

Isolation of Two Riboflavin Producer Yeasts from Environment and Optimization of Vitamin Production

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ABSTRACT

Riboflavin that commonly known as vitamin B₂ is an important B vitamins for maintaining human health and it has been widely used in the fields of feed and food additives and pharmaceuticals. Certain microorganisms have the potential for natural production of vitamins and some fungi, bacteria and yeasts can produce riboflavin. So the main purpose of current study was isolation the yeasts which are capable of producing riboflavin and then investigation of the effect of different sources of carbon and nitrogen on riboflavin production. Microorganisms are capable of producing vitamins, which are essential nutrients in the energy production. So in this study, different samples from soil of sugarcane and bagasse were collected and cultivated in an enrichment medium for growth of yeasts. After purification, all colonies were examined for riboflavin production. Based on spectrophotometer and chromatographic analysis, 13 isolates could produce riboflavin. Sc1 isolate from sugarcane and B5 isolate from bagasse were selected as the best riboflavin producers by 303.09µg/ml and 295.87µg/ml respectively. Then Sc1 isolate was selected to study on the effect of carbon and nitrogen sources on production. Morphological, and biochemical characteristics of the strains were examined. The polymerase chain reaction was done by using universal specific primers. According to sequencing of about 500bp of gene and comparing the sequences with existing data in GenBank, Sc1 isolate belonged to *Trichosporon asahii* sp. and B5 isolate belonged to *Rhodosporidium diobovatom* sp.

KEYWORDS: Yeast isolation; Riboflavin production; Optimization; *Trichosporon asahii*; *Rhodosporidium diobovatom*

INTRODUCTION

Riboflavin or vitamin B₂ is a dietary need for humans as, they cannot synthesis the vitamins [1]. This vitamin is a precursor of the coenzymes flavinadenine dinucleotide (FAD) and flavin mononucleotide (FMN), that are required for reactions such as the enzymatic oxidation of carbohydrates. Vitamin shortage in humans is related to many symptoms as loss of hair, inflammation of the skin, vision decay, and growth failure. Also has been found that this vitamin be successful in the treatment of migraine and malaria [2-4]. So owing to its particular physiological role, riboflavin has been widely used in the fields of feed and food additives and pharmaceuticals [5].

Riboflavin is produced commercially by both chemical and biological synthesis, however, it was made using chemical processes, but in recent years biotechnological processes have become more popular using organisms [6-7]. This water soluble vitamin, is a unique vitamin that can be combined in large amounts by some fungi and bacteria so many microorganisms have been screened and studied for industrial production up to now. However among the bacteria, *Clostridium acetobutylicum* was one of the earliest organisms used to produce riboflavin but in recent years, riboflavin production of *B.subtilis* has gained increased interest from researchers because advanced genetic engineering technology are available for use in this strain for overproduction [5 and 8]. As well as bacteria, some yeasts of the genus *Candida* were found to produce riboflavin. But both groups, for producing of high levels of riboflavin should be overcome to iron restraint. On the other hand, unlike the yeast and Bacteria, iron is not an inhibitor factor for the two yeast like molds, *Eremothecium ashbyii* and *Ashbya gossypii*, which are natural overproducers of riboflavin. It is important to know, despite iron restraint is a main problem but many advantages such as high growth rates, less costly and complex growth media were seen in developing bacterial and yeasts fermentation for riboflavin production. Though there are many reports about riboflavin producer organisms, but currently the Gram-positive bacteria *Bacillus subtilis*, the yeast *Candida famate*, and the filamentous fungus *Ashbya gossypii* [8-9].

It's important to know that for by introducing a productive strain, designing an enough fermentation medium is of crucial importance to improve the productivity of riboflavin fermentation process because medium composition can significantly affect product concentration, volumetric production, and the ease and cost of product separation [10]. The conventional method that has been used for optimization of riboflavin production is the one-factor-at-a-time, which a single factor is varied while fixing all others at a specific level [11 and 12].

The purpose of current study was isolation the yeasts which are capable of producing riboflavin and then investigation of the effect of different sources of carbon and nitrogen on riboflavin production.

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MATERIALS AND METHODS

Yeast strains

Different samples from soil of sugarcane plant and bagasse in Khuzestan province of Iran were collected. First, 5 grams of samples were suspended in 45ml distilled water and shaken at 130rpm and 28° C for 24h, then 2ml of supernatant was suspended in 18ml YM broth medium containing 0.3% yeast extract, 0.3% malt extract, 0.5% peptone and 1% dextrose, and shaken at 130rpm and 28° C for 3 days. A known amount of them were spread on YGC agar (Yeast extract Glucose Chloramphenicol agar) and the plates were incubated at 28° C for 3-4 days [13-14]. Different colonies from the plates were examined for riboflavin production. The yeast strains were maintained in YM agar medium at 4° C.

Riboflavin production

One loop of the cells of the yeast strains was transferred to 50ml of YPD medium containing 2% dextrose, 2% peptone and 1% yeast extract and was aerobically cultivated at 28° C for 48 h. At a point when the culture reached a high cell density ($OD_{600nm}=1.5$), 0.2 ml of the culture was transferred to 50ml of the production medium that contained 2% of sucrose, 0.5% of $(NH_4)_2SO_4$, 0.1% of KH_2PO_4 , 0.05% of $MgSO_4 \cdot 7H_2O$, 0.01% of $CaCl_2 \cdot 2H_2O$, 0.01% of NaCl and 0.2% of yeast extract and grown by shaken at 170 rpm and 28° C for 5 days [15]. The culture was centrifuged at 5000rpm and 4° C for 5min and the supernatant obtained was used as the crude riboflavin solution for determination of riboflavin.

Determination and Quantification of riboflavin

The amount of riboflavin in the supernatant was measured quantitatively at 440nm by using a spectrophotometer and riboflavin from sigma which served as standard [5 and 16].

Because of this wavelength isn't specific for riboflavin, identification of it in production medium was done by thin layer chromatography (TLC) using a solvent system of n-butanol-acetic acid-water (4:1:5 v/v) [17]. Spots giving a yellow fluorescence were identified as riboflavin as compared to a standard sample. Also the supernatant obtained at the end of incubation was filtered through a 0.22 μ filter before High Performance Liquid Chromatography (HPLC) analysis. For this method, C18 column with a mobile phase comprised of methanol: water (30:70) at a constant flow rate of 1ml/min was used. A UV detector that set on 254nm was employed for the detection of peaks [18-19].

Identification of yeast

The routine biochemical identification of the yeast was performed by using the methods as described by Kurtzman and Fell [20]. For molecular identification, the isolates were cultured in YPD medium and shaken at 200rpm and 30° C for 24h. The DNA extraction was performed using the methods described by Haffmann [21]. Then 5.8S-ITS fragments were enlarged using ITS1 (3'TCCGTAGGTGAACCTGCGG'5) and ITS4 (3' TCCTCCGCTTATTGATATGC'5) primers, and The PCR conditions were done by initial denaturing for 5 min at 94° C and followed by 35 cycles of 30 S at 94° C, 30 S at 56.6° C and 45 S at 72° C, plus one additional cycle with a final 10min chain elongation at 72° C [22]. The sequence of isolates was aligned with selected sequences obtained from Gen Bank databases.

Optimization of carbon and nitrogen sources

To determine the best sources for production, different carbon and nitrogen sources were evaluated. As carbon sources, sucrose was replaced by lactose, carboxy methylcellulose and starch. As nitrogen sources, peptone, trypton, yeast extract, meat extract and potassium nitrate were tested. After selection of the best sources, different concentrations were added to production medium. These concentrations were 1, 1.5, 2, 3, 4, 6% and 0.1, 0.3, 0.5, 0.7, 1, 1.2% for best carbon and nitrogen source respectively.

Determination of dry weight

The yeast growth was separated by centrifugation, washed and dried at 60° C to constant weight. This test was done to comparison of riboflavin and biomass production in a 10 days period.

RESULTS

Screening of riboflavin producing yeast

Thirty three yeasts were isolated from the Iranian samples. According to the spectrophotometer and chromatographically analysis, 13 isolates were detected as riboflavin producers. Based on standard curves, results showed that among all the yeast producers, Sc1 from soil of sugarcane by 303.09 μ g/ml and B5 from bagasse by 295.87 μ g/ml produced highest amount of riboflavin from 1% sucrose and 0.2% yeast extract. Optimization of carbon and nitrogen sources for Sc1 showed that with 2% lactose and 0.7% yeast extract, maximum riboflavin concentration (1200.543 μ g/ml) was obtained.

Time-course production

The changes in the growth and riboflavin yields of Sc1 during the different fermentation periods (4 to 10 days) were recorded. Fig 1 and 2 shows that a significant growth was observed after 5 days of incubation. On the other hand, riboflavin reached its highest level after 7 days. These results draw that first, organism must form enough biomass yield, then flavinogenesis occurs.

Phenotypic identification of yeast

The morphological and biochemical data of found out strains are described and represented in Table 1, Fig 3 and 4.

Phylogenetic analysis of yeast

According to Kurtzman and Fell, routine identification methods, that depend on phenotype, usually lead to inaccurate interpretations of species interaction. A more accurate method for deciding inter- and intraspecific relationships is Sequence analysis of phylogeny for microbial taxonomy. Therefore, a partial sequence of 5.8S-ITS of the yeast strains were determined and aligned by using BLAST analysis (<http://www.ncbi.nlm.nih.gov/BLAST>). A phylogenetic tree was built by using Neighbor joining analysis of MEGA4 software package (Fig 5 and 6). Therefore, the yeast strains B5 and Sc1 were closely related to *Rhodospiridium diobovatum* and *Trichosporon asahii* ; And considering isolation of them from bagasse and sugarcane we have named them *Rhodospiridium diobovatum* B5 (GenBank accession no: JQ343907) and *Trichosporon asahii* Sc1 (GenBank accession no: JQ343908) respectively.

DISCUSSION

Many microorganisms have been studied for industrial riboflavin production. Among them, *A. gossypii*, a filamentous fungus, *C.famata*, yeast, and the genetically engineered *B. subtilis* have been commercially used to produce riboflavin [8]. This is the first study to report that *R.diobovatum* and *T.asahii* can produce riboflavin and can isolate yeast from new samples. We know that because of limit of our knowledge about biodiversity of yeast, many niches in environment have remained unexplored [23-25]. So, however, based on previous reports; *R.diobovatum* seemed to be restricted to marine environment but in this research isolation of yeast from bagasse was done.

Also, though *T.asahii* was recognized as the cause of superficial fungal infections in many researches [26-28], but recently it was found that *T.asahii* with *Candida valida* and *Rhodotorula glutinis* could be effected growth of *Rhizoctonia solani* that is a fungal root pathogen and may protect sugar beetroot [29-31]. This research showed that this species could produce other useful metabolites such as riboflavin.

It is interesting to know that however, since the first description of the riboflavin production process, much research has been conducted to develop defined synthetic media that increase riboflavin production but few studies about carbon and nitrogen sources were done. *Candida flareri*, *Candida guilliermondii*, *Candida robusta*, *Candida ghoshii*, *Debaryomyces subglobosus*, and *Torulopsis famata* are some riboflavin overproducer yeasts that Carbon sources for these producers are various such as glucose, fructose, mannose, sucrose, xylose, arabinose, and acetate. Nitrogen sources for them are different too. Some nitrogen sources are preferred by some yeast strains while sometimes harmful, to others. In our case, preliminary tests using 0.7% yeast extract and 2% lactose showed the best riboflavin production. Yeast extract is a complex raw material that because of it's low cost and rich content of amino acids, peptides, growth factors, vitamins, and trace elements commonly used in fermentation processes [32]. Nishio and Kamikubo (1971) found out that yeast extract and casamino acid stimulated production by *Pichia guilliermondii*, while other organic nutrients did not affect riboflavin biosynthesis [33]. Suzuki et al (2009) showed that best riboflavin production was done with 0.5% yeast extract [34]. Nonetheless, it is important to know that each microorganism has it's own regulatory mechanism, So more studies are necessary to optimize the fermentation medium.

In conclusion, *R.diobovatum* and *T.asahii* are comparable with other riboflavin producer strains and their vitamin production is higher than some of them. So, these strains can be used for improvement of riboflavin production.

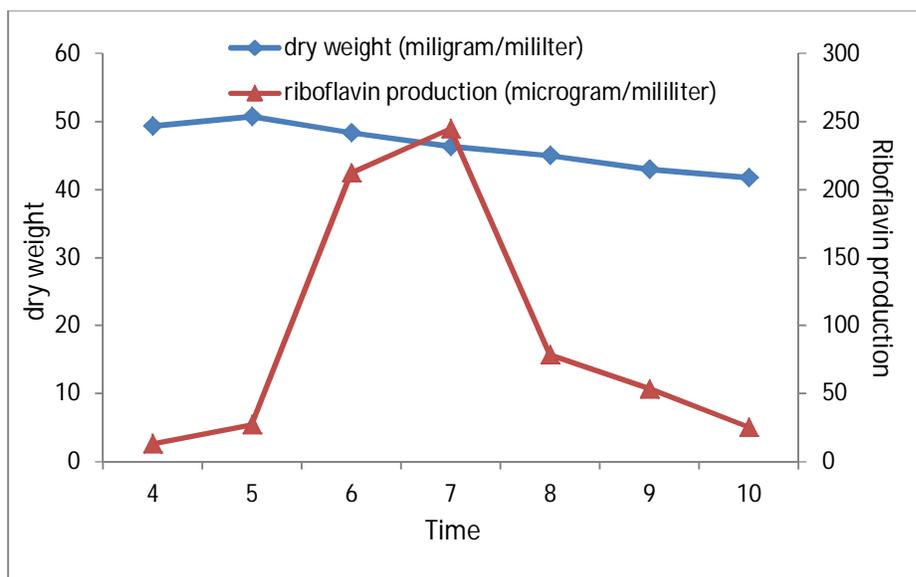


Fig 1: Biomass and riboflavin production for Sc1 with 2% lactose in a 10 days period

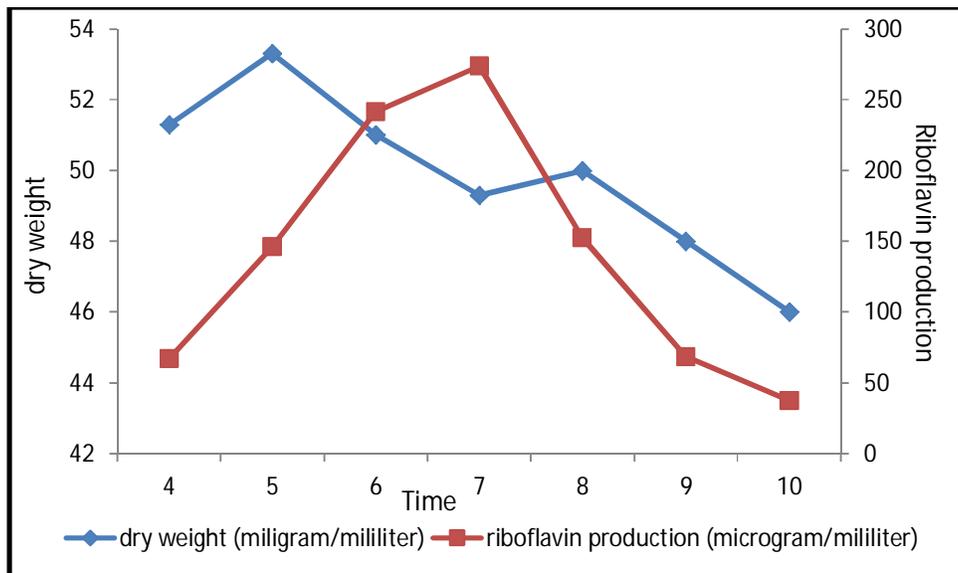


Fig 2: Biomass and riboflavin production for Sc1 with 0.7% yeast extract in a 10 days period

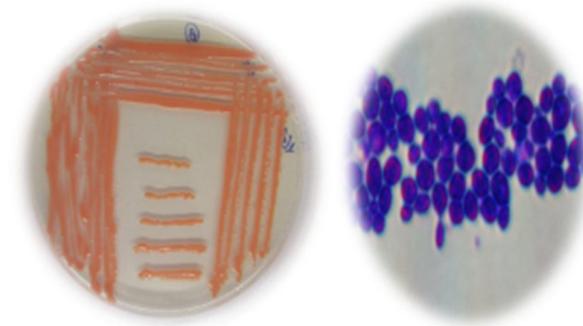


Fig 3: colony and microscopic photo of B5 strain

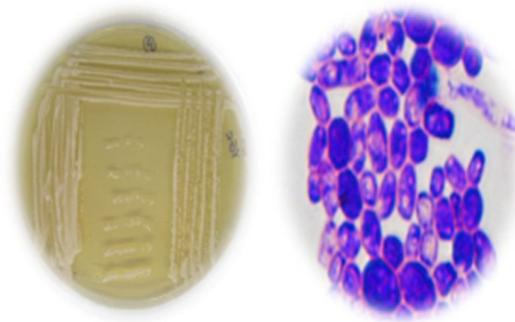


Fig 4: colony and microscopic photo of Sc1 strain

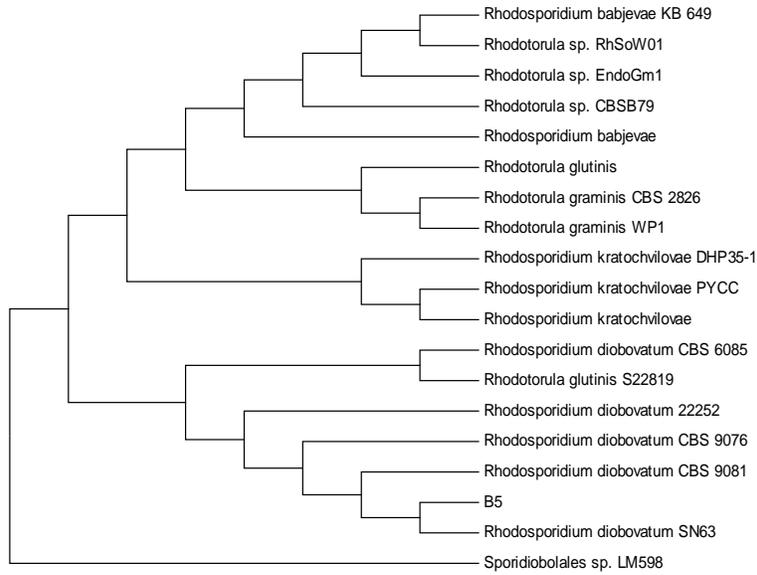


Fig 5: Phylogenetic tree of B5 strain (neighbor joining method)

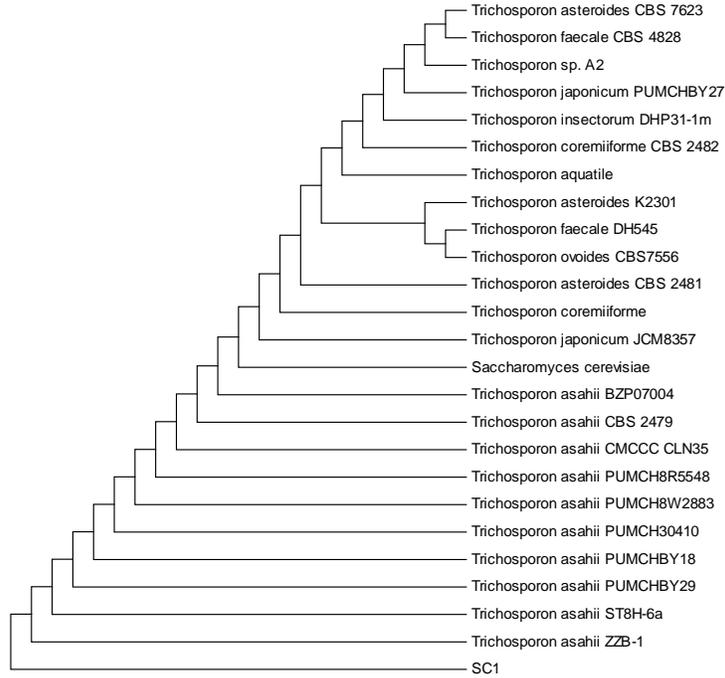


Fig 6: Phylogenetic tree of Sc1 strain (neighbor joining method)

Table 1: Morphological and biochemical characteristics of Sc1 and B5 strains

Characteristic		Sc1	B5
Morphology		White, dry colony with farinose at the center	Red, mucoid colony, raised and border entire
Fermentation	Maltose	-	-
	Galactose	-	-
	Lactose	-	-
	Glucose	-	-
	Sucrose	-	-
Assimilation	Manitol	+	+
	Raffinose	-	+
	Salicin	+	+
	Inositol	-	-
	Inulin	-	+
	Trehalose	+	+
	Arabinose	+	+
Gelatin hydrolysis		-	-

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The authors declare that they have no conflicts of interest in the research.

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