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OPTIMIZATION OF RIBOFLAVIN PRODUCTION USING *BACILLUS CEREUS* HDS07: A STRAIN ISOLATED FROM *AGARICUS BISPORUS*

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ABSTRACT

The current study focused on the production and optimization of riboflavin from *Bacillus cereus*, a strain isolated from *Agaricus bisporus*. Seven different strains were isolated from *Agaricus bisporus* and screened for riboflavin production. Among the 7 strains, only 4 strains were identified as riboflavin producers by riboflavin assay medium (RAM). To determine the potency of the strains, selected strains were exposed to roseoflavin – an analogue of riboflavin. The potent strain was identified through morphological, biochemical, and molecular characterization and the strain name was designated as HDS07. Estimation of riboflavin was done by UV Spectrophotometry. Riboflavin production was done in Chemically Defined Medium (CDM) and De Man, Rogosa and Sharpe *agar* (MRS) using a strain *Bacillus cereus* HDS07. To enhance riboflavin production, the medium was optimized with different parameters like carbon, nitrogen sources, pH, temperature, and inoculum size. The potent strain HDS07 was identified as *Bacillus cereus* by 16S rDNA sequencing and NCBI-Gen Bank accession number - MK177597 was obtained. Riboflavin production from *Bacillus cereus*-HDS07 was more in the MRS medium than that of CDM. It was found to be 2.97 mg/L and 1.8 mg/L correspondingly. The maximum riboflavin (3.48 mg/L) was obtained from *Bacillus cereus*-HDS07 under the culture conditions; glucose, glycine, pH-6, 30°C, and 3% inoculum size. The current study emphasizes that the isolated potent strain *Bacillus cereus*-HDS07 from *Agaricus bisporus* could be used as a starter for the industrial production of riboflavin.

Keywords: *Bacillus cereus*, riboflavin, *Agaricus bisporus*, optimization

INTRODUCTION

Riboflavin (vitamin B_2) is an important nutrient that acts as a co-factor to FMN and FAD (coenzymes) that involved in oxidative metabolic pathways. **(Sybesma *et al.*, 2003).** This vitamin is also important for cell energy metabolism, and riboflavin has been demonstrated to boost the efficacy of conventional therapy in a variety of disorders, including cisplatin-induced intestinal epithelial cell death and *Staphylococcus aureus* infection **(Krymchantowski *et al.*, 2002).** Riboflavin deficiency can impair the mouth's mucocutaneous surfaces, causing inflammation in the lips (cheilitis) and tongue (glossitis) **(Thakur *et al.*, 2016; Bhusan *et al.*, 2021).**
Chemical or biological synthesis can be used to produce riboflavin in the industry. However, because of the advantages of biotechnical processes, which include cost-effectiveness, pollution and waste minimization, and the utilization of renewable resources, it has fully shifted from chemical synthesis and toward microbial fermentation. **(Stahmann *et al.*, 1994; Kalingan *et al.*, 1997; Lin *et al.*, 2001).** The predominant strains that may produce larger quantities of riboflavin and are used for industrial riboflavin production are the bacterium *Bacillus subtilis*, yeast *Candida famata* and fungi *Eremothecium gossypii* **(Kato *et al.*, 2006)**. Recombinant strains of the Gram-positive bacterium *Bacillus subtilis* and the filamentous fungus *Eremothecium gossypii* have also proven successful in producing riboflavin. *Bacillus* species are used in commercial and industrial biotechnological applications because the US Food and Drug Administration (FDA) has declared many of them harmless, and *Bacillus* species including *B. subtilis* and *B. cereus* grows fast, enabling continuous production cycles. **(Stahmann *et al.*, 2000; Singh *et al*, 2020).**
The synthesis of riboflavin can be affected by a variety of fermentation parameters such as pH, carbon and nitrogen, incubation temperature, and so on. The composition of the media culture is among the essential components in microbiological process, particularly riboflavin synthesis. **(Perkins *et al.*, 1999).** However, after numerous cycles of production, the bacteria lose their capacity to make riboflavin. As a result, industrialists are anticipating the arrival of the innovative riboflavin-producing strain. The current research focuses on the production of riboflavin by bacteria isolated from mushroom (*Agaricus bisporus*) samples. *Bacillus cereus* HDS07, a potent isolated strain that was evaluated for riboflavin synthesis. The main purpose of this study was to locate a low-cost, readily available source that could meet a microbe's basic requirements without compromising the quality of the end product.

METHODOLOGY

Requirements

MRS, *standard riboflavin, roseoflavin, glucose, lactose, sucrose, maltose, galactose, yeast extract, tryptone, glycine, NH_4Cl, $(NH_4)_2SO_4$ were procured from* Hi-Media Laboratories (Mumbai, India). Riboflavin assay media (RAM) was procured from Difco (US).

Isolation and screening for riboflavin-producing strain

The *Agaricus bisporus* (mushroom) was selected as a sample source for riboflavin production. Because 100g of fresh mushroom contains 0.9mg of riboflavin. *Agaricus bisporus* was collected from Anaicut, Vellore region and it was washed with sterile water for the isolation process. It was crushed and pasted using mortar and pestle. A gram of homogenized *Agaricus bisporus* sample was serially diluted with 0.85% saline up to 10^{-9} dilutions. The spread plate technique was used to obtain isolated colonies. After 24h of incubation, the isolated strains were screened for riboflavin production. A specific medium, riboflavin assay medium (RAM) was used for screening which contains minerals, nucleic acid, and vitamins except for riboflavin. The isolated colonies were inoculated in RAM broth. After 24 h of incubation, the potent strain was identified by the growth using a UV spectrophotometer at 600nm **(Stahmann *et al.*, 2000**).

Selection of potent strain by roseoflavin (an analogue of riboflavin)

RAM (Riboflavin assay medium) plates were supplemented with different concentrations of roseoflavin (10, 50, 100, and 200 mg/L). The selected isolated strains (HDS01, HDS02, HDS05 and HDS07) were inoculated in RAM plate supplemented with roseoflavin. The plates were incubated for 24h at 37 °C **(Hugenholtz *et al.*, 2002).**

Estimation of riboflavin

Riboflavin estimation was done by the method of Sauer et al. (1996). The cultured broth was analysed for the existence of riboflavin. It was centrifuged for 10 min at 5000 g. The presence of riboflavin was determined in the collected supernatant. The supernatant was treated with sodium hydroxide (1M) and potassium phosphate

buffer (pH-6.0). The mixture was used for taking OD at 444nm in a UV spectrophotometer. The standard graph was plotted using the riboflavin standard (Sigma Aldrich, Mumbai) and determined the concentration of riboflavin in cultured broth **(Sauer *et al.*, 1996).**

Identification of potent strain

Morphological and biochemical tests were done to identify an isolated and selected potent strain. Further, it was characterized by 16S rDNA sequencing. The obtained sequences were submitted to NCBI- Gen Bank and generated accession number **(Thakur *et al.*, 2015).**

Production of riboflavin

The riboflavin was produced in Chemically Defined Medium (CDM) and MRS. The selected potent pure culture was inoculated in both CDM and MRS medium. The inoculated medium was incubated in a shaker at 37℃ for 24h. After incubation, the culture broth was centrifuged and the supernatant was used for the estimation of riboflavin in UV spectrophotometer at 444nm. Production of riboflavin was done in triplicates and the standard deviation was plotted with error bars **(Otto *et al.*, 1983).**

Optimization of riboflavin production

To enhance the production of riboflavin, production medium was optimized. Different parameters were selected for the optimization of riboflavin production. Carbon and nitrogen supplies, inoculum size, pH and temperature were the five factors used for enhanced production of riboflavin. Carbon sources (20g/L) – glucose, maltose, galactose, lactose and sucrose were used. Nitrogen sources (3g/L) like yeast extract, tryptone, glycine, ammonium sulphate and ammonium chloride were selected for the optimization process. pH - 3 to 7, temperature - 20℃ to 60℃, and Inoculum size from 0.5% to 3% have been chosen for the optimization of riboflavin production **(Devi *et al.*, 2015; Thakur *et al.*, 2020).**

RESULTS AND DISCUSSION

Isolation of bacteria and screening for riboflavin production

Totally 7 different strains were observed after 24 h of incubation and it was designated as HDS01 to HDS07. Table (1) represents the colony morphology of isolated strains. Among 7 strains, only 4 (HDS01, HDS02, HDS05, and HDS07) have grown well in the Riboflavin assay medium (RAM). The remaining 3 isolates (HDS03, HDS04, and HDS06) have not grown in RAM which shows that colonies could not able to produce riboflavin. Figure 1 shows the growth and riboflavin concentration of selected strains. Comparatively, an isolate HDS07 was showing the maximum level of growth in the Riboflavin assay medium (RAM) and riboflavin concentration was found to be 1.8 mg/L of riboflavin.

Table 1 Colony morphology of isolated strains

S. No	Morphology	Isolated colonies from *Agaricus bisporus*						
		HDS01	**HDS02**	**HDS03**	**HDS04**	**HDS05**	**HDS06**	**HDS07**
1	Colour	dull white	creamy	Reddish orange	White	creamy	light	dull grey
2	Form	spherical	circular	round	Irregular	round	irregular	circular
3	Elevation	flat	low convex	convex	Flat	flat	convex	low
4	Margin	irregular	undulate	entire	entire	entire	undulate	irregular
5	Size	large	medium	medium	large	medium	medium	large
6	Consistency	mucoid	brittle	friable	mucoid	mucoid	friable	brittle
7	Opacity	opaque	translucent	opaque	transparent	opaque	opaque	opaque

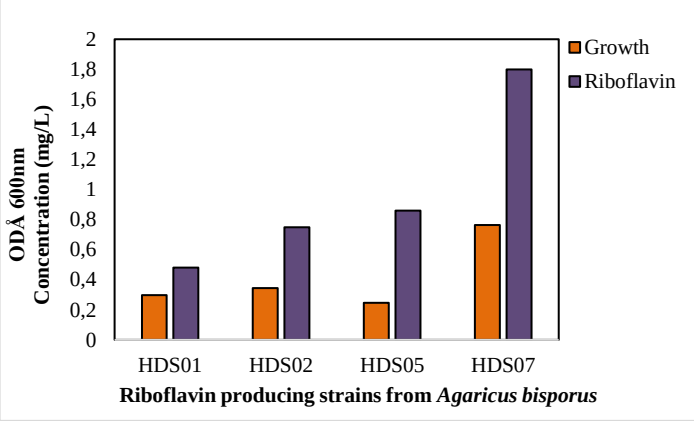


Figure 1 Growth and riboflavin concentration of selected colonies on RAM.

Selection of potent strain

From the four selected strains (HDS01, HDS02, HDS05, and HDS07), only a single strain HDS07 has grown well against different concentrations of roseoflavin (an analogue of riboflavin). The strain HDS07 shows the potency of riboflavin overproduction (Figure 2).

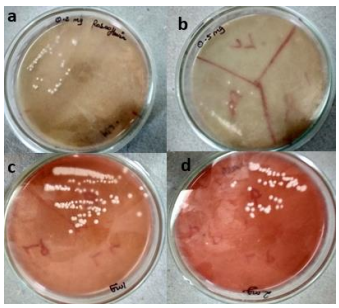


Figure 2 Potent strain *Bacillus cereus* – HDS07 grown against roseoflavin (an analogue of riboflavin). (a) 10, (b) 50 (c) 100 (d) 200 mg/L of roseoflavin in RAM medium.

Identification of potent strain

Based on morphological and molecular characterization, potent strain -HDS07 was identified as *Bacillus cereus* (Table 2). Figure 3 represents the growth and morphological identification of *Bacillus cereus.* Gen Bank accession number of *Bacillus cereus* – HDS07 from NCBI was MK177597 and the phylogram was created using MEGA software. (Figure 4)

Table 2 Results of morphological characterization of a potent strain *Bacillus cereus* – HDS07.

S.No:	Characteristics	HDS07
1	Gram's staining	+
2	Shape	Rod
3	Indole	_
4	Methyl red	_
5	Voges Proskauer	+
6	Nitrate reduction	+
7	Citrate	+
8	Catalase	+
9	Motility	+
10	Glucose	+
11	Spore	+
12	Oxidase	_
13	Pigment	_

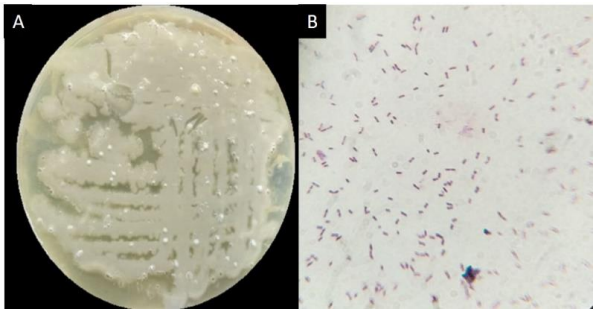
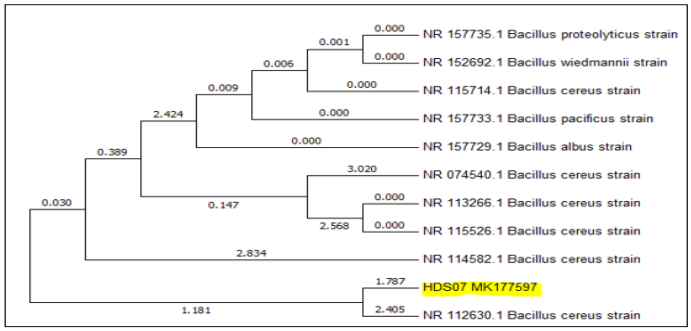


Figure 3 (A). Growth of *Bacillus cereus* HDS07 on MRS agar plate. (B). Microscopic image of Gram-stained *Bacillus cereus* HDS07 – Gram positive rod.



Phylogenetic analysis of *Bacillus cereus* HDS07 using 16S rDNA sequence along with sequences obtained from NCBI GenBank for similarity check.

Figure 4 Phylogram of *Bacillus cereus* – HDS07

Production and estimation of riboflavin from *Bacillus cereus* – HDS07

Bacillus cereus – HDS07 was producing 1.8mg/L of riboflavin in CDM and 2.79mg/L in MRS medium (Figure 5). Comparatively, MRS medium inoculated with *Bacillus cereus* – HDS07 produced more riboflavin than the Chemically Defined Medium (CDM). (Figure 6)

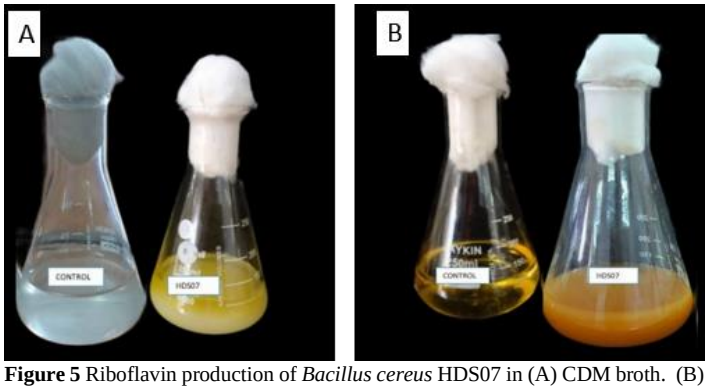


Figure 5 Riboflavin production of *Bacillus cereus* HDS07 in (A) CDM broth. (B) MRS broth

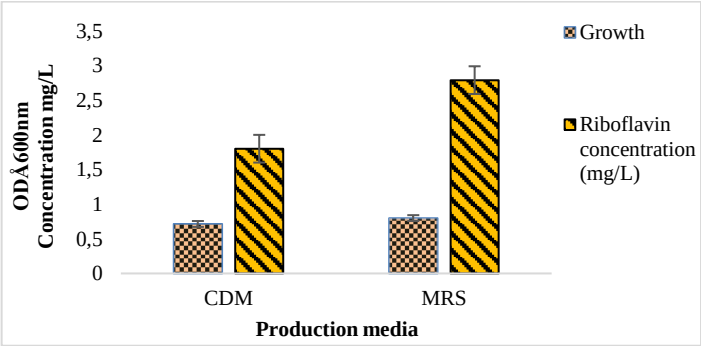


Figure 6 Growth and riboflavin concentration in CDM and MRS from *B. cereus* - HDS07.

Optimization

Effect of Carbon

The impact of carbon source on riboflavin production has been studied by inoculating *Bacillus cereus* – HDS07 in medium enriched with various carbon sources. Maximum level of growth and yield of riboflavin (2.95mg/L) was obtained in medium supplemented with glucose. The growth and riboflavin concentration of *Bacillus cereus* – HDS07 in medium supplemented with lactose was 0.99 and 2.56mg/L respectively. *Bacillus cereus* – HDS07 producing moderate level of growth and riboflavin yield in medium with galactose and maltose. It was found to be 1.98 and 1.7mg/L of riboflavin respectively. Minimum yield of riboflavin (0.74mg/L) was produced in medium added with sucrose by *Bacillus cereus* – HDS07.

Effect of Nitrogen

The influence of nitrogen source on riboflavin synthesis was obtained by incubating *Bacillus cereus* – HDS07 with supplement of different nitrogen sources. Among the tested nitrogen, maximum riboflavin yield (2.83 mg/L) and the growth of *Bacillus cereus* – HDS07 was observed in a medium with glycine. Riboflavin yield was moderate in medium added with yeast extract and it was found to be 2.48 mg/L of riboflavin. The amount of riboflavin was 2.44 mg/L when tryptone was fortified as a nitrogen source in production medium. When the ammonium chloride was added in production medium, the growth and the riboflavin production of *Bacillus cereus* – HDS07 was 2.01 mg/L. The minimum yield of riboflavin, 1.56mg/L was obtained by *Bacillus cereus* – HDS07 in medium incorporated with ammonium sulphate.

Effect of temperature

To study the influence of temperature, the medium inoculated with *Bacillus cereus* – HDS07 was incubated under different temperature. Temperature-30°C was found to be optimal for the riboflavin production. The growth of *Bacillus cereus* – HDS07 and riboflavin production (2.62mg/L) was higher under 30°C. *Bacillus cereus* – HDS07 was producing the yield of 2.48 mg/L of riboflavin incubated at 50°C. The growth of strain and the riboflavin production was moderate at 40°C and was found to be 0.98 and 2.43mg/L respectively. The amount of riboflavin (1.6mg/L) and the cell doubling rate have been slow down during the medium incubated at 60°C. Less amount of riboflavin (1.14mg/L) was measured in a medium incubated at 20°C.

Effect of pH

To determine the effect of pH in riboflavin production, medium with different pH was inoculated with *Bacillus cereus* – HDS07. The study revealed that the *Bacillus cereus* – HDS07 was growing well and the yield of riboflavin (3.48mg/L) also maximum in pH 7 (Neutral). Moderate level of growth by *Bacillus cereus* – HDS07 and riboflavin production (2.62mg/L) was recorded in medium with pH-6. The amount of riboflavin obtained from pH 4 and 5 medium was less, with 1.14 and 1.16 mg/L, respectively. A minimum amount of riboflavin (0.88mg/L) was recorded by *Bacillus cereus* – HDS07 in a medium with acidic pH-3.

Effect of Inoculum size

The production of riboflavin is determined by the growth of strains. The influence of inoculum size on riboflavin synthesis was investigated using various inoculum size. As the percentage of inoculum increases, so does the concentration of riboflavin. The maximum level 3% inoculum was inoculated in production medium, 3.48mg/L of riboflavin from *Bacillus cereus* – HDS07 was obtained. When the percentage of inoculum was 2.5 and the riboflavin yield was found to be 2.83mg/ L. The production medium was then inoculated with 2% of *Bacillus cereus* – HDS07, and the amount of riboflavin was found to be 1.98mg/L. Minimum yield of riboflavin was obtained in medium added with 0.5 and 1 percent of the inoculum. Amount of riboflavin was found to be 0.88 and 1.24mg/L respectively.

The current research focused on riboflavin production and optimization using an isolated potent strain from an *Agaricus bisporus* (mushroom) source. According to the present study, the strain HDS07 inoculated in MRS medium produced more riboflavin than the strain inoculated in CDM (Chemically Defined Medium). Furthermore, the current paper provides more information on the many aspects of riboflavin augmentation. The culture medium must provide all of the essential nutrients necessary by microorganisms for riboflavin synthesis, hence the way of life medium plays an important role in riboflavin biosynthesis. To reduce the manufacturing cost, the production medium must contain low-cost carbon and nitrogen sources. The development parameters, such as duration of incubation, pH, and temperature, were also preserved at optimal levels for increased riboflavin production. In large-scale industries, medium quality and culture were to be increased before to the inoculation.

In previously reported study, enhanced riboflavin production have been obtained from *Bacillus subtilis* which is genetically modified using *B. cereus* ATCC14579. The *rib* operon of ATCC14579 was cloned with Pn promotor and it was expressed in *Bacillus subtilis.* The heterologous rib operon in *Bacillus subtilis* has been produced 4.3g/L of riboflavin **(Yunxia *et al*., 2010).** In current study, riboflavin production from *Bacillus cereus* HDS07 producing more riboflavin without any genetically modification. Moreover, this is the first report that riboflavin production from *Bacillus cereus* without any genetically modification of strains. Recombination of rib genes in *Bacillus cereus* – HDS07 from mushroom could may increase the rate of riboflavin production. According to **Abd-lla *et al*., (2016),** *Bacillus subtilis* and *Bacillus tequilensis* have been used for riboflavin synthesis and glycine has been used as a nitrogen source for riboflavin overproduction. In another study, riboflavin production (12.8mg/L) has been enhanced in *B. subtilis* subsp. *subtilis* (ATCC 6051) by the addition of different minerals in the production medium using the statistical method (RSM). Chu et al. (2021) stated the significance of microbial synthesis methods to increase the yield of riboflavin. To enhance the production of riboflavin, **Wang *et al*., (2011)** cloned Zwf and gnd genes from *Corynebacterium* to *Bacillus subtilis* and it has been further improved by site- directed mutagenesis. The production rate of riboflavin from *Bacillus subtilis* mutant strain was found to be 33% increased than the wild strain **(Wang *et al*., 2011).** In the future study, rib genes from isolated potent *Bacillus cereus* HDS07 will be used for cloning to enhance riboflavin production.

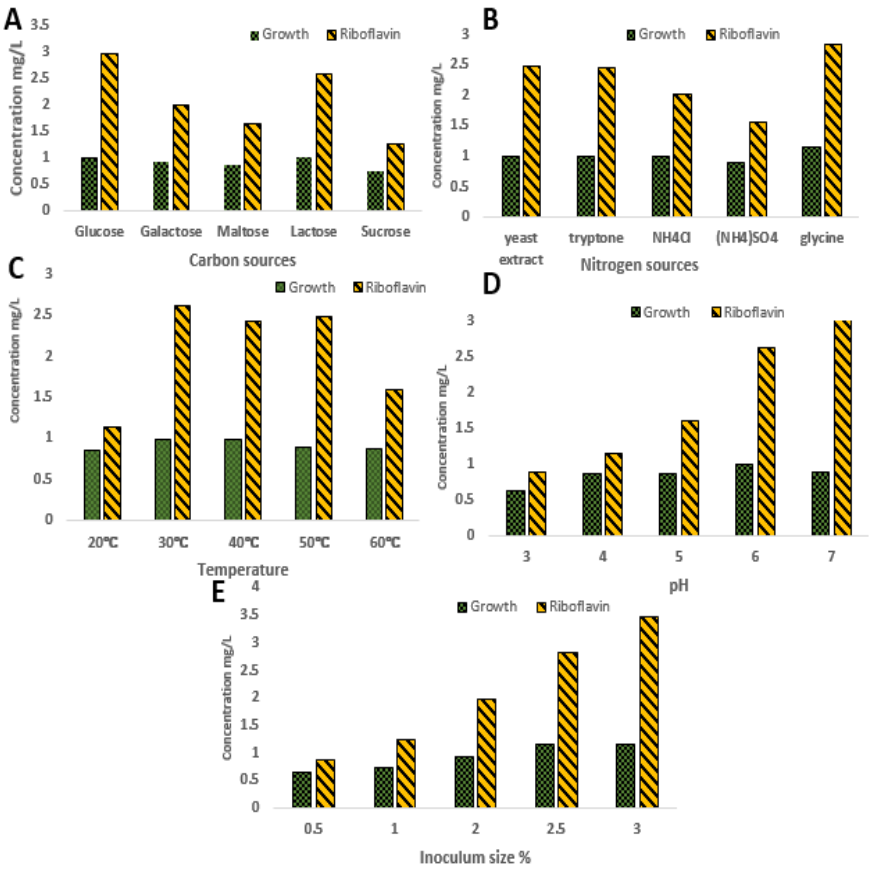


Figure 7 Factors influencing riboflavin production (A) sources of carbon (B) sources of nitrogen (C) Temperature (D) pH (E) size of Inoculum.

In continuation of the present study, the statistical experimental design – Response Surface Methodology (RSM) would enhance the riboflavin production from *Bacillus cereus* – HDS07 isolated from *Agaricus bisporus.*

To boost riboflavin production in this study, the medium should have a variety of carbon sources. *Bacillus cereus* – HDS07 produced riboflavin, using glucose as the main carbon source. Nitrogen, in addition to carbon, is essential for cell formation and proliferation. The best source of nitrogen was examined and found to be glycine. *Bacillus cereus* – HDS07 grew and produced riboflavin more efficiently at pH-6. Furthermore, the potent strain *Bacillus cereus* – HDS07 was cultivated at temperatures 40°C, producing 2.43 mg/L of riboflavin, indicating a higher quantity of riboflavin production in the current investigation. So far, very few studies have been conducted in riboflavin optimization in *Bacillus cereus*. When compared to the unoptimized medium, the *Bacillus cereus*-HDS07 strain isolated from *Agaricus bisporus* produced 2.3 times more riboflavin (3.48 mg/L) in the optimized medium.

CONCLUSION

The study's main purpose was to figure out the process parameters for increasing riboflavin production in *Bacillus cereus*-HDS07. Media optimization can improve riboflavin yield, so commercial manufacturing of riboflavin employing *Bacillus cereus*-HDS07 strain in large scale with improved medium components can enhance the yield of riboflavin. Considering, the importance of riboflavin for both dietary fortification and medical purposes, these conditions would enhance riboflavin production. Riboflavin is a vitamin that is routinely manufactured in industries and is one of the most important vitamins utilized in everyday life. It would be useful to society if this vitamin could be produced at a low cost. Recombination and cloning of the riboflavin gene (RIBA) could be used in future studies to enhance riboflavin synthesis**.**

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