at 200 rpm . Sampling was done under aseptic conditions through silicone tubing by peristaltic pump and foaming was prevented by using 0.2% soybean oil as an antifoaming agent [11, 16] and after sampling, the sample tubing was placed into alcohol to keep sterility. Fermentor vessel was covered with aluminum foil to prevent light degradation of riboflavin produced.

3.3. Sampling

For fermentor studies; at every 24 hour intervals for fermentor studies 70 ml sample, for shake flask studies 30ml or 100 ml sample were taken from fermentation medium under aseptic conditions and pH, riboflavin, sugar and biomass were determined. Analysis were done as triplicate. During the sampling from fermentor, first 20 ml proportion of the sample was discarded to remove broth left in the sampling system.

For shake flask studies; at eavey 24 hour intervals 30 ml or 100 ml samples were taken from fermentation media depending on determined parameters.

3.4. Analysis

Table 7 summarizes substrate types, initial concentrations used and determined parameters.

Table 7. Substrate types used, initial substrate concentration and determined parameters for each run.

			Determined Parameters			
Run No	Substrate Used	Substrate Conc. (g/L)	pH	Sugar	Ribofl.	Biomass
SF.1	Galactose	20	√	√	√	-
SF.2	Lactose	20	√	√	√	-
SF.3	Glucose	20	√	√	√	-
SF.4	Glucose	40	√	√	√	-
SF.5	Glucose	60	√	√	√	-
SF.6	Lactose	20	√	√	√	√
SF.7	Glucose	20	√	√	√	√
F.1	Glucose	20	√	√	√	√
F.2	Glucose	20	√	√	√	√

SF; Shake flask runs
F ; Fermentor runs
√ ; Determined parameters
- ; Undetermined parameters

3.4.1. pH Measurement

pH of the sample were determined by using a Jenway 3010 model pH-meter.

3.4.2. Comparison of pretreatment methods for riboflavin determination

In order to release bound vitamin four methods were compared. The methods used were illustrated in Table 8.

Table 8. Pretreatment methods used to release bound vitamin.

Methods	
1	Heat treatment (120°C, 1hr.)
2	Heat treatment and adjusting medium pH to 8.5
3	Heat and ultrasonic disintegrator treatment (10 amplitude, 3min.)
4	pH adjustment, ultrasonic disintegrator, and heat treatment

3.4.3. Determination of riboflavin

After pH measurement, three 10 ml samples were placed into centrifuge tubes which were covered with aluminum foil. Riboflavin present both in the 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 [24]. After heating, mycelium was separated by centrifugation for 30 minutes at 500 rpm. Then supernatants of centrifuged samples were used to detect riboflavin and sugar concentrations.

The concentration of riboflavin produced was determined at 445 nm by means of Novaspec II Model Spectrophotometer [18, 19] from calibration curve.

A calibration curve was prepared by using riboflavin solutions in culture media ranging in concentrations of $1x10^{-6}$ to $1x10^{-5}$ g/ml.

3.4.4. Determination of sugar

The reducing sugar contents of the samples were determined by using Dinitrosalicylic acid (DNS) method [25].

In the determination of the reducing sugar, 1 ml of sample was mixed with 3 ml of DNS reagent in a test tube. Test tube was waited in the boiling water bath for 5 minutes. Then, 16 ml of distilled water was added and mixed thoroughly. The absorbance value of colored sample was measured at 575 nm by using Novaspec II model spectrophotometer in turn, the concentration of consumed sugar was determined from calibration curve.

The calibration curve was obtained by preparing sugar solutions in inoculation media in concentrations of 0.002 to 0.01 g/ml. The above procedure was applied for sugar solutions and corresponding absorbances were obtained. Standard curve of each sugar (glucose, lactose, galactose) was plotted separately.

Preparation of DNS reagent; 2.65 g DNS reagent (3,5-Dinitrosaliciylic acid, $C_7H_4N_2O_7$) and 4.95 g NaOH were dissolved in 354 ml distilled water

and mixed. Then, 76.5 g sodium potassium tartarate, 2.075 g sodium metabisülfite and 1.9 ml of phenol were added to the solution and mixed.

Reducing sugars reduce the nitro groups in DNS acid in to amine form. Obtained compound is dark red. The intensity of the color was related with the concentration of reducing sugars entering in to reaction.

3.4.5. Cell dry weight analysis

40 ml sample obtained from fermentation media was put into ultrasonic disintegrator for 3 minute at 10 amplitude to homogenize solution by disintegrating cell flocks and then placed into centrifuge tubes and centrifuged for 30 minutes. After centrifugation, supernatant was discarded and precipitate was dissolved in 10 ml distilled water and centrifuged again in order to remove riboflavin residue from the precipitate. After 30 minutes of centrifugation, supernatant was again discarded and precipitate resuspended in 10 ml distilled water. Then absorbance was read at 500 nm [18, 19]. The cell dry weight was determined from the calibration curve.

For dry weight; 5 days old *Ashbya gossypii* was filtered from 0.45μm autoclavable Gelman type filter and dried in an oven at 105°C for 3 hours to obtain dry biomass concentration in stock suspension. 5 g cell was taken and completed to 20 ml with distilled water and placed into ultrasonic disintegrator for 3 minutes at 10 amplitude to homogenize the cells. This stock suspension was diluted several times, and absorbance value of each dilution was determined at 500 nm. Dry biomass weight versus absorbance value was plotted to obtain a calibration curve.

CHAPTER 4
RESULTS AND DISCUSSION

4.1. Comparison of the pretreatment methods for riboflavin dermination

Riboflavin present both in the solution and bound to the mycelium in the fermentation broth [26]. In order to release the bound vitamin, four methods were compared. Found riboflavin concentrations were illustrated in Table 9.

Table 9. Riboflavin concentrations for two different fermentation broth after pretreatment.

	Riboflavin Conc. (g/L)	
Methods	Sample 1	Sample 2
1	0.0178	0.0171
2	0.0182	0.0191
3	0.0169	0.0158
4	0.0178	0.00928

There is little information concerning the effect of pretreatment methods on riboflavin determination. Although in many studies only heat treatment method was used for this purpose, in some there was no pretreatment method to release bound vitamin from mycelium into the medium.

pH adjustment was used as a pretreatment method because; it was found in literature that the medium pH certainly had a striking effect on the culture morphology in *Eremothecium ashbyii* which the metabolic changes occurring in medium of shake cultures of *Eremothecium ashbyii* are very similar to those observed with *Ashbya gossypii* [9], a high pH may encourage the riboflavin release from the cells. For example, as the culture pH increased, hyphae became less even, and cells more bloated and pleimorphic until at pH 8.5 they were almost granular and highly irregular. At

pH 3.5 although thin and regularly branched, the hyphae were pelleted and aggregated. The lack of riboflavin elaboration by *Eremothecium ashbyii* at pH 3.5, when it grows as aggregated mycelial clumps, suggested that there might be some relationship between morphology and riboflavin synthesizing ability. Therefore it would appear that *Eremothecium ashbyii* prefers more acidic conditions for maximum riboflavin elaboration [27]. In the riboflavin fermentations with *Eremothecium ashbyii* and *Ashbya gossypii*, the both pH increase and riboflavin formation is terminated at approximately 9.5 [17]. pH has less effect on biological activities than does temperature because the cell able to regulate its internal hydrogen ion concentration in the face of adverse external medium may also have an effect on structure and permeability of cell membrane [26].

Ultrasonic disintegrator was used to disintegrate the cell in turn as a pretreatment method to release bound vitamin to the medium. Ultrasonic oscillators break cells by shearing. Rapidly moving bubbles in the sonic field at the tip cause high shear forces capable of breaking the tough cell walls. The tip of the sonic probe inserted just below the surface of the cell suspension. Treatment was applied to the sample at 10 amplitude for 3 minute. In literature there is no information concerning the effect of this method on riboflavin release as a pretreatment method.

After 8 days fermentation the pretreatment methods were applied to fermentation broth and samples were analyzed for riboflavin concentration to see the effects of pretreatment on the release of bound riboflavin. Experiments repeated twice and the results were average of two measurements. ANOVA test (one-way) were used to compare these methods.

ANOVA test showed that four methods were not significantly different from each other at $\alpha = 0.05$ level (Table 10).

Table 10. ANOVA test results of pretreatment methods for riboflavin determination.

Source of variation	Sum of squares	d.f	Mean square	F-ratio	Sig. level
Between groups	0.0021504	3	0.0007168	1.019	0.475
Within groups	0.0028138	4	0.0007034		

This study sheeds some light in this regard indicating that these four pretreatment methods has approximately same effect on the release of bound vitamin from mycelium. Because of shorter application time, throughout this study the first method which is the only method found in literature was used to release bound vitamin to the medium.

4.2. Riboflavin production

Plots of the experimental data for all runs were given in Figures 8 through 16.

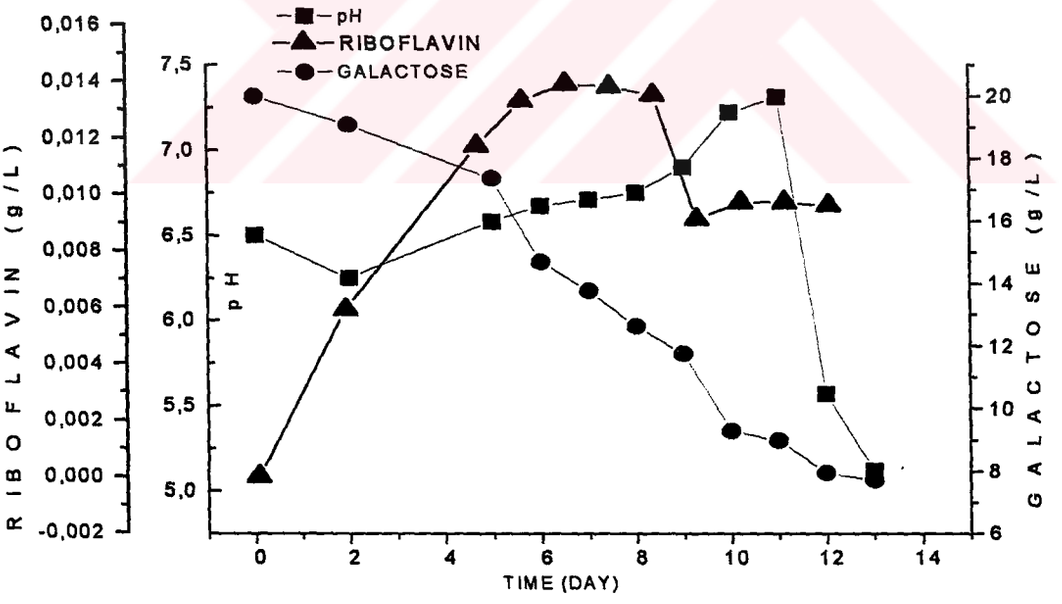

Figure 8. Plot of galactose, riboflavin concentration and pH versus fermentation time for Run No SF.1.

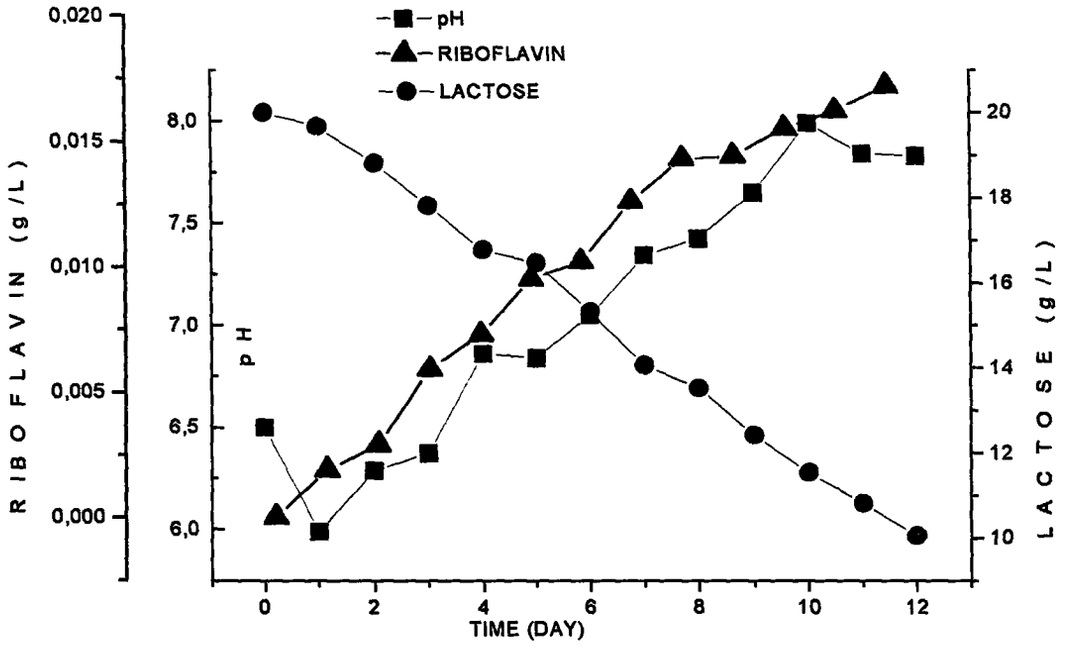

Figure 9. Plot of lactose, riboflavin concentration and pH versus fermentation time for Run No SF.2.

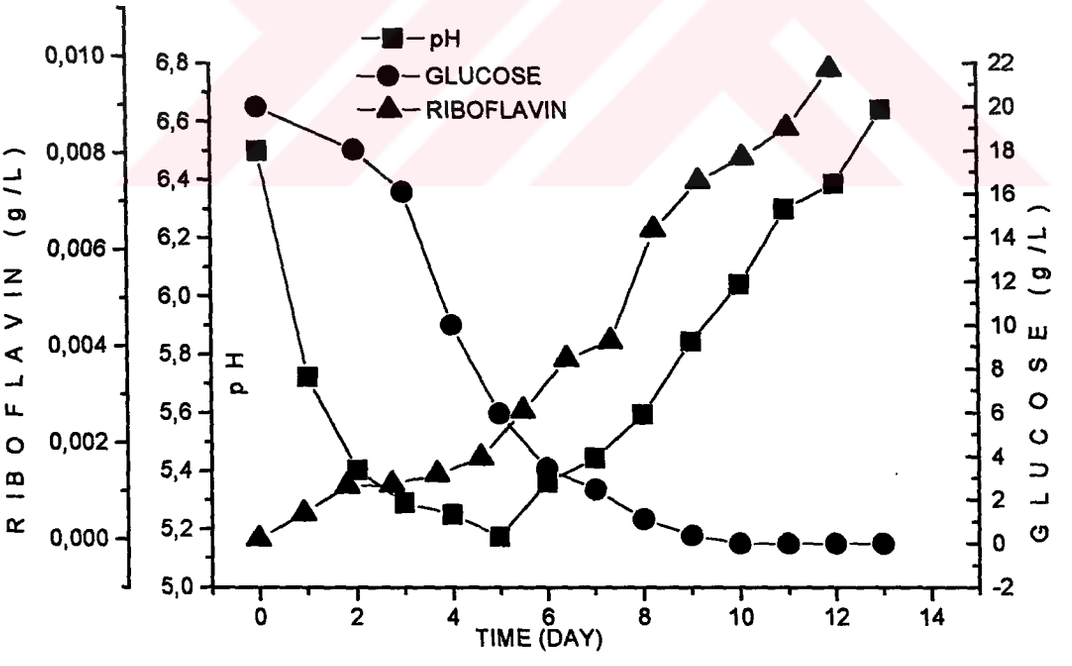

Figure 10. Plot of glucose, riboflavin concentration and pH versus fermentation time for Run No SF.3.

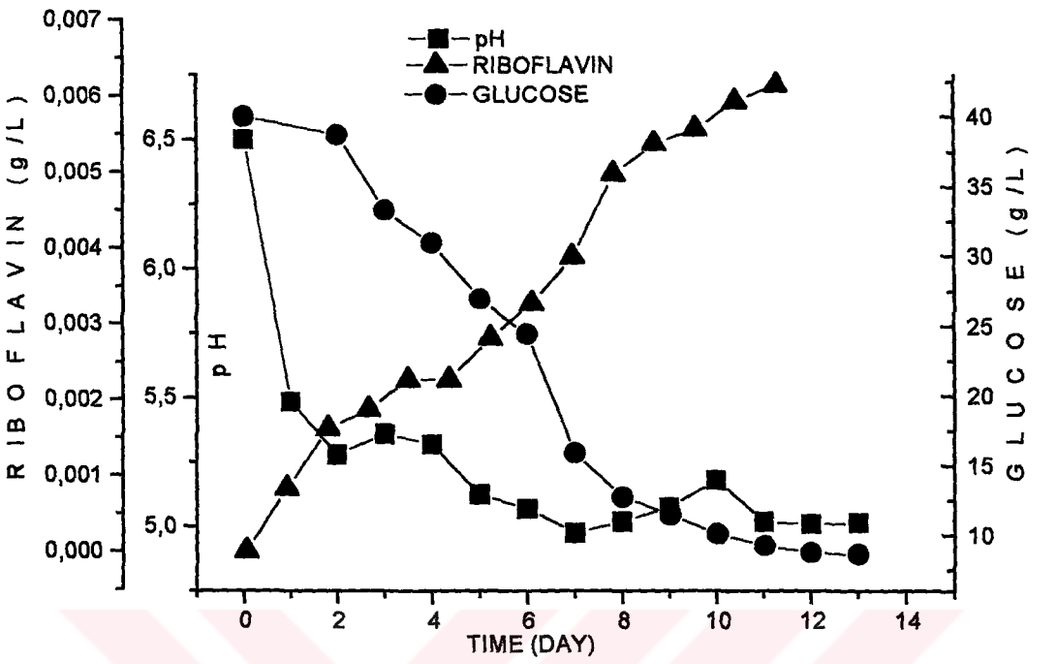

Figure 11. Plot of glucose, riboflavin concentration and pH versus fermentation time for Run No SF.4.

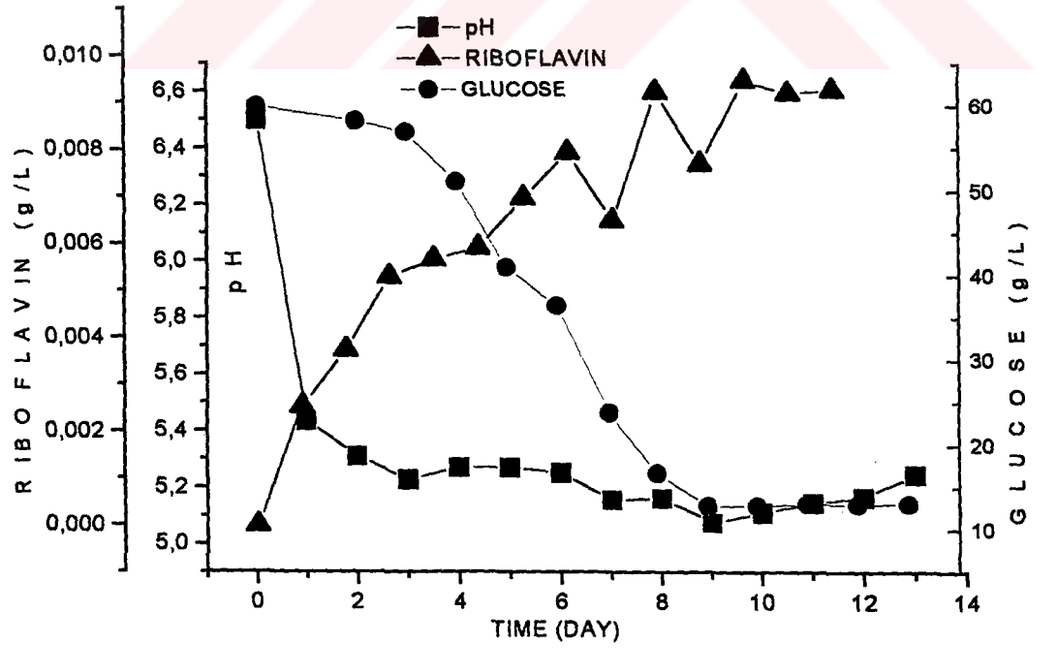

Figure 12. Plot of glucose, riboflavin concentration and pH versus fermentation time for Run No SF.5.

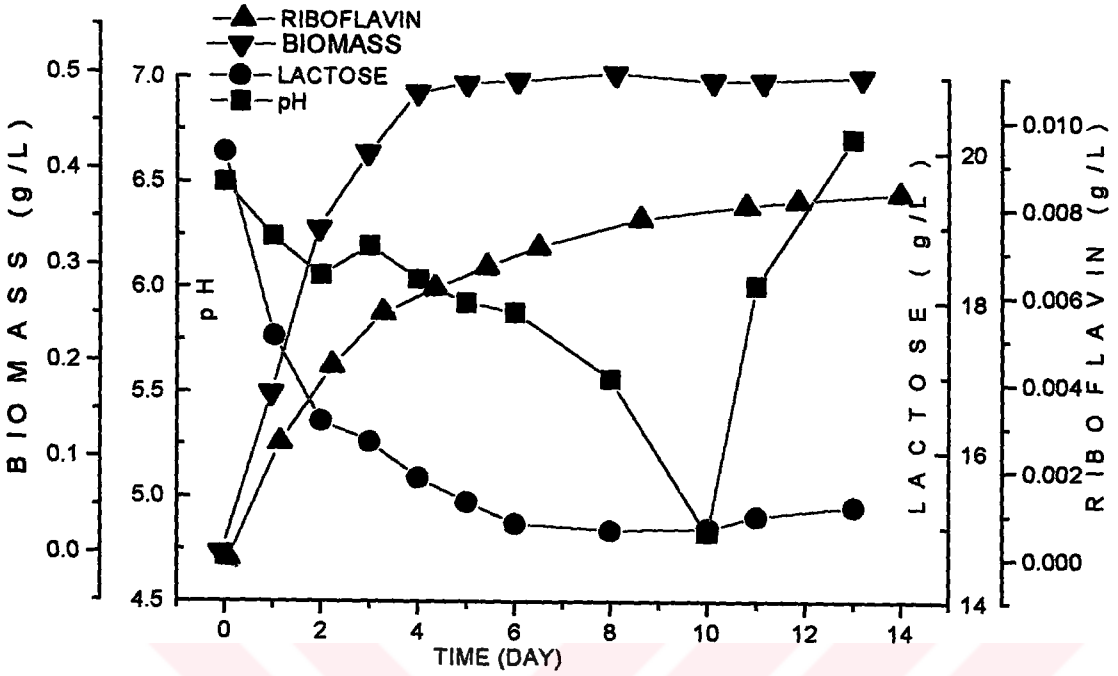

Figure 13. Plot of lactose, riboflavin, biomass concentration and pH versus fermentation time for Run No SF.6.

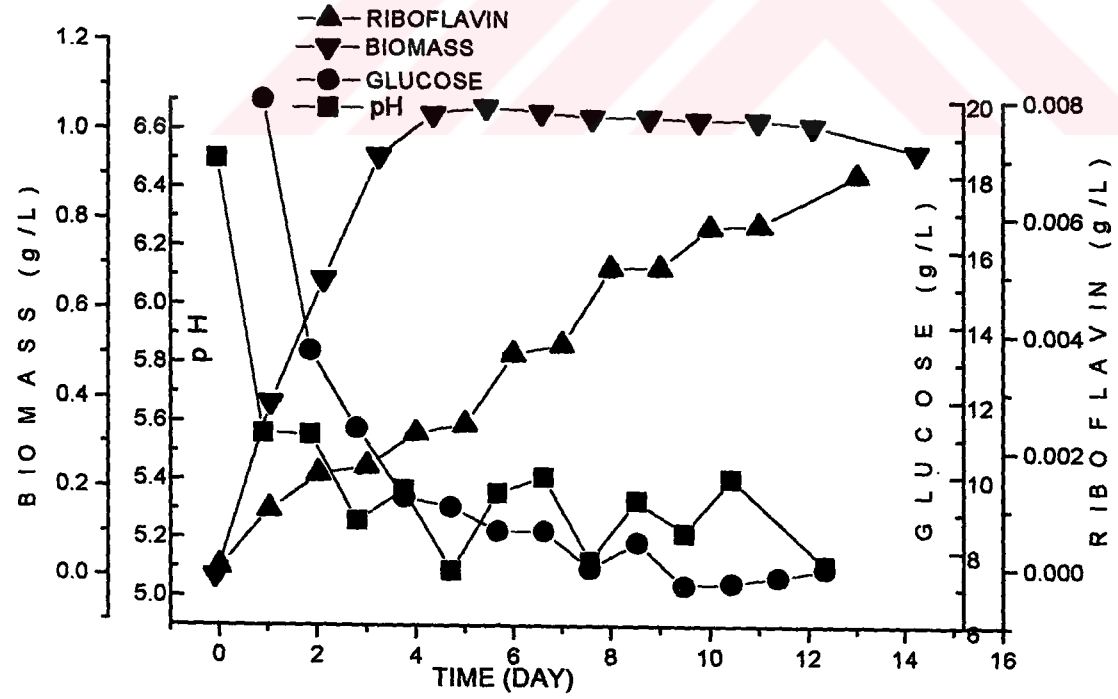

Figure 14. Plot of glucose, riboflavin, biomass concentration and pH versus fermentation time for Run No SF.7.

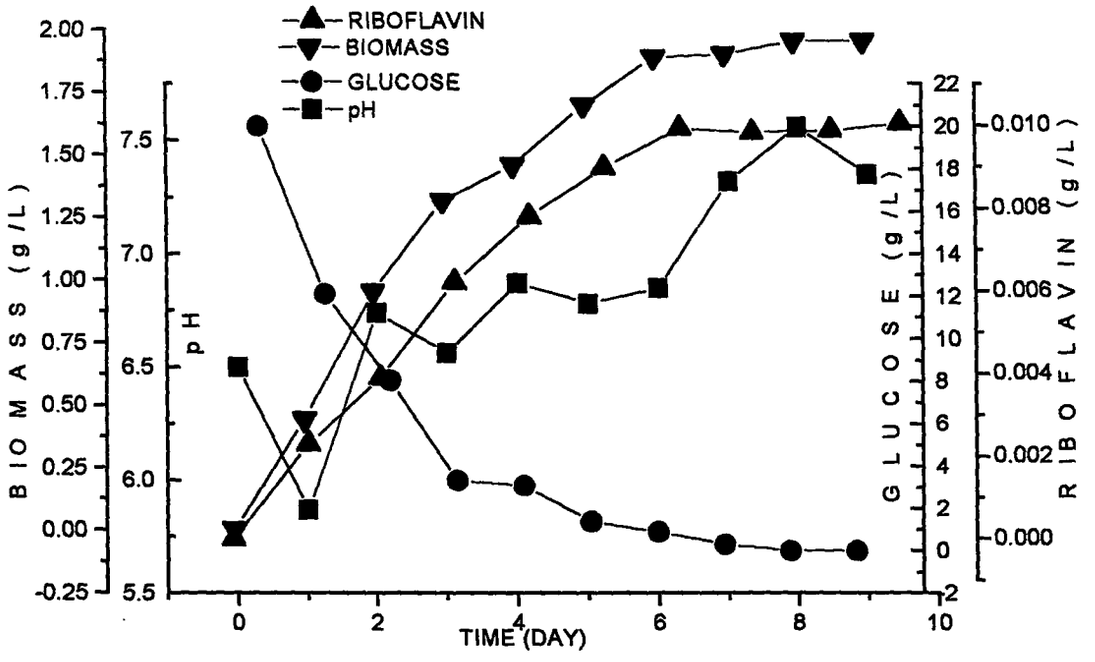

Figure 15. Plot of glucose, riboflavin, biomass concentration and pH versus fermentation time for Run No F.1.

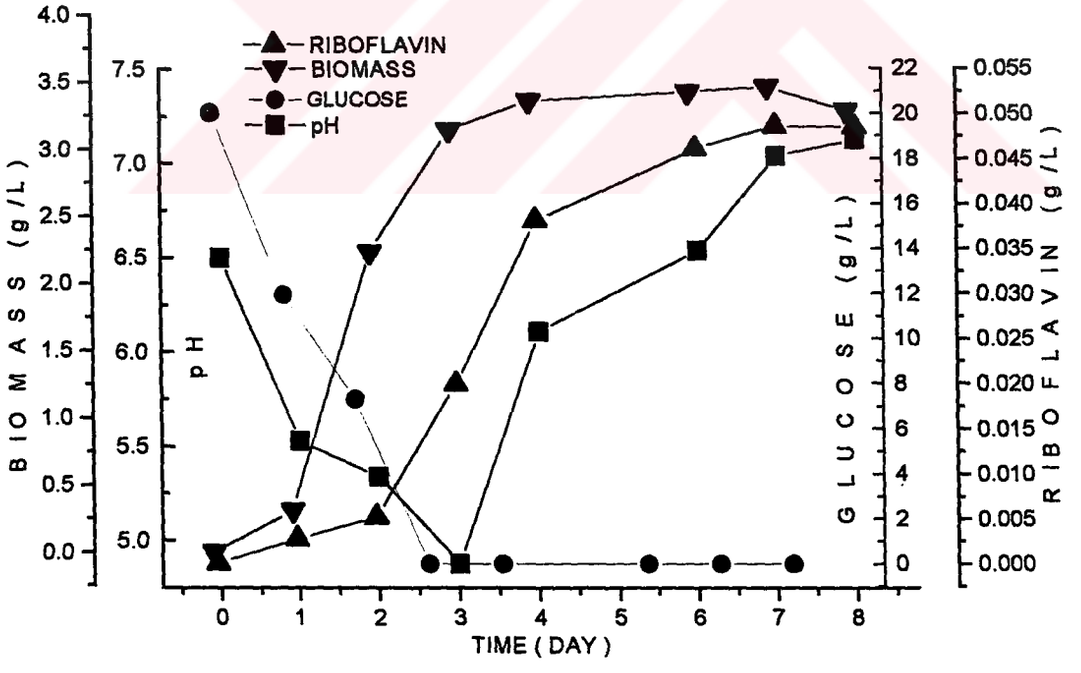

Figure 16. Plot of glucose, riboflavin, biomass concentration and pH versus fermentation time for Run No F.2.

4.3. Effect of medium pH change on riboflavin production

For all runs initial medium pH was 6.5. pH was not controlled throughout this study but pH values were measured and recorded.

The changes in pH of fermentation medium for all runs were illustrated in Figure 17. During fermentation, pH decreased and then raised with time up to 7. But changes in run no SF.4, SF.5, SF.7 showed different patterns that the initial drop in pH was not then followed by a substantial pH increase as in the other runs and noticed in the earlier studies.

Özbaş and Kutsal [17] suggest that, in riboflavin fermentations in *Eremothecium ashbyii* and *Ashbya gossypii*, the pH of the medium decreases with the time and drops to approximately 4.5. At this point the microorganisms are growing rapidly but only synthesize insignificant amount of riboflavin. Then both riboflavin production and pH increase and riboflavin formation is terminated at approximately 9.5. It was found that during biomass production in *Eremothecium ashbyii,* this decrease appeared to be the result of initial accumulation of pyruvate in the culture medium, which was then utilized [22]. Özbaş and Kutsal [17] also demonstrated that highest riboflavin productivity in both *Eremothecium ashbyii* and *Ashbya gossypii* occurred at an initial pH of 6.5, although they did not report subsequent changes in the medium pH over the fermentation period.

Another study by Seviour et al. [22] indicated that the initial drop in pH, corresponding to the phase of biomass production, was not then followed by a substantial pH increase as in this study. This can be related to the much lower riboflavin levels with this medium compared to the other studies with higher riboflavin yield. However these pH profiles may also be explained in terms of the different nitrogen sources used. For example, it was found that pH increase to the eventual utilization of complex organic nitrogen sources present in the medium [28].

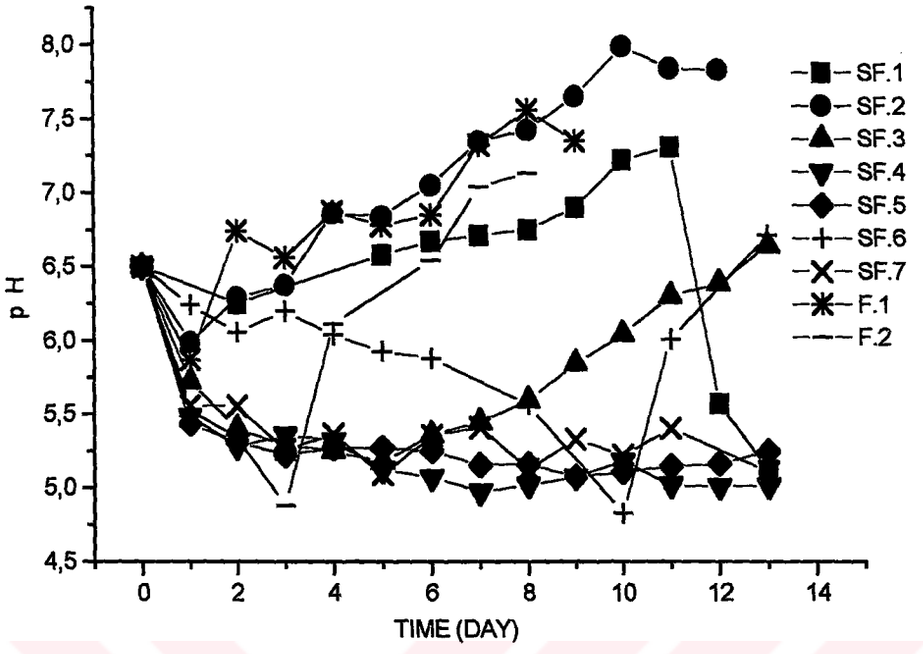

Figure 17. pH change of the medium during fermentation for all Runs.

4.4. Effect of substrate on riboflavin production

Substrate types and their concentrations used were tabulated in Table 7. The changes in substrate concentrations during fermentation for all runs were plotted against the time (Figure 18).

4.4.1. Shake flask studies

It can be seen from Figure 18 that glucose (20 g/L) only in Run No SF.3 was utilized completely after 10 days fermentation when the pH was reached to 6.41 after reduction of initial pH from 6.5 to 5.2. But in run no SF.7 glucose (20 g/L) consumption did not show the same pattern of in SF.3 as the other runs did. After fermentation was completed, residual substrate concentrations present in the culture media for run no SF.1, SF.2, SF.3, SF.4, SF.5, SF.6, SF.7, SF.8 was 8.794, 10.051, 0, 8.64, 13.052, 15.274 and 7.528 (g/L) respectively.

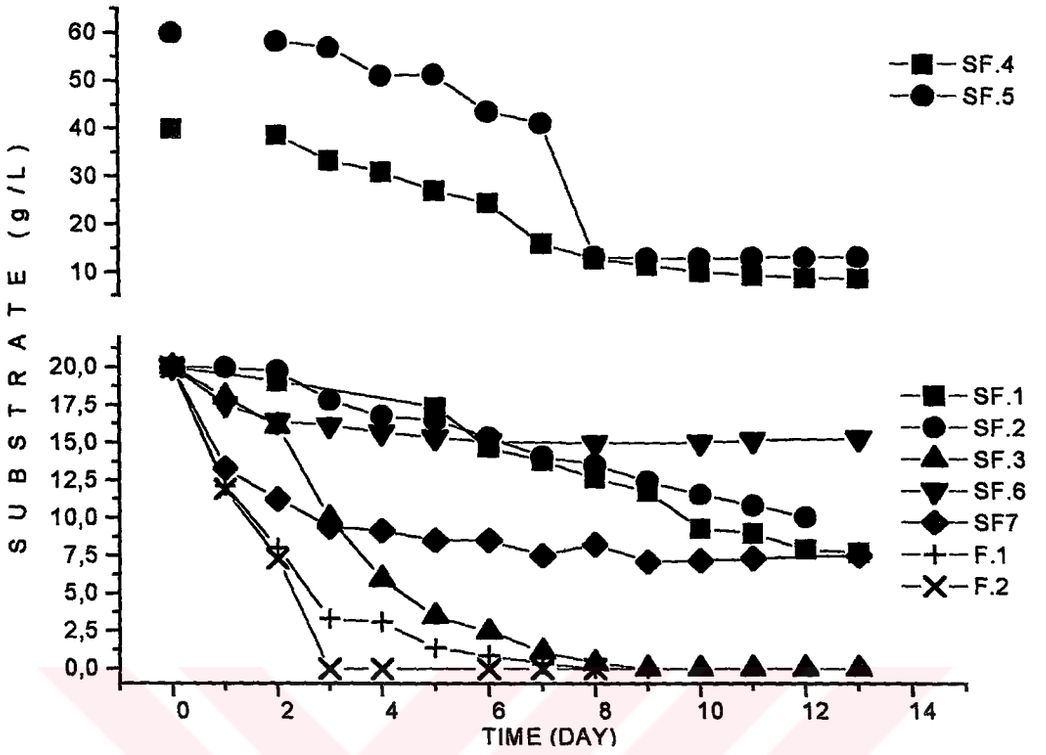

Figure 18. Changes in the substrate concentration during fermentation for all Runs.

The initial glucose concentration effects on the riboflavin production were investigated at the level of 20, 40 and 60 (g/L) and corresponding final riboflavin concentrations were 0.00973, 0.00615, 0.00928 (g/L) respectively. As it can be seen from Figure 18 that for Run No SF.3 glucose (20 g/L) was completely consumed and riboflavin was produced at a higher level than the others. Riboflavin concentration versus time plot of glucose concentrations were shown in Figure 19. Özbaş and Kutsal [15] also investigated the effects of initial glucose concentration from 10 to 30 (g/L) and they concluded that the optimum glucose concentration was 20 (g/L) for riboflavin production.

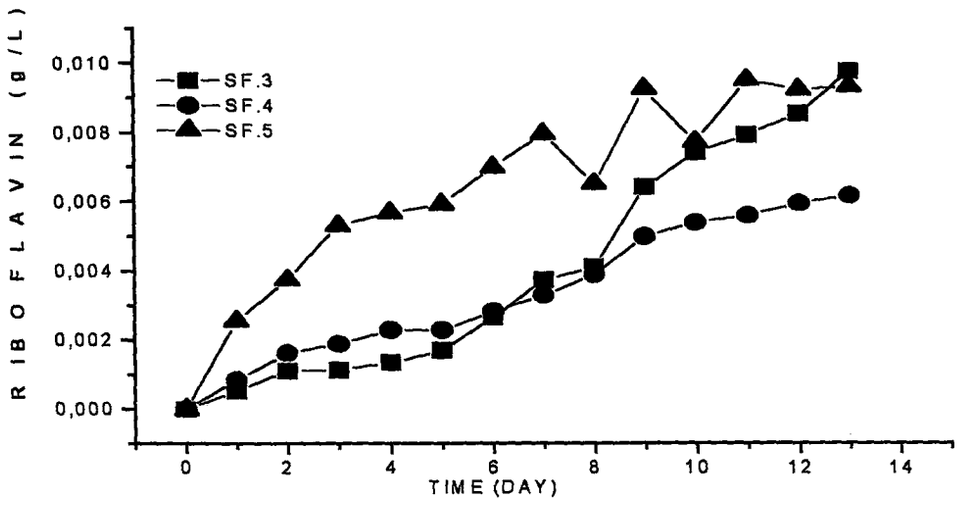

Figure 19. Effects of initial glucose concentrations (20, 40, 60 g/L) on riboflavin production (Run No SF.3, SF.4, SF.5 respectively).

It was important to note that in run no SF.7 obtained riboflavin concentration was lower than run no SF.3 and sugar consumption and pH profile also showed different pattern as noticed in SF.3 and earlier studies. In contrast to Run No SF.3, in Run No SF.7 glucose was not completely consumed until pH reaches its minimum level.

Normally maximum riboflavin production was observed when an initial drop in pH and followed by a substantial increase. The decrease in pH appeared to be related to the consumption of glucose in turn initial accumulation of pyruvate in the culture medium. pH changes of medium due to the accumulation and subsequent re-utilization of pyruvate may be important. Formation of biomass and riboflavin release into the medium initially closely followed each other, but then riboflavin levels in the medium continued to increase after biomass production slowed down. Initial drop in pH corresponds to the growth phase [22]. It was reported that almost all the glucose in the medium is utilized during the first 24 hr of the fermentation while the pH drops from 6.3 to 4.5 and no significant amounts of riboflavin are formed; thereafter up to 90-130 hr of fermentation riboflavin is rapidly

synthesized and pH of the medium gradually rises [13]. There were differences in the fermentation patterns obtained in this study when they were compared with literature in that sugar consumption were not completed in first day of fermentation. It may be due to the decrease in the activity of *Ashbya gossypii* because of long storage time.

The effects of the types of the substrates on the riboflavin production were investigated. During fermentation galactose, lactose, glucose (20 g/L) was used as a substrate in culture media. Figure 20 represents riboflavin production versus time for different substrates. Riboflavin concentration at the end of fermentation for galactose, lactose and glucose was 0.0097, 0.0172, 0.00973 (g/L) respectively. Washed suspensions of *Ashbya gossypii* completely metabolize glucose to the carbondioxide and water, probably via conventional glycolytic and tricarboxylic acid pathways [11]. Although it was reported that commercial glucose appeared to be the best source for riboflavin production, significant differences in final riboflavin concentration were not observed in this work.

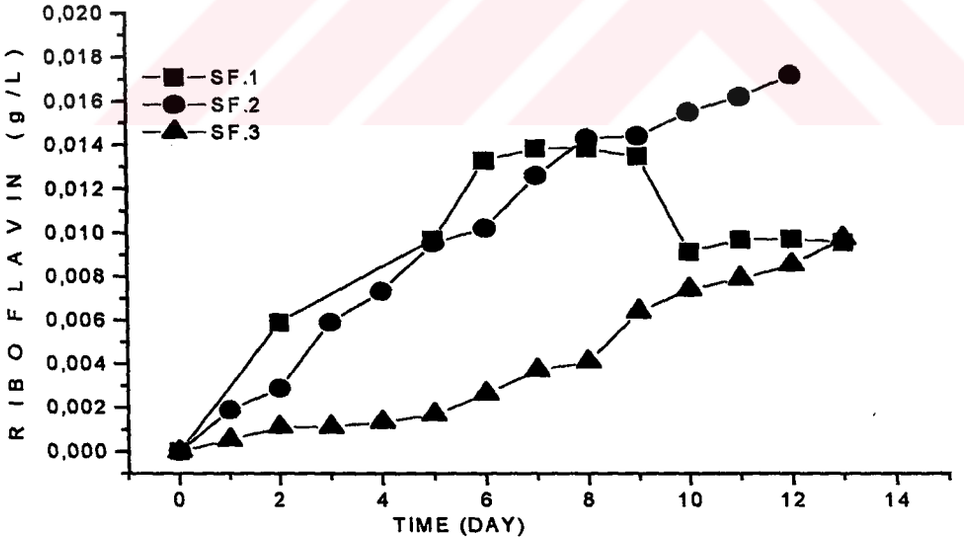

Figure 20. Effects of initial substrate (galactose, lactose and glucose) on riboflavin production.

There have been many studies on the effect of initial substrate on riboflavin production. For example Özbaş and Kutsal [20] studied the effects of glycerol, sunflower oil, whey and various combinations of these substrate on riboflavin production and growth. Hockenhull [11] explained the ability of various carbohydrates to support growth of *Ashbya gossypii* (Table 5). As it can be seen from the Table 5, glucose is the best carbon source than the lactose and galactose for supporting the growth of *Ashbya gossypii.* But there was no information about the produced riboflavin concentrations of these sources. As it was indicated before there were no significant differences between final riboflavin concentrations obtained from fermentations where lactose, galactose and glucose used as substrate.

4.4.2. Fermentor studies

0.25 vvm aeration and 200 rpm agitation rates were used in a batch stirred tank reactor using glucose (20 g/L) as a substrate. Optimum temperature and initial medium pH was 30°C and 6.5 respectively.

Glucose was completely consumed after 7 days for run no F.1 and 3 days for run no F.2 and change of glucose versus time was illustrated in Figure 21. During the fermentation of F.1, the initial pH of the culture media decreased down to 5.87, microorganism grew rapidly but produced insignificant amounts of riboflavin. Then both riboflavin formation increased and pH reached to 7.56. But in fermentation of F.2 pH decreased down to 4.88 at which sugar completely consumed and raised to 7.13 and obtained riboflavin concentration was much higher than that of F.1.

At the end of fermentation for Run No F.1 and F.2 obtained riboflavin concentrations were 0.0101 and 0.0484 (g/L) respectively. Plot of riboflavin versus time for F.1 and F.2 were represented in Figure 22.

In fermentor studies agitation rate used was lower than optimum value which was found as 250-300 rpm in literature [11, 14, 19]. Because of foaming problems and the fermentor used in this work agitation rate was set at 200 rpm.

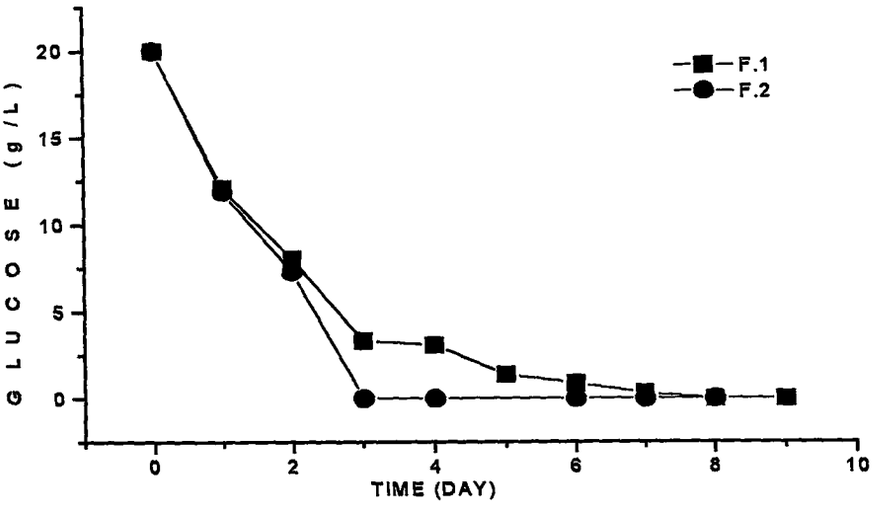

Figure 21. Consumption of glucose during fermentation in fermentor for run numbers F.1 and F.2.

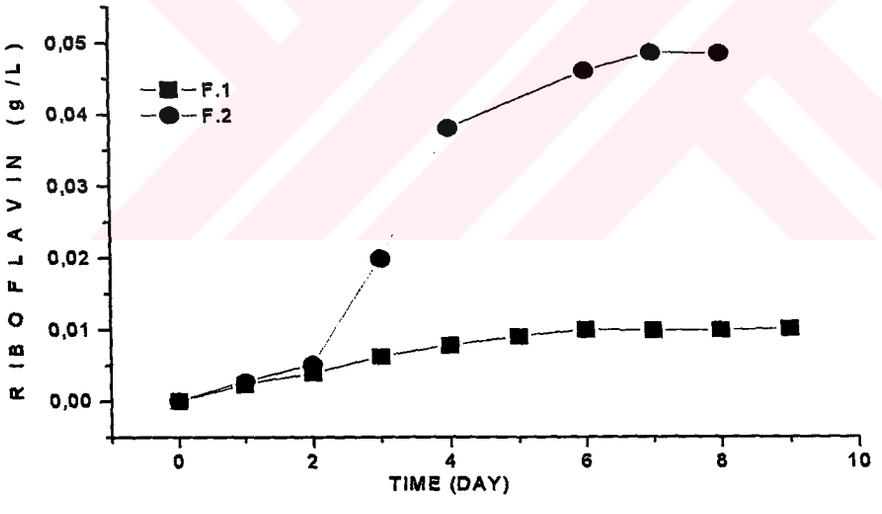

Figure 22. Plot of riboflavin versus time for Run No F.1and F.2.

It may be concluded in the light of above facts that during fermentation pH change and sugar consumption profile of the culture medium was very

important as other parameters such as optimum temperature, aeration rate etc. to obtain higher riboflavin production.

4.5. Product yields

For each run carbon fraction of all determined parameters and carbon yields of riboflavin were calculated and presented in Tables 12 through 23.

As it can be seen from the tables, carbon fraction of substrate (S_C), product (P_C) and for Run No SF.6, SF.7, F.1, and F.2 also biomass (X_C) were determined. From these fractions; (S_C/S_{C0})%, ($P_C/(S_{C0}-S_C)$)%, (P/S_{C0})% and for run no SF.6, SF.7, F.1 and F.2 ($X_C/(S_{C0}-S_C)$)% were calculated. Maximum yields were obtained from run no F.2 seen in the Tables 22, 23. Maximum riboflavin yield (P/S_{C0})% obtained from fermentor studies (run no F.2) was 0.605. In shake flasks, Özbaş and Kutsal [20] found the riboflavin yield (P/S_{C0})% as 3.1 in glucose containing media. Obtained riboflavin yield (P/S_{C0})% from this study in glucose containing media for shaking flasks was 0.106.

Table 11. Carbon fraction of sugar, riboflavin and riboflavin yield for Run No SF.1.

TIME (Day)	P_C	S_C	(S_C/S_{C0}) (%)	($P_C/(S_{C0}-S_C)$) (%)	(P/S_{C0}) (%)
0	0,00000	8,0000	100,0000	0,0000	0,0000
2	0,00320	7,6444	95,4300	0,8763	0,0740
5	0,00637	6,9416	86,7700	0,6023	0,1470
6	0,00721	5,8732	73,4150	0,3391	0,1660
7	0,00752	5,5006	68,8700	0,3020	0,1730
8	0,00750	5,0552	63,1900	0,2548	0,1730
9	0,00732	4,7028	58,7850	0,2220	0,1690
10	0,00495	3,7144	46,4300	0,1156	0,1140
11	0,00526	3,5896	44,8700	0,1192	0,1210
12	0,00527	3,1748	39,6850	0,1092	0,1210
13	0,00519	3,0846	38,5450	0,1055	0,1190

Table 12. Carbon fraction of sugar, riboflavin and riboflavin yield for Run No SF.2.

TIME (Day)	P_C	S_C	(S_C/S_{C0}) (%)	$(P_C/(S_{C0}-S_C))$ (%)	(P/S_{C0}) (%)
0	0,00000	8,4200	100,0000	0,0000	0,0000
1	0,00103	8,2824	98,3660	0,7499	0,0226
2	0,00157	7,9216	94,0803	0,3159	0,0344
3	0,00320	7,4993	89,0650	0,3480	0,0701
4	0,00396	7,0644	83,9000	0,2924	0,0867
5	0,00516	6,9356	82,3700	0,3475	0,1128
6	0,00554	6,4522	76,6300	0,2815	0,1211
7	0,00684	5,9205	70,3150	0,2737	0,1496
8	0,00776	5,6949	67,6350	0,2849	0,1698
9	0,00782	5,2276	62,0850	0,2449	0,1710
10	0,00842	4,8600	57,7200	0,2364	0,1841
11	0,00880	4,5540	54,0850	0,2275	0,1924
12	0,00934	4,2315	50,2550	0,2230	0,2043

Table 13. Carbon fraction of sugar, riboflavin and riboflavin yield for Run No SF.3.

TIME (Day)	P_C	S_C	(S_C/S_{C0}) (%)	$(P_C/(S_{C0}-S_C))$ (%)	(P/S_{C0}) (%)
0	0,00000	8,0000	100,0000	0,0000	0,0000
2	0,00060	7,2160	90,2000	0,0762	0,0012
3	0,00061	6,4396	80,4950	0,0390	0,0140
4	0,00073	3,9992	49,9900	0,0182	0,0168
5	0,00092	2,3812	29,7450	0,0163	0,0211
6	0,00144	1,3812	17,2650	0,0217	0,0331
07	0,00202	0,9863	12,3288	0,0288	0,0465
8	0,00223	0,4481	5,6018	0,0295	0,0513
9	0,00348	0,1448	1,8102	0,0442	0,0800
10	0,00402	0,0000	0,0000	0,0502	0,0925
11	0,00429	0,0000	0,0000	0,0536	0,0988
12	0,00462	0,0000	0,0000	0,0578	0,0990
13	0,00528	0,0000	0,0000	0,0660	0,1060

Table 14. Carbon fraction of sugar, riboflavin and riboflavin yield for Run No SF.4.

TIME (Day)	P_c	S_c	(S_c/S_{c0}) (%)	$(P_c/(S_{c0}- S_c))$ (%)	(P/S_{c0}) (%)
0	0,00000	16,0000	100,0000	0,0000	0,0000
2	0,00088	15,4800	96,7500	0,1692	0,0101
3	0,00102	13,3280	83,3000	0,0382	0,0118
4	0,00123	12,3852	77,4075	0,0341	0,0142
5	0,00123	10,7880	67,4250	0,0236	0,0142
6	0,00153	9,7784	61,1150	0,0246	0,0176
7	0,00178	6,3620	39,7630	0,0185	0,0205
8	0,00211	5,0964	31,8525	0,0193	0,0243
9	0,00270	4,5832	28,6448	0,0237	0,0311
10	0,00292	4,0352	25,2202	0,0244	0,0336
11	0,00302	3,7065	23,1654	0,0246	0,0348
12	0,00322	3,5025	21,8934	0,0258	0,0371
13	0,00334	3,4560	21,6000	0,0266	0,0384

Table 15. Carbon fraction of sugar, riboflavin and riboflavin yield for Run No SF.5.

TIME (Day)	P_c	S_c	(S_c/S_{c0}) (%)	$(P_c/(S_{c0}-S_c))$ (%)	(P/S_{c0}) (%)
0	0,00000	24,0004	100,0000	0,0000	0,0000
2	0,00203	23,3004	97,0850	0,2903	0,0156
3	0,00288	22,7792	94,9133	0,2357	0,0221
4	0,00308	20,4272	85,1133	0,0862	0,0236
5	0,00321	16,3718	68,2159	0,0421	0,0247
6	0,00379	14,5634	60,6817	0,0402	0,0291
7	0,00432	9,5147	39,6445	0,0298	0,0331
8	0,00353	6,6732	27,8050	0,0204	0,0271
9	0,00501	5,1220	21,3417	0,0265	0,0385
10	0,00418	5,1208	21,3367	0,0221	0,0321
11	0,00515	5,2132	21,7217	0,0274	0,0395
12	0,00500	5,2064	21,6933	0,0266	0,0383
13	0,00504	5,2208	21,7533	0,0268	0,0387

Table 16. Carbon fractions of sugar, riboflavin and biomass for Run No SF.6.

TIME (Day)	P_c	X_c	S_c	(S_c/S_{c0}) (%)
0	0,00000	0,0000	8,4200	100,000
1	0,00143	0,0767	7,3877	87,7398
2	0,00239	0,1562	6,9103	82,0700
3	0,00305	0,1922	6,7933	80,6800
4	0,00336	0,2213	6,5924	78,2950
5	0,00362	0,2259	6,4563	76,6781
6	0,00387	0,2272	6,3361	75,2500
8	0,00422	0,2309	6,2948	74,7603
10	0,00438	0,2272	6,3063	74,8973
11	0,00444	0,2272	6,3766	75,7200
13	0,00453	0,2292	6,4303	76,3699

Table 17. Biomass and riboflavin yields for Run No SF.6.

TIME (Day)	$(X_c/(S_{c0}-S_c))$ (%)	$(P_c/(S_{c0}-S_c))$ (%)	(P/S_{c0}) (%)
0	0,0000	0,0000	0,0000
1	7,4269	0,1389	0,0314
2	10,3435	0,1583	0,0523
3	11,8145	0,1873	0,0666
4	12,1089	0,1836	0,0734
5	11,5047	0,1844	0,0792
6	10,9025	0,1858	0,0847
8	10,8633	0,1985	0,0923
10	10,7493	0,2073	0,0958
11	11,1135	0,2173	0,0971
13	11,5213	0,2276	0,0990

Table 18. Carbon fractions of sugar, riboflavin and biomass for Run No SF.7.

TIME (Day)	P_c	X_c	S_c	(S_c/S_{c0}) (%)
0	0,00000	0,000	8,0000	100,0000
1	0,00053	0,1795	5,3270	66,5852
2	0,00086	0,3068	4,5040	56,0000
3	0,00094	0,4345	3,7720	47,1500
4	0,00125	0,4780	3,6701	45,8758
5	0,00133	0,4856	3,4116	42,6450
6	0,00198	0,4804	3,4106	42,6321
7	0,00207	0,4751	3,0114	37,6419
8	0,00279	0,4751	3,2975	41,2182
9	0,00279	0,4727	2,8384	35,4795
10	0,00317	0,4727	2,8612	35,7650
11	0,00319	0,4674	2,9249	36,5607
13	0,00365	0,4398	3,0114	37,6419

Table 19. Biomass and riboflavin yields for Run No SF.7.

TIME (Day)	$(X_c/(S_{c0}-S_c))$ (%)	$(P_c/(S_{c0}-S_c))$ (%)	(P/S_{c0}) (%)
0	0,0000	0,0000	0,0000
1	6,7135	0,0199	0,0123
2	8,7748	0,0247	0,0199
3	10,2771	0,0222	0,0216
4	11,0395	0,0288	0,0288
5	10,5824	0,0290	0,0306
6	10,4666	0,0432	0,0456
7	9,5229	0,0416	0,0478
8	10,1023	0,0592	0,0641
9	9,1582	0,0540	0,0641
10	9,1989	0,0616	0,0729
11	9,2101	0,0628	0,0734
13	8,8161	0,0733	0,0841

Table 20. Carbon fractions of sugar, riboflavin and biomass for Run No F.1.

TIME (Day)	P_C	X_C	S_C	(S_C/S_{C0}) (%)
0	0,00000	0,0000	8,0000	100,0000
1	0,00125	0,2042	4,8464	60,5800
2	0,00211	0,4403	3,2080	40,1000
3	0,00337	0,6075	1,3280	16,6000
4	0,00423	0,6727	1,2356	15,4450
5	0,00487	0,7840	0,5456	6,8200
6	0,00539	0,8727	0,3504	4,3800
7	0,00534	0,8778	0,1204	1,5050
8	0,00536	0,9027	0,0000	0,0000
9	0,00546	0,9032	0,0000	0,0000

Table 21. Biomass and riboflavin yields for Run No F.1.

TIME (Day)	$(X_C/(S_{C0}-S_C))$ (%)	$(P_C/(S_{C0}-S_C))$ (%)	(P/S_{C0}) (%)
1	6,4753	0,0396	0,0288
2	9,1879	0,0441	0,0486
3	9,1057	0,0505	0,0775
4	9,9443	0,0625	0,0974
5	10,5175	0,0653	0,1121
6	11,4087	0,0704	0,1240
7	11,1402	0,0677	0,1229
8	11,2844	0,0670	0,1234
9	11,2901	0,0682	0,1256

Table 22. Carbon fractions of sugar, riboflavin and biomass for Run No F.2.

TIME (Day)	P_c	X_c	S_c	(S_c/S_{c0}) (%)
0	0,00000	0,0000	8,0000	100,0000
1	0,00147	0,1428	4,7636	59,5450
2	0,00277	1,0323	2,9156	36,4450
3	0,01075	1,4521	0,0000	0,0000
4	0,02063	1,5519	0,0000	0,0000
6	0,02489	1,5833	0,0000	0,0000
7	0,02634	1,6013	0,0000	0,0000
8	0,02628	1,5191	0,0000	0,0000

Table 23. Biomass and riboflavin yields for Run No F.2.

TIME (Day)	$(X_c/(S_{c0}-S_c))$ (%)	$(P_c/(S_{c0}-S_c))$ (%)	(P/S_{c0}) (%)
1	4,4110	0,0453	0,0338
2	20,3026	0,0545	0,0638
3	18,1508	0,1344	0,2475
4	19,3982	0,2579	0,4750
6	19,79109	0,3122	0,5750
7	20,0162	0,3292	0,6063
8	18,9882	0,3285	0,6050

Carbon fraction of residual sugar, yield for riboflavin produced and biomass growth according to consumed substrate at the end of fermentation for each run were illustrated in Table 24. Although high amount of residual lactose (SF.2, SF.6) was determined at the end of fermentation time, riboflavin yields obtained were higher than those of other runs. Sugar consumption and riboflavin yield for Run No SF.3 and F.1 showed approximately same results. Sugar was completely consumed in Run No SF.3, F.1 and F.2 but although same glucose level was used in SF.7 38%

residual sugar was obtained at the end of fermentation. In Run No SF.4 and SF.5 carbon fractions of residual sugar and riboflavin yields were approximately the same.

Sugar was consumed at very low levels for product formation and biomass growth in Run No SF.6, SF.7, F.1 and F.2. As it is seen from Table 24 approximately 10-20 % of substrate carbon was used for growth and product formation. Remaining 80-90 % of substrate carbon assumed to be consumed for maintenance requirements of the cell and released as carbondioxide.

Measurements for Run No SF.6 and SF.7 were done by using different flasks for each day analysis during fermentation. This may be result in differences between determined data of riboflavin, biomass, sugar and pH.

In Run No F.2, riboflavin produced was higher than Run No F.1. This may be differences in the glucose consumption and pH pattern during fermentation. In Run No F.1, all glucose was consumed after 8 days, pH decreased from 6.5 to 5.87 and than raised to 7.35. In Run No F.2, after 3 days pH decreased from 6.5 to 4.88 where all glucose was completely consumed and than raised to 7.13. As it can be seen from the results pH and sugar consumption patterns for Run No F.2 was much suitable than F.1 for riboflavin production.

Table 24. Carbon yield of substrate, riboflavin and biomass obtained at the end of fermentation for each run.

RUN NO	(S_c/S_{c0}) (%)	$(P_c/(S_{c0}-S_c))$ (%)	$(X_c/(S_{c0}-S_c))$ (%)
SF.1	38,5450	0,1055	-
SF.2	50,2550	0,2230	-
SF.3	0,0000	0,0660	-
SF.4	21,6000	0,0266	-
SF.5	21,7533	0,0268	-
SF.6	76,3699	0,2276	11,5213
SF.7	37,6419	0,0733	8,8161
F.1	0,0000	0,0682	11,2900
F.2	0,0000	0,3285	18,9900

4.6. Determination of kinetic parameters

4.6.1. Modeling of the growth curve and determination of biomass production kinetics

Biomass concentrations were determined daily for run no SF.6, SF.7, F.1 and F.2. Biomass concentration is, since the early days of bioprocessing, of promant interest to scientists as well as engineers. It is a simple measure for the available quantity of biocatalyst. It is definitely an important key variable because it determines -among others- the rates of the growth and/or product formation. All mathematical models used to describe microbial or cell growth or product formation contain biomass as the most important state variable. The observation of constant biomass concentration is a first criterion for assuming a population to be in steady state. The measure of a mass is important with respect to calculate mass balance; however, the elemental composition of biomass is normally ill defined. Another reason for determining bio-mass is the need for a reference when calculating specific rates [29].

Growth curves of *Ashbya gossypii* were drawn and also modeled. Prediction of growth curve of *Ashbya gossypii* with model equations and its comparison of experimental data were represented in Figures 23 through 26.

In order to build these models, growth has to be measured and modeled. Growth shows a phase in which the specific growth rate starts at a maximum value (μ_{max}) in a certain period of time, resulting in a lag time (λ). In addition, growth curves contain a final phase in which rate decreases and finally reaches zero, so that an asymptote (A) is reached. To describe such a curve and to reduce measured data to a limited number of interesting parameters, investigators need adequate models. A number of growth models are found in the literature such as the model of Gompertz, Richards, Stannard et al. , Schnute, Logistic model, Boltzman model and others. These models describe only the number of microorganism and do not include the consumption of substrate as a model based on the monod equation would do [30].

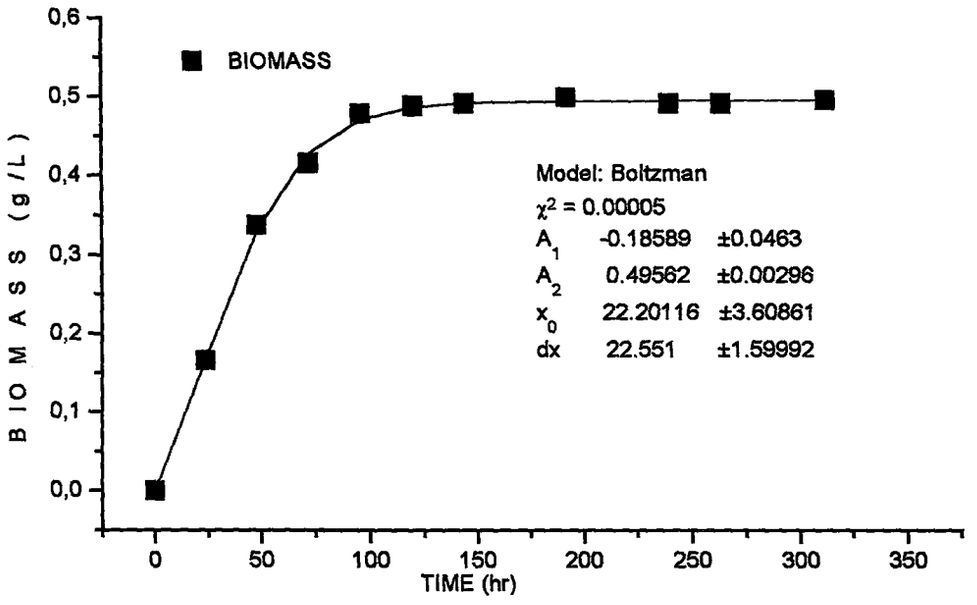

Figure 23. Growth curve of *Ashbya gossypii* with model curve fit for Run No SF.6.

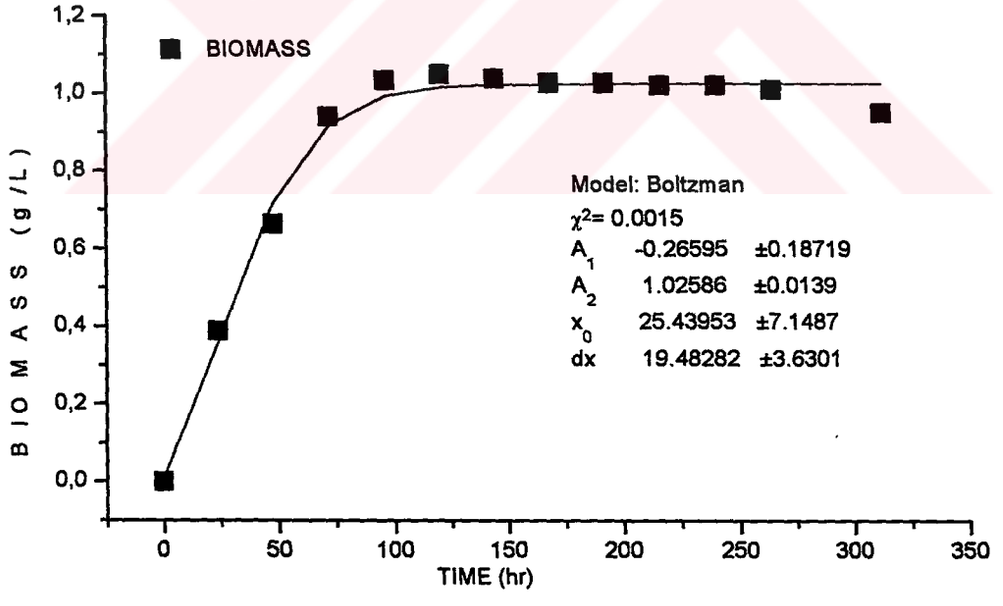

Figure 24. Growth curve of *Ashbya gossypii* with model curve fit for Run No SF.7.

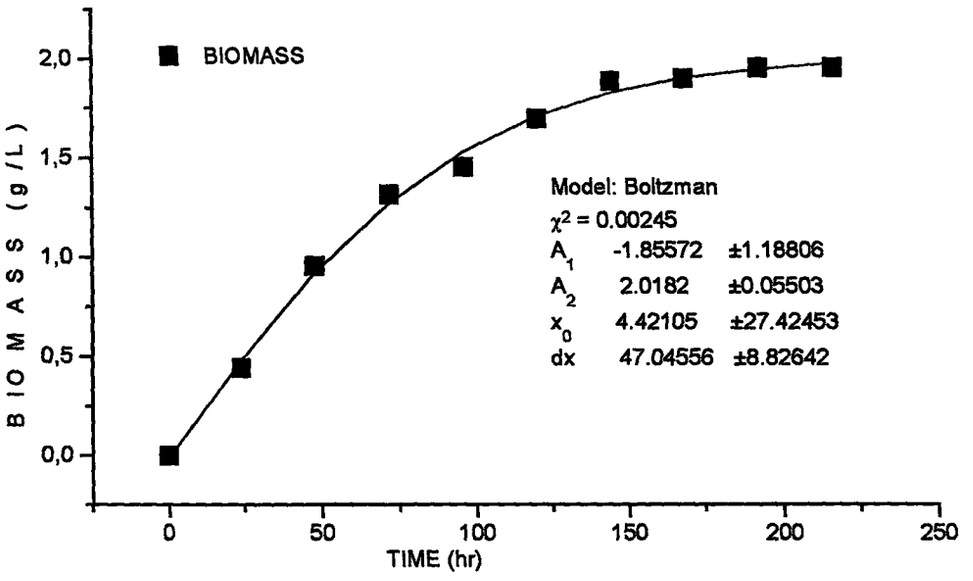

Figure 25. Growth curve of *Ashbya gossypii* with model curve fit for Run No F.1.

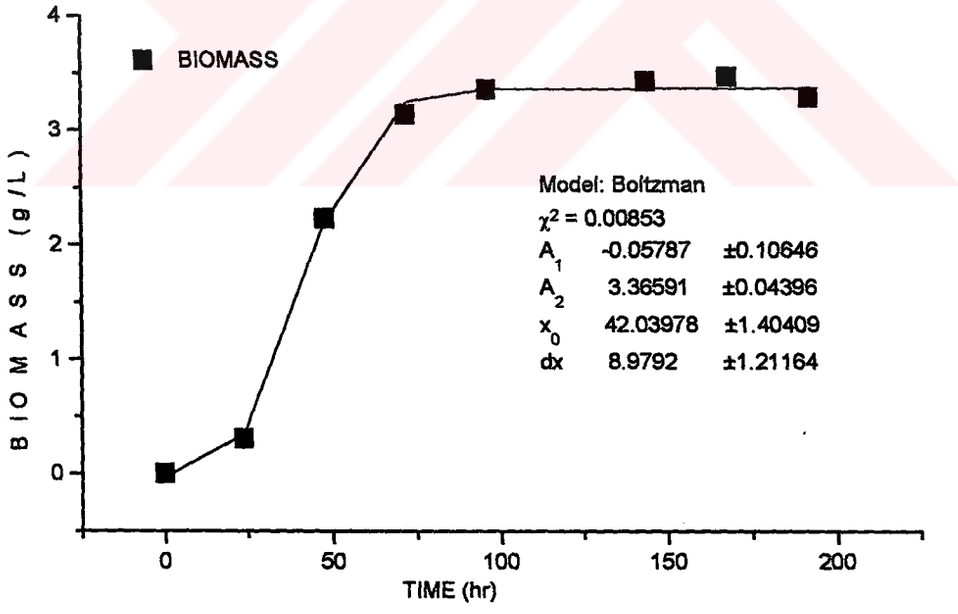

Figure 26. Growth curve of *Ashbya gossypii* with model curve fit for Run No F.2.

As it can be seen from Figures Boltzman equation was found to be a suitable model fit for growth curve of *Ashbya gossypii*.

Equation was;

$$x = (A_1 - A_2)/(1 + e^{(t - to)/dt}) + A_2 \qquad (1)$$

The plot of biomass concentration against the time gave a sigmoidal curve. Figure 27 describes a model curve with equation constants derivation.

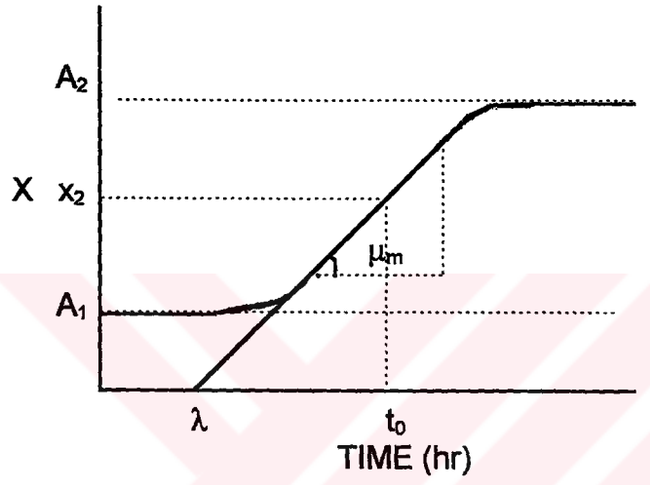

Figure 27. A growth curve of microorganism.

Obtained constants for model fits were represented on the Figures. χ^2 indicates the goodness of the fits which should have small value.

Models are used to describe the behavior of microorganisms under different physical or chemical conditions such as, pH and water activity. These models allow the prediction of microbial safety or shelflife of products, the detection of critical parts of the production and distribution chains [30].

At the end of the lag phase the population of the microorganism is still well adjusted to its environment. The cells then multiply, and cell mass or the number of living cells doubles regularly with time. The equations [30]

$$(dx)/(dt) = \mu x \qquad (2)$$

with $x = x_0$ at $t = t_{lag} = 0$

describe the increase in cell numbers during this period. Thus the rate of increase in x is proportional to the integrated form of Equation (2).

$$\ln(x/x_0) = \mu(t - t_{lag}) \text{ or } x = e^{\mu(t - t_{lag})} \quad (3)$$

$$\ln x = \ln x_0 + \mu t \quad (4)$$

Expressions for rate are usually nonlinear. The specific growth rate, μ, is defined as [31]

$$\mu = (1/x)(dx/dt) \quad (5)$$

Specific growth rates of *Ashbya gossypii* were determined by using Equation 5 for run no SF.5, SF.6, F.1 and F.2. All data obtained from model fits.

If the value of μ is constant, Equation 5 represents the so-called exponential growth, where growth is proportional to the mass of cells present [31].

Equation 4 implies that the plot of lnx versus time is a straight line provided that μ is a constant. Intercept of the line was $\ln x_0$ and slope was μ_{max}, when the organisms grow exponentially [29, 30]. Besides the lag period and asymptotic value, another valuable parameter of the growth curve is the maximum specific growth rate (μ_{max}) [30].

μ_{max} and x_0 for run no SF.6, SF.7, F.1 and F.2 was determined by deciding subjectively which part of the curve is linear and then determining the slope of this curve section eventually by linear regression. Figures 28 through 31 represent linear parts of lnx versus time for determination of μ_{max} and x_0.

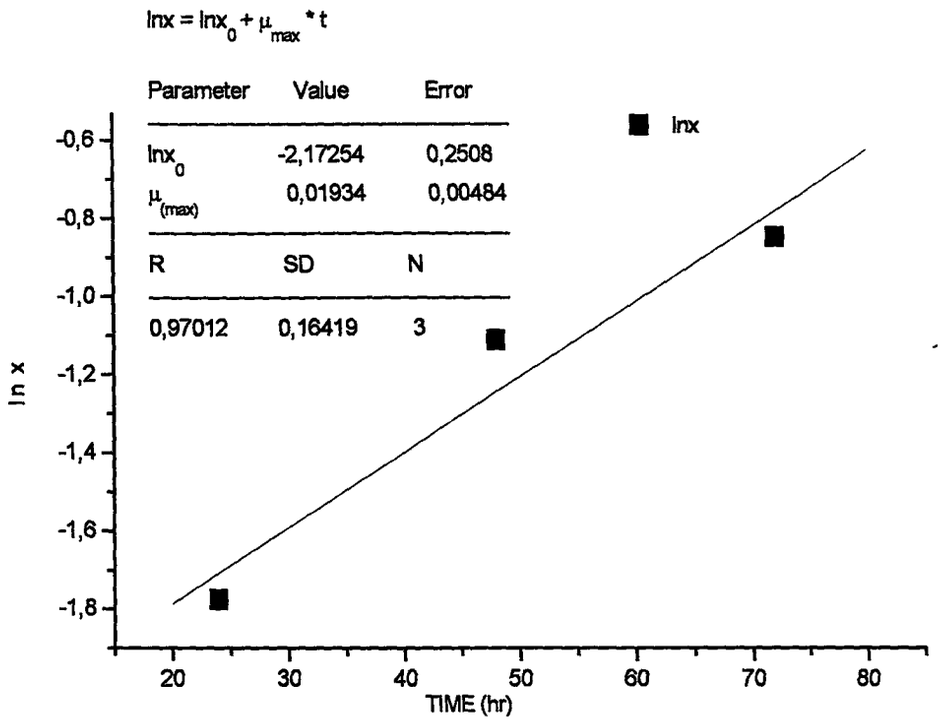

Figure 28. Plot of lnx versus time for Run No SF.6.

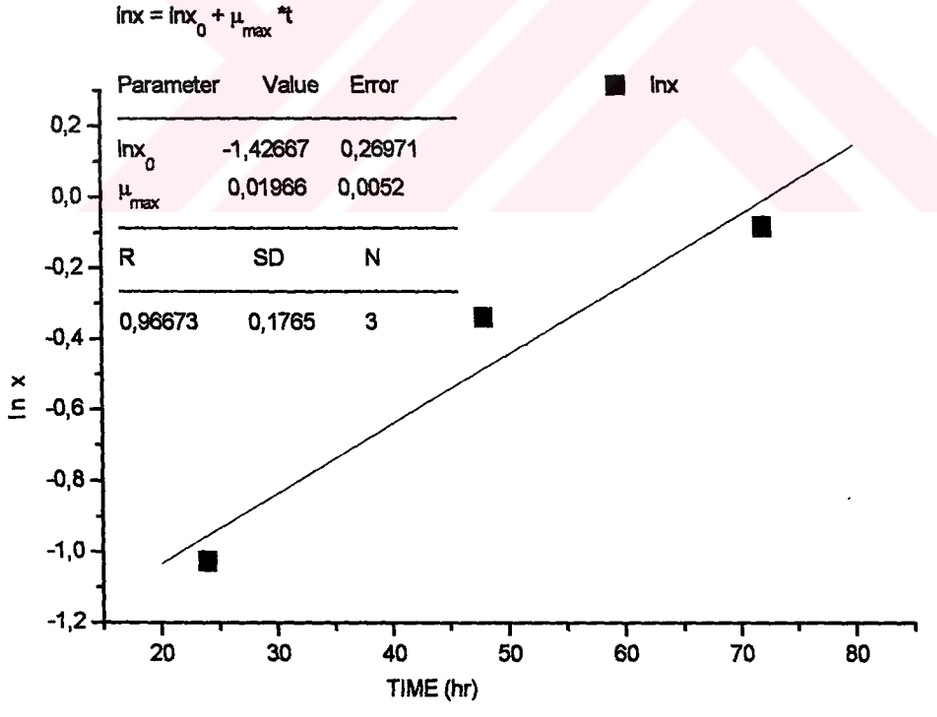

Figure 29. Plot of lnx versus time for Run No SF.7.

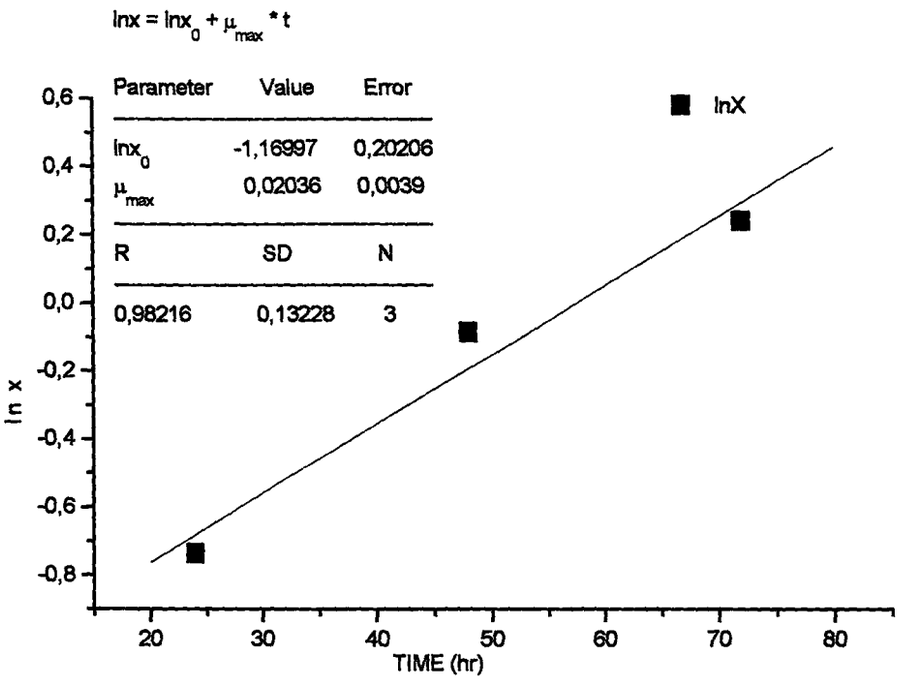

Figure 30. Plot of lnx versus time for Run No F.1.

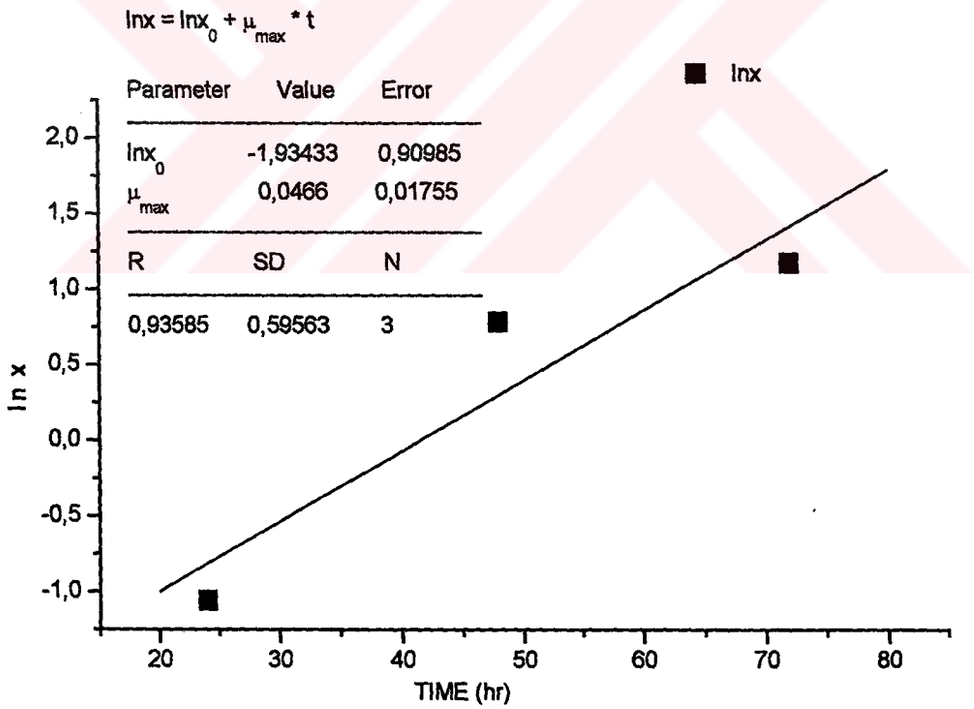

Figure 31. Plot of lnx versus time for Run No F.2.

All obtained parameters were represented in Table 25 through 28 for each run.

Table 25. Determined biomass parameters for Run No SF.6.

TIME (hr)	x	lnx	dx/dt	μ	μ_{max}
24	0.1694	-1.7754	0.006879	0.0406150	0.01933
48	0.3295	-1.1101	0.005472	0.0166700	
72	0.4287	-0.8470	0.002958	0.0068950	
96	0.4714	-0.7520	0.001217	0.0025830	
120	0.4871	-0.7192	0.000442	0.0009020	
144	0.4926	-0.7080	0.000138	0.0002790	
168	0.4951	-0.7031	0.000029	0.0000570	
240	0.4953	-0.7026	0.000004	0.0000070	
264	0.4954	-0.7025	0.000000	0.0000020	

Table 26. Determined biomass parameters for Run No SF.7.

TIME (hr)	x	lnx	dx/dt	μ	μ_{max}
24	0.3581	-1.0269	0.0146780	0.0409908	0.01967
48	0.7127	-0.3388	0.0118090	0.0165713	
72	0.9202	-0.0832	0.0057520	0.0062508	
96	0.9936	-0.0064	0.0020100	0.0020233	
120	1.0161	0.0159	0.0006200	0.0006104	
144	1.0230	0.0227	0.0001820	0.0001779	
168	1.0248	0.0245	0.0000530	0.0000517	
192	1.0254	0.0251	0.0000150	0.0000150	
216	1.0256	0.0252	0.0000040	0.0000042	
240	1.0256	0.0253	0.0000013	0.0000013	

Table 27. Determined biomass parameters for Run No F.1.

TIME (hr)	x	lnx	dx/dt	μ	μ_{max}
24	0.479	-0.735	0.0192500	0.4016667	
48	0.919	-0.085	0.0165416	0.0180000	0.02038
72	1.274	0.242	0.0128333	0.0100830	
96	1.534	0.428	0.0091250	0.0059580	
120	1.712	0.538	0.0061250	0.0035833	
144	1.829	0.604	0.0039583	0.0021667	
168	1.902	0.643	0.0024583	0.0012917	
192	1.947	0.666	0.0015412	0.0007917	
216	1.975	0.681	0.0011667	0.0005833	

Table 28. Determined biomass parameters for Run No F.2.

TIME (hr)	x	lnx	dx/dt	μ	μ_{max}
24	0.347	-1.059	0.0463150	0.1335188	
48	2.202	0.789	0.0603675	0.0274118	0.04663
72	3.247	1.178	0.0241371	0.0074339	
96	3.358	1.211	0.0023916	0.0007142	
144	3.366	1.214	0.0000900	0.0000268	
168	3.366	1.214	0.0000013	0.0000003	
192	3.366	1.214	0.0000004	0.0000001	

Maximum specific growth rates obtained for Run No SF.6, SF.7, F.1 and F.2 were 0.01933, 0.01960, 0.02038, 0.04663 and riboflavin yield (P/S_{c0}%) determined were 0.0990, 0.08413, 0.1256, 0.6050 respectively. Higher riboflavin yield was obtained at higher maximum specific growth rate from Run No F.2. It can be seen from the results that riboflavin production was parallel with maximum specific growth rate so higher riboflavin yield was obtained at higher maximum specific growth rate.

Özbaş and Kutsal [20] determined the riboflavin yield (P/S_{c0} %) and maximum specific growth rate as 3.1, 0.1538 (hr^{-1}) respectively. Riboflavin yields and maximum specific growth rates obtained in this study were lower than literature values, probably due to the lower activity of culture used.

4.6.2. Product formation kinetics

Riboflavin production kinetics of *Ashbya gossypii* has been studied in synthetic media. Riboflavin versus time was plotted against the time and modeling of riboflavin production was done. Figures 32 through 35 represents the comparison of experimental data with model fit. Model fits obtained for Run No F.1 and F.2 were better than Run No SF.6 and SF.7. Intervals of parameters in Boltzman equation were much narrover which indicates better fit. This was expected result because Boltzman model fits better to sigmoidal shape curves than the others.

When a substrate is stoichiometrically converted to a single product, P, the rate of product formation is related to the rate of growth by;

$$(dP/dt) = \alpha * (dx/dt) \qquad (6)$$

provided

α = stochiometric constant

This is the so called growth-associated model [31]. Product is formed from primary metabolism. The products are referred to as growth associated products, as their rate of production parallels the growth of cell population [32].

For cases where the rate of product formation is only depend on cell concentration, the cell has a constitutive enzyme system that the product formation rate, then

$$(dP/dt) = \beta * x \qquad (7)$$

β = proportionality constant [31].

Secondary metabolites are termed non-growth-associated products, and their kinetics do not depend on the rate of growth of culture [32]. Production rate is proportional to the cell concentration rather than growth rate [31].

An intermediate class of products can also be identified, where product formation kinetic lies between the two classes above. Such products are partially growth associated.

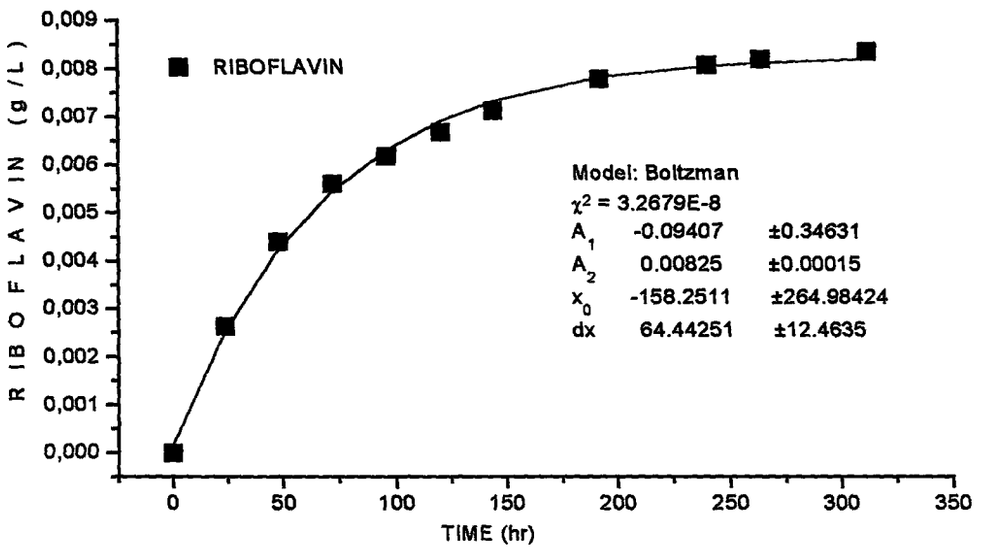

Figure 32. Comparison of model fit with experimental data for Run No SF.6.

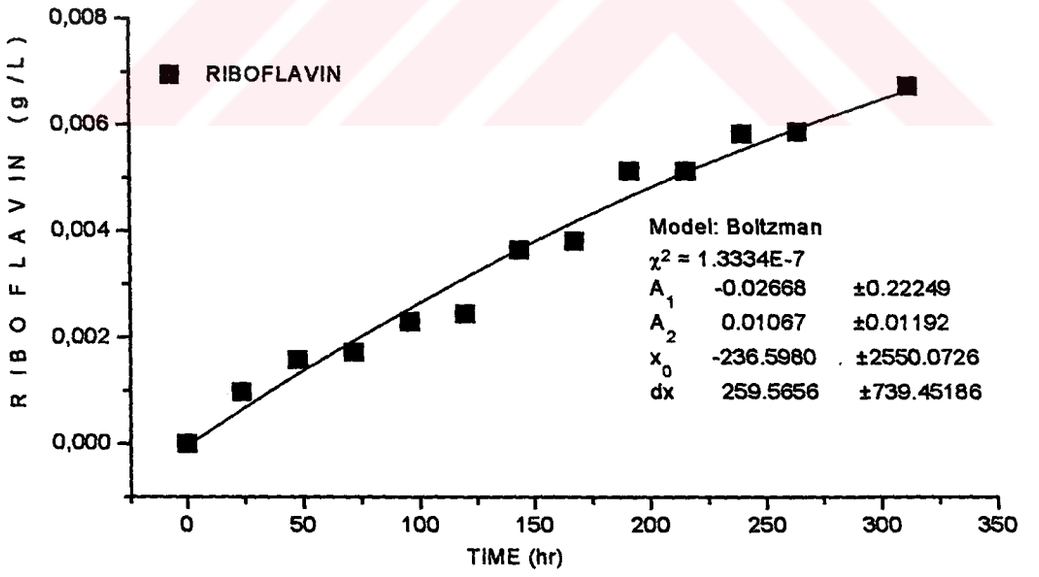

Figure 33. Comparison of model fit with experimental data for Run No SF.7.

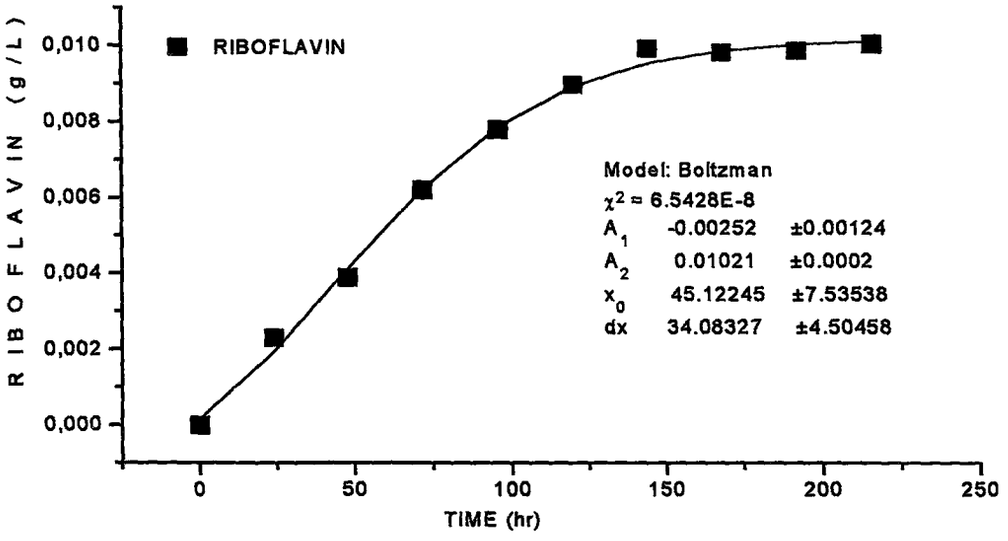

Figure 34. Comparison of model fit with experimental data for Run No F.1.

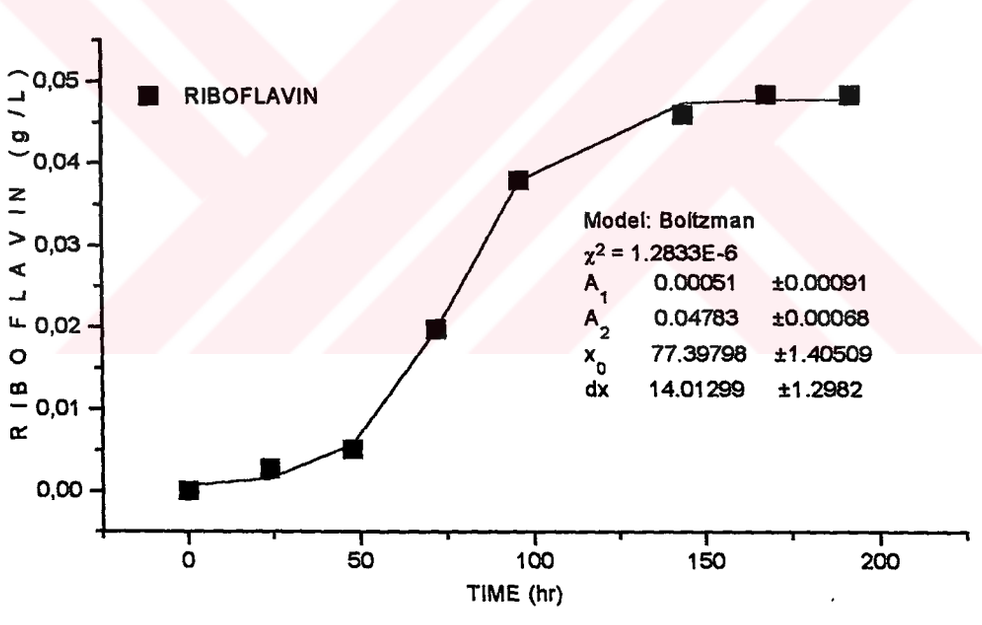

Figure 35. Comparison of model fit with experimental data for Run No F.2.

The development of constitutive rate expression for these classes of product formation arise from the studies on the formation of lactic acid by

Lactobacillus delbrueckii [32]. It was proposed that Equations 5 and 6 be combined to express the productivity of lactic acid fermentations

$$(dP/dt) = \alpha * (dx/dt) + \beta * x$$

$$(1/x) * (dP/dt) = \alpha * (1/x) * (dx/dt) + \beta \qquad (8)$$

α = growth associated (dimensionless)

β = non-growth associated (hr^{-1})

This is an expected form when product is the result of energy yielding metabolism, as in several anaerobic fermentations. In this case the first and second terms in Equation 8 may be identified with energy used for growth and for maintenance [29].

The product formation kinetic parameters α and β in Equation 8 describe the specific rate of product formation [33].

Figures 32 through 35 show the experimental data and model curves obtained. After modeling the data, α and β constants were obtained by linear regression analysis from equation 8. Table 29 through 30 represent the obtained data from experimental values for Run No F.1, F.2.

Table 29. Riboflavin data obtained from experimental data and determined parameters for Run No F.1.

TIME (hr)	P	dP/dt	1/X*(dP/dt)	1/X*(dX/dt)
24	0,0023	0.00007917	0.00018291	0.0449208
48	0,0039	0.00008333	0.00008583	0.0190833
72	0,0062	0.00008333	0.00006167	0.0079791
96	0,0078	0.00005833	0.00003958	0.0054667
120	0,0090	0.00004583	0.00002583	0.0053167
144	0,0099	0.00001667	0.00000916	0.0022375
168	0,0098	0.00000000	0,00000000	0.0007125
192	0,0099	0.00000417	0.00000250	0.0005875
216	0,0101	0.00000833	0.00000416	0.0000208

Table 30. Riboflavin data obtained from experimental values and determined parameters for Run No F.2.

TIME (hr)	P	dP/dt	1/X*(dP/dt)	1/X*(dX/dt)
24	0,0027	0.0001083	0.0003438	0.1502500
48	0,0051	0.0003583	0.0001596	0.0264167
72	0,0198	0.0006875	0.0002179	0.0074583
96	0,0380	0.0004625	0.0001379	0.0015416
144	0,0460	0.0001375	0.0000396	0.0004583
168	0,0485	0.0000500	0.0000146	-0.0008333
192	0,0484	-0.0000042	-0.0000013	-0.0022500

α, β obtained from experimental data and μ_{max} from model fits for Run No F.1 and F.2 were illustrated in Table 31.

Table 31. Determined kinetic parameters for Run No F.1and F.2.

Run No	α (*10^3)	β (*10^5)	μ_{max}
F.1	4.03	0.704	0.02038
F.2	1.83	8.250	0.04663

As it can be seen from Table 31, α was higher than β values. Since the maximum specific growth rate of *Ashbya gossypii* was approximately 0.020380 (hr^{-1}) for Run No F.1 and 0.04663 (hr^{-1}) for Run No F.2, comparison of the α and β values shows that in most cases, riboflavin production is growth associated in contrast to literature.

When product formation is desired goal, the maximum specific growth rate should be as large as possible. Both product yield and specific rate of product formation increase with increasing values of maximum specific growth rate.

In order to well understand the pH change, growth, riboflavin production and sugar consumption patterns, sample analysis should be done within 2 or 3 hour intervals at 4 or 5 days of fermentation periods instead of daily analysis during long period of fermentations.

CHAPTER 5

CONCLUSION

In this study riboflavin was produced by using *Ashbya gossypii* NRRL Y-1056 in shake flasks and fermentor.

Four pretreatment methods were compared for riboflavin determination. Lactose, galactose and glucose were studied separately as carbon source and effect of initial glucose concentrations on riboflavin production were determined at 20, 40, 60 (g/L) concentration levels. Modeling of growth, riboflavin production and sugar consumption were done and specific growth rate (μ), maximum specific growth rate (μ_{max}) and product formation parameters were calculated.

There were no significant differences between four pretreatment methods (heat treatment at 120 °C, 1 hr; heat treatment and adjusting pH medium to 8.5; heat and ultrasonic disintegrator treatment 10 amplitude, 3 minute; pH adjustment, ultrasonic disintegrator, and heat treatment) which were used to release bound riboflavin from mycelia.

Riboflavin produced by using galactose, lactose and glucose separately as energy and carbon source was 0.00955, 0.0172, 0.00973 (g/L) respectively. As it can be seen from results, there were no significant differences between final riboflavin concentrations when galactose, lactose and glucose were used as substrate.

At the initial glucose concentrations of 20, 40, 60 (g/L), riboflavin obtained were 0.00973, 0.00615, 0.00928 (g/L)respectively. For riboflavin production optimum glucose concentration was found to be 20 (g/L).

Effect of medium pH change and substrate consumption pattern on riboflavin production were investigated. During fermentation, the pH of the medium decreases with time and drops to approximately 4.5 and then both riboflavin production and pH increase. The decrease in pH appeared to be

related to the consumption of glucose in turn initial accumulation of pyruvate in the culture medium.

Concentrations of riboflavin produced by using glucose as a substrate in shake flask and fermentor were 0.00973 and 0.0484 (g/L) respectively. Riboflavin produced in fermentor was approximately 5 times higher than the shake flask.

According to carbon yield of substrate, riboflavin and biomass obtained at the end of fermentation approximately 10-20 % of substrate carbon was used for growth and product formation. Remaining 80-90 % of substrate carbon assumed to be consumed for maintenance requirements of the microorganism and released as carbondioxide.

Boltzman model was found to be a suitable model to fit growth curve of *Ashbya gossypii*.

Maximum specific growth rates (μ_{max}) for Run No SF.6, SF.7, F.1 and F.2 were found to be 0.0193, 0.0197, 0.0204 and 0.0461 respectively. These values were lower than the values given in literature.

Product formation kinetic parameters α (growth associated) were 0.00403, 0.00183 for Run No F.1 and F.2 respectively. Similarly, β (non growth associated) was calculated as 0.000007, 0.0000825 for Run No F.1 and F.2 respectively. In contrast to literature results, riboflavin production was mainly growth associated.

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APPENDICES

APPENDIX A

Table A. 1. Data of Run No SF.1.

TIME (Day)	pH	GALACTOSE (g/L)	RIBOFLAVIN (g/L)
0	6.50000	20.00000	0.00000
2	6.25000	19.08600	0.00590
5	6.58000	17.35400	0.01174
6	6.67000	14.68300	0.01328
7	6.71000	13.77400	0.01385
8	6.75000	12.63800	0.01382
9	6.90000	11.75700	0.01348
10	7.22000	9.28600	0.00912
11	7.31000	8.97400	0.00968
12	5.57000	7.93700	0.00970
13	5.12000	7.70900	0.00955

Table A. 2. Data of Run No SF.2.

TIME (Day)	pH	LACTOSE (g/L)	RIBOFLAVIN (g/L)
0	6.50000	20.00000	0.00000
1	5.99000	19.67319	0.00190
2	6.29000	18.81605	0.00290
3	6.37500	17.81300	0.00590
4	6.86000	16.78000	0.00730
5	6.84000	16.47400	0.00950
6	7.05000	15.32600	0.01020
7	7.34500	14.06300	0.01260
8	7.42500	13.52700	0.01430
9	7.65000	12.41700	0.01440
10	7.99000	11.54400	0.01550
11	7.84000	10.81700	0.01620
12	7.83000	10.05100	0.01720

Table A. 3. Data of Run No SF.3.

TIME (Day)	pH	GLUCOSE (g/L)	RIBOFLAVIN (g/L)
0	6.50000	20.00000	0.00000
1	5.72211	-	0.00053
2	5.40333	18.04000	0.00110
3	5.29000	16.09900	0.00112
4	5.25000	9.99800	0.00134
5	5.17500	5.94900	0.00169
6	5.36000	3.45300	0.00265
7	5.44500	2.46575	0.00372
8	5.59354	1.12035	0.00410
9	5.84364	0.36204	0.00640
10	6.04090	0.00000	0.00740
11	6.29804	0.00000	0.00790
12	6.38500	0.00000	0.00851
13	6.64000	0.00000	0.00973

Table A. 4. Data of Run No SF.4.

TIME (Day)	pH	GLUCOSE (g/L)	RIBOFLAVIN (g/L)
0	6.50000	40.00000	0.00000
1	5.48500	-	0.00083
2	5.28000	38.70000	0.00162
3	5.36000	33.32000	0.00188
4	5.32000	30.96300	0.00227
5	5.12500	26.97000	0.00227
6	5.07000	24.44600	0.00282
7	4.97500	15.90500	0.00328
8	5.02000	12.74100	0.00388
9	5.07500	11.45793	0.00498
10	5.18000	10.08806	0.00538
11	5.02000	9.26614	0.00557
12	5.01000	8.75734	0.00593
13	5.01500	8.64000	0.00615

Table A. 5. Data of Run No SF.5.

TIME (Day)	pH	GLUCOSE (g/L)	RIBOFLAVIN (g/L)
0	6.50000	60.00000	0.00000
1	5.43500	-	0.00255
2	5.31000	58.25100	0.00374
3	5.22500	56.94800	0.00530
4	5.27000	51.06800	0.00567
5	5.27000	40.92955	0.00592
6	5.25000	36.40900	0.00698
7	5.15500	23.78669	0.00795
8	5.16000	16.68297	0.00650
9	5.07500	12.80500	0.00923
10	5.11000	12.80200	0.00770
11	5.14500	13.03300	0.00948
12	5.16500	13.01600	0.00920
13	5.24500	13.05200	0.00928

Table A. 6. Data of Run No SF.6.

TIME (Day)	pH	BIOMASS (g/L)	LACTOSE (g/L)	RIBOFLAVIN (g/L)
0	6.50000	0.00000	20.00000	0.00000
1	6.24000	0.16595	17.54795	0.00264
2	6.05667	0.33800	16.41400	0.00440
3	6.19667	0.41600	16.13600	0.00561
4	6.03667	0.47900	15.65900	0.00618
5	5.92667	0.48900	15.33562	0.00667
6	5.88000	0.49178	15.05000	0.00713
8	5.56333	0.49971	14.95205	0.00777
10	4.83000	0.49178	14.97945	0.00807
11	6.01000	0.49178	15.14400	0.00818
13	6.70667	0.49618	15.27397	0.00834

Table A. 7. Data of Run No SF.7.

TIME (Day)	pH	BIOMASS (g/L)	GLUCOSE (g/L)	RIBOFLAVIN (g/L)
0	6.50000	0.00000	20.00000	0.00000
1	5.56000	0.38845	13.31703	0.00098
2	5.55500	0.66400	11.26000	0.00159
3	5.26000	0.94051	9.43000	0.00173
4	5.36500	1.03464	9.17515	0.00230
5	5.09000	1.05100	8.52900	0.00245
6	5.35500	1.03973	8.52642	0.00365
7	5.41000	1.02828	7.52838	0.00382
8	5.12500	1.02828	8.24364	0.00513
9	5.33000	1.02319	7.09589	0.00513
10	5.22000	1.02319	7.15300	0.00583
11	5.40500	1.01174	7.31213	0.00587
13	5.11000	0.95196	7.52838	0.00673

Table A. 8. Data of Run No F.1

TIME (Day)	pH	BIOMASS (g/L)	GLUCOSE (g/L)	RIBOFLAVIN (g/L)
0	6.50000	0.00000	20.00000	0.00000
1	5.87000	0.44200	12.11600	0.00230
2	6.74000	0.95300	8.02000	0.00389
3	6.56000	1.31500	3.32000	0.00620
4	6.87000	1.45600	3.08900	0.00779
5	6.78000	1.69700	1.36400	0.00897
6	6.85000	1.88900	0.87600	0.00992
7	7.32000	1.90000	0.30100	0.00983
8	7.56000	1.95400	0.00000	0.00987
9	7.35000	1.95500	0.00000	0.01005

Table A. 9. Data of Run No F.2

TIME (Day)	pH	BIOMASS (g/L)	GLUCOSE (g/L)	RIBOFLAVIN (g/L)
0	6.50000	0.00000	20.00000	0.00000
1	5.53000	0.30900	11.90900	0.00270
2	5.34000	2.23434	7.28900	0.00510
3	4.88000	3.14300	0.00000	0.01980
4	6.11000	3.35900	0.00000	0.03800
6	6.54000	3.42700	0.00000	0.04600
7	7.04000	3.46600	0.00000	0.04850
8	7.13000	3.28800	0.00000	0.04840

APPENDIX B

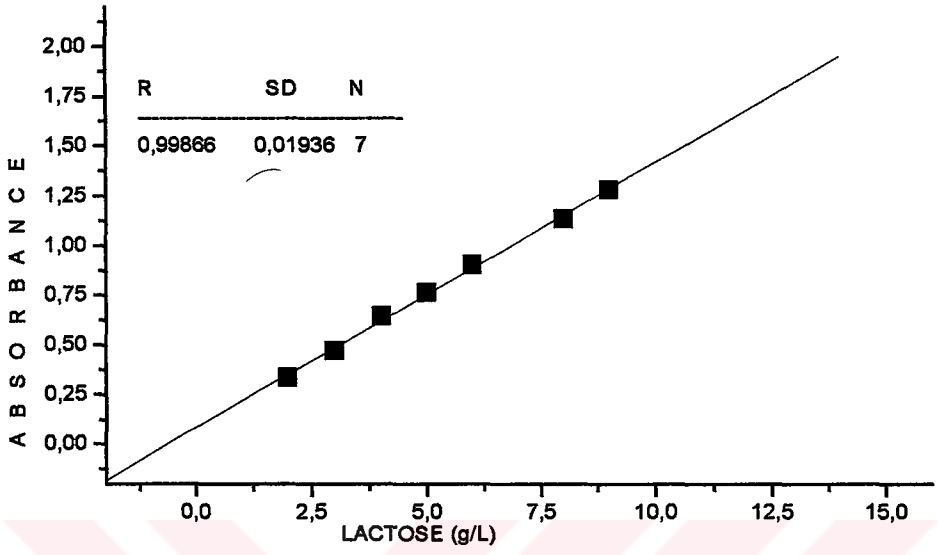

Figure B.1. Standard curve for lactose (DNS method).

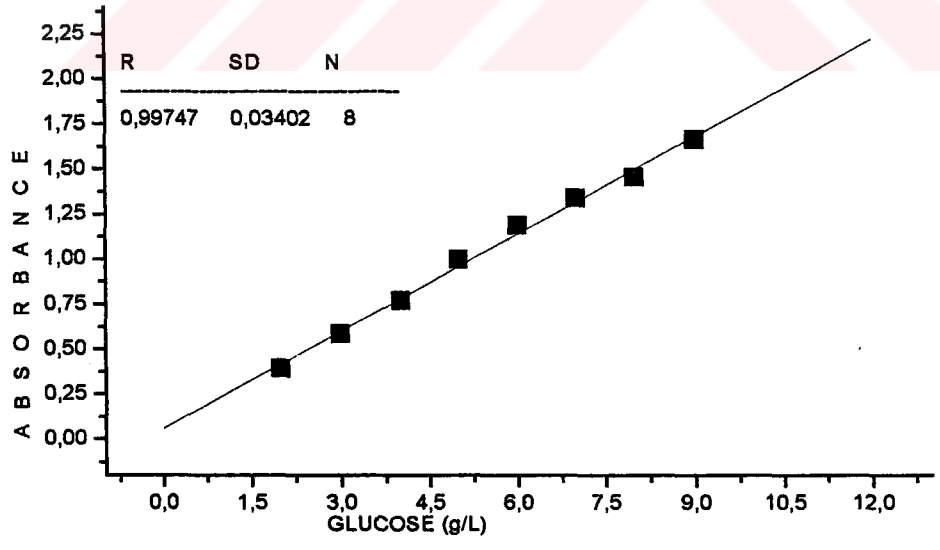

Figure B.2. Standard curve for glucose (DNS method).

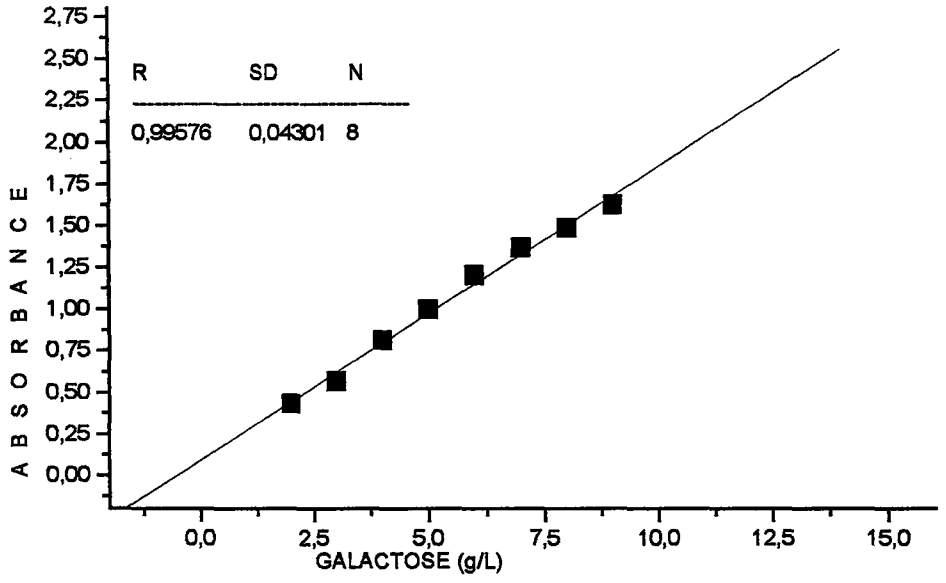

Figure B.3. Standard curve for galactose (DNS method).

APPENDIX C

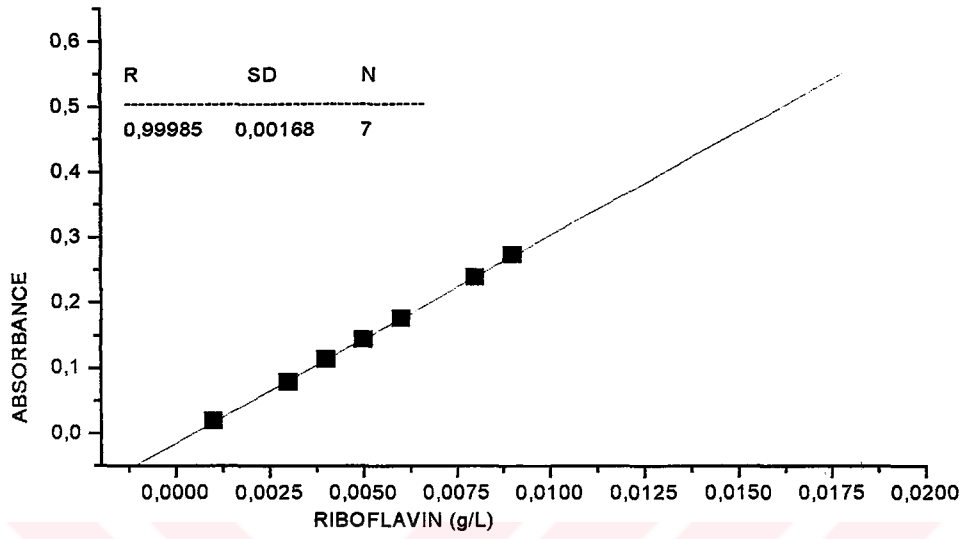

Figure C.1. Standard curve for riboflavin.

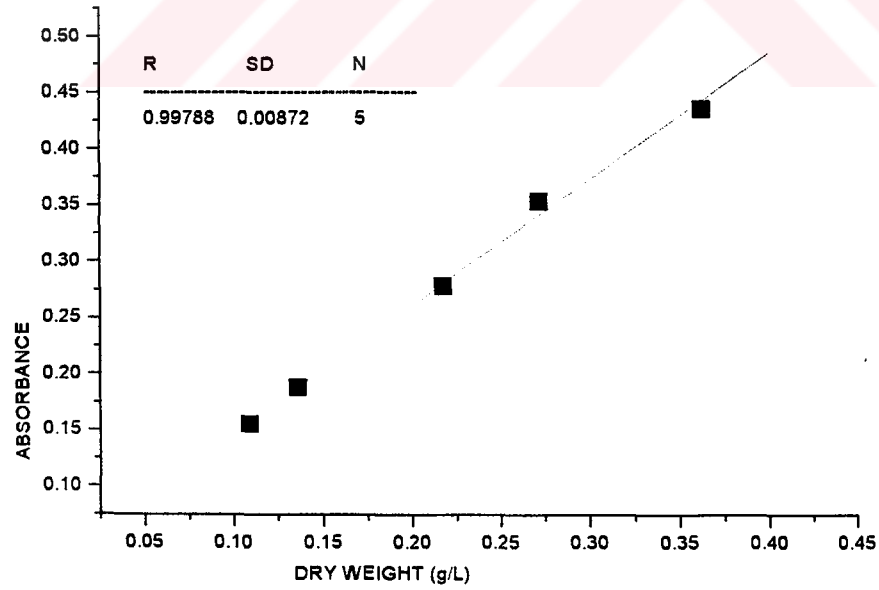

Figure C.2. Standard curve for biomass.