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# Production of Amylase by *Arthrobacter kerguelensis* VL-RK_09 Isolated from Mango Orchards

**Rajesh Kumar Munaganti[1], Vijayalakshmi Muvva[1*] and Krishna Naragani[1]**

*[1]Department of Botany and Microbiology, Acharya Nagarjuna University, Guntur-522 510 A.P., India.*

***Authors' contributions***

*This work was carried out in collaboration between all authors. Author RKM performed the statistical analysis, wrote the protocol, wrote the first draft of the manuscript and managed the analyses of the study. Author VM designed the study. Author KN managed the literature searches. All authors read and approved the final manuscript.*

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## ABSTRACT

**Objectives:** To optimize the cultural parameters for improved production of amylase by *Arthrobacter kerguelensis* VL-RK_09 isolated from Mango orchards of Vissannapet, Krishna District, A.P., India.
**Methods:** The strain *A. kerguelensis* was screened initially for amylase production on Inorganic salts starch agar medium (ISP-4). The enzyme assay was performed as per the procedure described by Bernfield (1955). One amylase unit equals to that amount of enzyme needed to release 1 mg of reducing sugar (maltose as standard) for 15 min at 37°C. Attempts were also made to optimize cultural parameters such as pH, temperature, carbon and nitrogen sources affecting the production of amylase by the strain.
**Results:** Maximal yields of amylase were recorded after 4 days of incubation in Inorganic salts starch medium with initial pH 7.0 and temperature 35°C. ISP-4 broth amended with sorghum flour (2%) and yeast extract (0.5%) with initial pH 7.0 inoculated with *Arthrobacter kerguelensis* VL-RK_09 and incubated at 30°C for 96 h resulted in improved production of amylase from initial 4.0 U to 10.4 U/mL.
**Conclusion:** This is the first report on the production and optimization of amylase by *A. kerguelensis* and further studies on purification and characterization of the enzyme are in progress.

**Corresponding author: Email: profmuvva@gmail.com;*

*Keywords: Mango orchards; Arthrobacter kerguelensis; amylase; optimization.*

## 1. INTRODUCTION

Amylases are enzymes, capable of hydrolyzing α-1,4-glucosidic linkages in polysaccharides containing three or more 1,4-α-linked glucose units to give diverse products including dextrins and progressively smaller polymers. Amylases are among the most important enzyme classes having approximately 25-30% of the world enzyme market [1,2] derived from several plants, animals and microbes. Amylase application has widened in many fields such as clinical, medical and analytical chemistry as well as in starch saccharification and in the textile, food, fermentation, paper, brewing, baking and distilling industries [3-5]. The α-amylases also have an anti-staling effect in bread baking and improve the softness retention of baked goods there by increasing the shelf life of these products [6-8].

Microbial source of amylase is preferred over other sources because of its plasticity, vast availability, low cost, large productivity, chemical stability and environmental protection [9-11]. For decades, microbial α-amylases have been widely used in the industry and they rank first in terms of commercial exploitation among various extracellular enzymes [12]. Most of them are metallo enzymes, which require calcium ions ($Ca^{2+}$) for their activity, structural integrity and stability. They belong to family 13 (GH-13) of the glycoside hydrolase group of enzymes [13]. The major advantage of using microorganisms as a source of amylases is their economical production capacity and easy to manipulate to obtain enzymes of desired characteristics. The microbial amylases meet industrial demands and a large number of them are available commercially and almost replaced chemical hydrolysis of starch in starch processing industry [14]. Extensive studies were carried out on the production of amylases by actinomycetes including *Arthrobacter psychrolactophilus* [15], *Streptomyces* spp. [16] and Streptomyces strain A3 [17].

The manipulation of growth conditions of microorganisms is a common strategy employed for high yields of the bioactive compounds such as enzymes. The present work describes the effects of different culture conditions for improved amylase production by *Arthrobacter kerguelensis* VL-RK_09 in shake flasks under controlled conditions.

## 2. MATERIALS AND METHODS

### 2.1 Microorganism

*Arthrobacter kerguelensis* VL-RK_09 was isolated from the soil samples of Mango orchards, Vissannapet, Krishna district of Andhra Pradesh, India. The strain was isolated on yeast extract malt extract dextrose agar (ISP-2), purified and preserved at 4°C. The strain was identified by 16S rDNA analysis and the gene sequence was deposited in the NCBI gene bank with accession number KJ787652.

### 2.2 Screening for Amylase Activity

The amylolytic activity of the strain was assessed by inoculating on Inorganic salts starch agar (ISP-4) [18] composed of 1% soluble starch, 0.01% (w/v) $K_2HPO_4$, 0.01% (w/v) $MgSO_4$. $7H_2O$, 0.01% (w/v) NaCl, 0.02% $(NH_4)_2SO_4$, 0.02% (w/v) $CaCO_3$, 0.001% (w/v) $MnCl_2$, 0.001% $ZnSO_4$. $7H_2O$, agar 2.0% (pH 7.2) and incubated at 30°C for 72 h. After incubation, the plate was flooded with Gram's iodine solution and left for 5 min. Zone of clearance or decolorization against the blue background is considered as the positive reaction for amylolytic activity.

### 2.3 Production Medium and Cultural Conditions

For selection of suitable media for growth and amylase activity, the strain *A. kerguelensis* VL-RK_09 was cultivated in four different media such as starch glycerol broth (M-1), starch peptone broth (M-2), starch arginine salts broth (M-3), inorganic salts starch broth (M-4) (ISP-4) and incubated at 35°C for 3 days. After incubation the growth and amylase activity were determined simultaneously. The medium in which maximum amylase production was recorded is selected for further study. In the present study, maximum growth and amylase production of the strain was recorded in ISP-4 broth.

### 2.4 Production Profile and Assay of Amylase

Inorganic salts-starch broth (ISP-4) was used for studying the amylolytic profile of *Arthrobacter kerguelensis* VL-RK_09. The culture suspension of the strain, prepared from one week old culture

inoculated into ISP-4 broth and fermentation was carried at 30°C for 7 days. Production of biomass as well as amylase activity was analyzed. Biomass was measured in terms of dry weight (mg $ml^{-1}$) and the culture filtrates collected at regular intervals of 24 h served as crude enzyme extract.

The enzyme assay was based upon the procedure described by Bernfield (1955) [14]. The reaction mixture containing 1 ml of 1% (w / v) starch solution and 1 ml of enzyme extract was incubated at room temperature for 15 min. The reaction was terminated by the addition of 2 ml Dinitrosalicylic (DNS) reagent and the tubes kept at zero time incubation served as control. The solution was heated in a boiling water bath for 5 min followed by the addition of 1 ml potassium sodium tartrate solution. After cooling, the volume of the solution was made up to 10 ml and optical density was read at 560 nm. One amylase unit equals to that amount of enzyme needed to release 1 mg of reducing sugar (maltose as standard) for 15 min at 37°C.

### 2.5 Optimization of Cultural Conditions for Improved Amylase Production by *A. kerguelensis* VL-RK_09

#### 2.5.1 Effect of pH on growth and amylase production

The effect of initial pH on growth and enzymatic activity of the strain was determined by inoculating it in inorganic salts-starch broth (ISP-4) with initial pH levels ranging from 4 to 10 and incubated at 30°C in shaking condition. Biomass and enzyme activity were measured after 96 h of incubation. The optimal pH achieved at this step was used for further study.

#### 2.5.2 Impact of temperature on growth and amylase production

To study the impact of temperature on growth and enzyme production, inorganic salts-starch broth (ISP-4) with initial temperatures ranging from 20°C to 40°C was inoculated with the strain and incubated for 96 h. Temperature at which the strain showed maximum amylolytic activity was fixed for further studies.

#### 2.5.3 Influence of carbon and nitrogen sources on amylase production by the strain VL-RK_09

The effect of carbon sources on growth and amylase production was studied by supplementing the production medium with different carbon sources such as dextrose, lactose, sucrose, maltose, mannitol, sorbitol, sorghum flour and rice flour each at a level of 1% (w / v). Influence of different levels of best carbon source (1-5% w / v) on enzyme production was examined. The impact of nitrogen sources on enzymatic yield was also investigated by adding different nitrogen sources like yeast-extract, urea, tyrosine, tryptone, potassium nitrate, peptone, beef extract, ammonium sulphate (each at a concentration of 0.2%) to the production medium containing an optimum amount of the superior carbon source. Besides, the concentration of nitrogen source (0.25 - 1.5%) supporting optimal yields of amylase was also recorded.

#### 2.5.4 Statistical analysis

Statistical data are recorded on biomass of the strain and enzyme production by using One-way Analysis of Variance (ANOVA).

## 3. RESULTS AND DISCUSSION

### 3.1 Identification of the Strain

The strain was identified as *Arthrobacter kerguelensis* VL-RK_09 based on morphological, cultural, physiological, biochemical and 16S rDNA analysis.

### 3.2 Screening for Amylase Activity

Screening of amylase was carried out on Inorganic salts starch agar (ISP-4). The production of amylase was evidenced by a clear zone around the colony against the dark blue background (Plate 1).

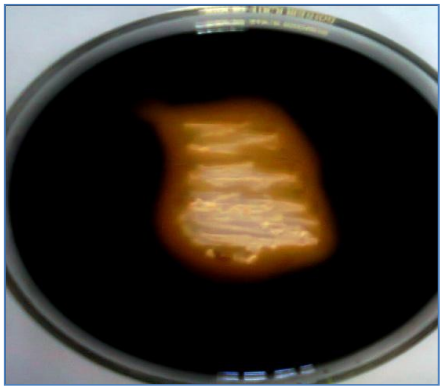

**Plate 1. Production of amylase by *Arthrobacter kerguelensis* VL-RK_09**

### 3.3 Production Profile and Assay of Amylase

Among the four different media tested, ISP-4 supported the maximum biomass and amylase activity by the strain *A. kerguelensis* VL-RK_09 (Fig. 1).

Growth pattern as well as amylolytic activity of the strain was determined in ISP-4 broth. The production of amylase by the strain began after 24 h of incubation, increased gradually, peaked at 96 h and then started declining (Fig. 2). High yield of amylase was reported after 48 h of incubation in *Streptomyces chaeonanensis, S. erumpens* and thermophilic *Streptomyces* sp. MSC702 [19-21]. The optimal incubation period for obtaining high yields of amylases was reported to be 96 h for *Streptomyces albidoflavus* [22] and *S. tendae* TK-VL_333 [23], while it was 72 h for *S. clavifer* [24].

### 3.4 Impact of Initial pH on Amylase Production by *Arthrobacter kerguelensis* VL-RK_09

The optimum pH for biomass and amylase production by the strain was found to be 7.0 (Fig. 3). These results are in conformity with that reported for *Streptomyces aureofaciens* 77 [25], *S. erumpens*, [20], *S. tendae* TK-VL_333 [23], *Streptomyces* sp. MSC702 [21] and *Rhodococcus* spp. [26]. Shang and Wang (1999) recorded high yields of amylases at pH 6.7 from *Streptomyces rimosus* [19]. Yassien and Asfour (2012) noted the optimal pH for the production of amylase as 6.0 for *S. clavifer* [24].

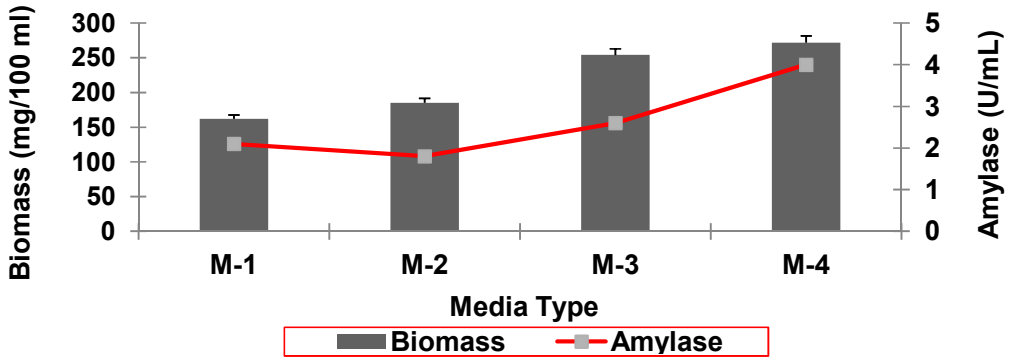


**Fig. 1. Growth and amylase production of *A. kerguelensis* VL-RK_09 cultured in different media**

*[M-1: Starch glycerol broth; M-2: Starch peptone broth; M-3; Starch arginine salts broth; M-4: Inorganic salts starch broth]*

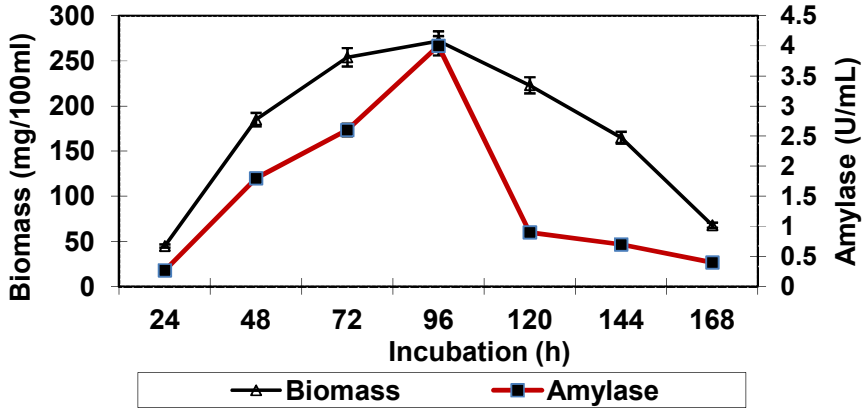


**Fig. 2. Production profile of amylase by *A. kerguelensis* VL-RK_09 in inorganic salts starch broth (ISP-4)**

*(Values are the means of three replicates ±SD)*

### 3.5 Effect of Temperature on Amylase Production by *Arthrobacter kerguelensis* VL-RK_09

The strain incubated at 30°C showed maximum yields of biomass and amylase (Fig. 4). The optimal temperature for amylase production was also reported to be 30°C for *Streptomyces albidoflavus* [22] and *S. tendae* TK-VL_333 [23] while it was 28°C for *S. aureofaciens* 77 [25]. *S. erumpens* and *Streptomyces* sp. D1 yielded optimal levels of amylase at 50°C [20,17]. The optimal temperature for amylase production was recorded as 45°C for *S. gulbargensis* [27]. Ayub et al. [28] recorded 60°C as the optimal temperature for the production of amylase by *Thermoactinomyces sacchari* .

### 3.6 Influence of Carbon and Nitrogen Sources on Amylase Production by *Arthrobacter kerguelensis* VL-RK_09

Different carbon and nitrogen sources were amended separately to the production medium to determine their effect on amylase production. Sorghum flour was found to be the best carbon source for amylase production by the strain followed by rice flour and lactose (Fig. 5). Utilization of carbon sources for the production of amylases by *Streptomyces* sp. was found to vary. High amounts of amylases were reported with starch for *Streptomyces aureofaciens* 77 and *S. albidoflavus* [25,22], while sorghum flour supported high yields of amylases from *S. tendae* TK-VL_333 [23].

In the present work, maximum yield of amylase was obtained with sorghum flour which may be useful for the development of cost-effective and high quality biotechnological processes for amylase production. As sorghum flour supported high yield of amylases, the effect of different concentrations of sorghum flour was further analyzed. High productivity of amylase by the strain was observed when cultured in the medium containing 2% sorghum flour (Fig. 6). Culture medium amended with 3% soluble starch favored high yields of amylase production by *S. aureofaciens* 77 [25], while sorghum flour at 3% supported amylase production by *S. tendae* TK-VL_ 333 [23].

The presence or absence of several amino acids and complex nitrogen sources in the medium influences the synthesis of amylase by microorganisms [21]. In the present study, yeast extract was found to be the best organic nitrogen source for high amylase production followed by peptone, tryptone and tyrosine. Potassium nitrate proved to be good inorganic nitrogen source for the production of amylase by the strain followed by ammonium sulphate (Fig. 7). Narayana and Vijayalakshmi [22] reported yeast extract as the best nitrogen source for high amylase production by *S. albidoflavus.* Natasha et al. [29] reported yeast extract as the best nitrogen source for amylase production by *Bacillus licheniformis* ATCC 9945. Peptone was reported to be the best organic nitrogen source for high amylase production by *Streptomyces tendae* TK-VL_333

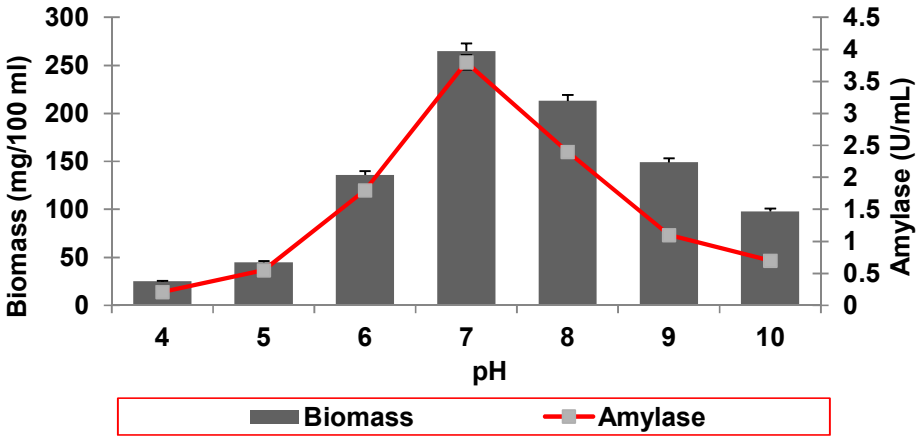


**Fig. 3. Impact of pH on growth and amylase production by *Arthrobacter kerguelensis* VL-RK_09**
*(Values are the means of three replicates ±SD)*

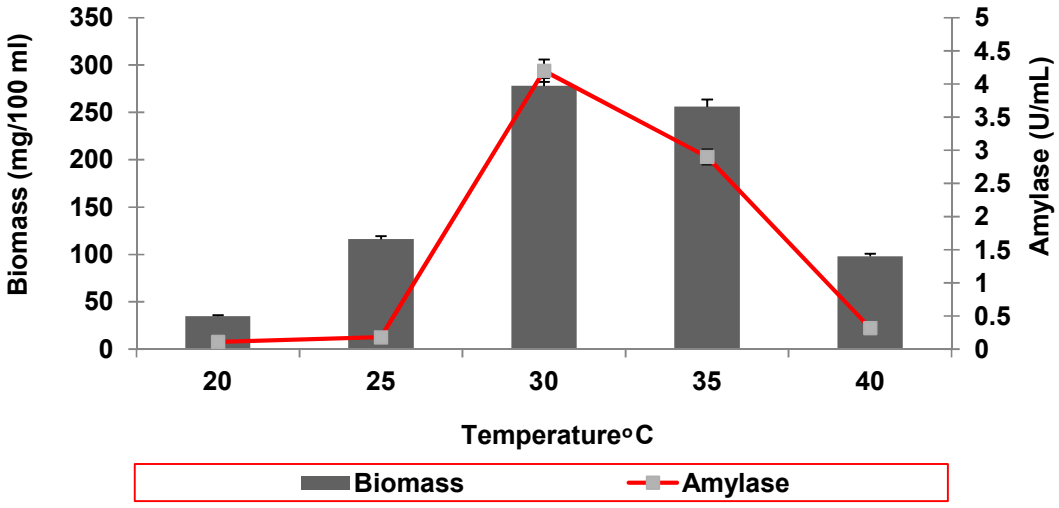


**Fig. 4. Effect of temperature on growth and amylase production by *Arthrobacter kerguelensis* VL-RK_09**
*(Values are the means of three replicates ±SD)*

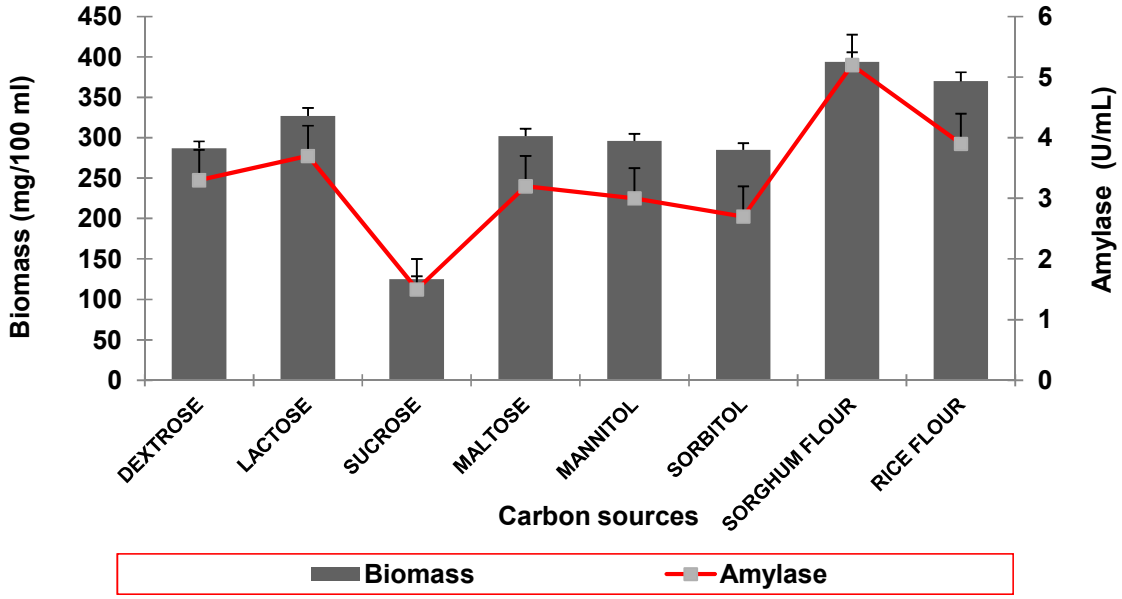


**Fig. 5. Influence of carbon sources on growth and amylase production by *Arthrobacter kerguelensis* VL-RK_09**
*(Values are the means of three replicates ±SD)*

and *Streptomyces* sp. MSC702 [22,21]. The production of amylase by the strain was tested by increasing the concentration of yeast extract in the medium from 0.25 to 2%. Yeast extract at 0.5% was found to support the production of high levels of the enzyme (Fig. 8).

Narayana and Vijayalakshmi [22] noted that 0.5% yeast extract as the optimal nitrogen source for amylase production by *Streptomyces albidoflavus.* Kavitha and Vijayalakshmi [23] reported peptone at 0.5% was suitable for high amylase production by *Streptomyces tendae* TK-

VL_333 [23]. ISP-4 broth amended with sorghum flour (2%) and yeast extract (0.5%) with initial pH 7.0 inoculated with *A. kerguelensis* VL-RK_09 and incubated at 30°C for 96 h resulted in the improved the production of amylase from initial 4.0 U to 10.4 U/mL.

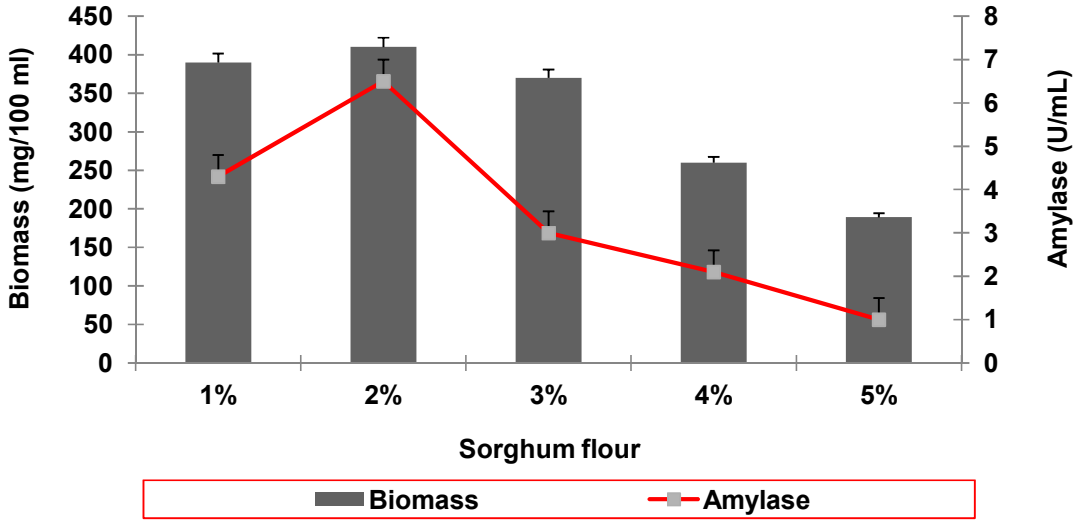


**Fig. 6. Effect of concentration of sorghum flour on growth and amylase production by *Arthrobacter kerguelensis* VL-RK_09**
*(Values are the means of three replicates ±SD)*

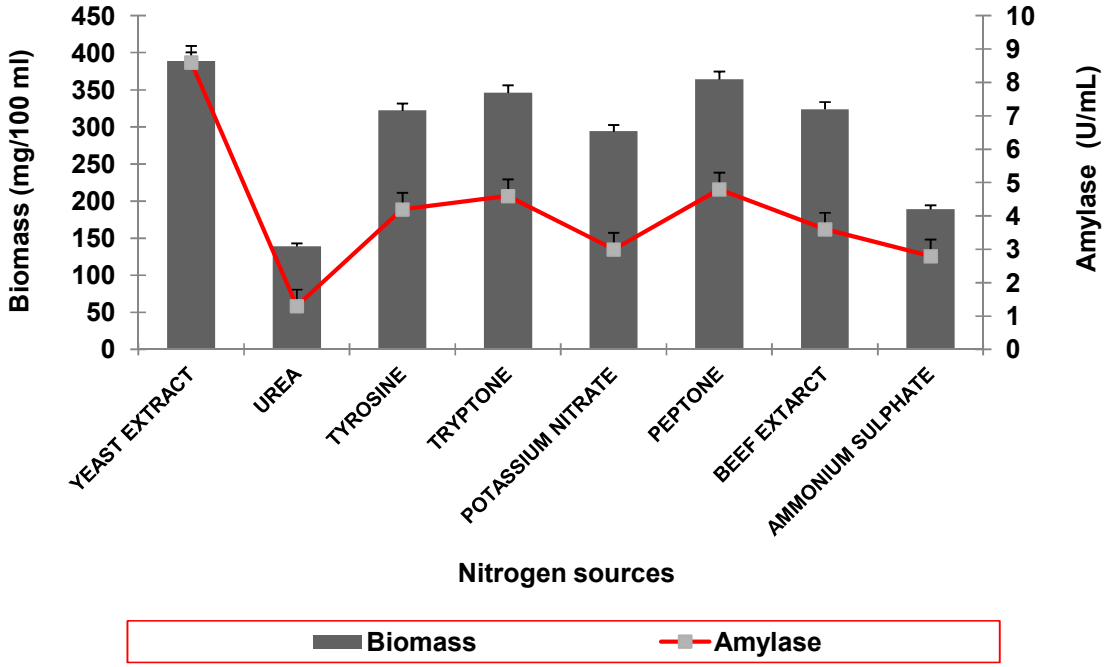


**Fig. 7. Effect of nitrogen sources on growth and amylase production by *Arthrobacter kerguelensis* VL-RK_09**
*(Values are the means of three replicates ±SD)*

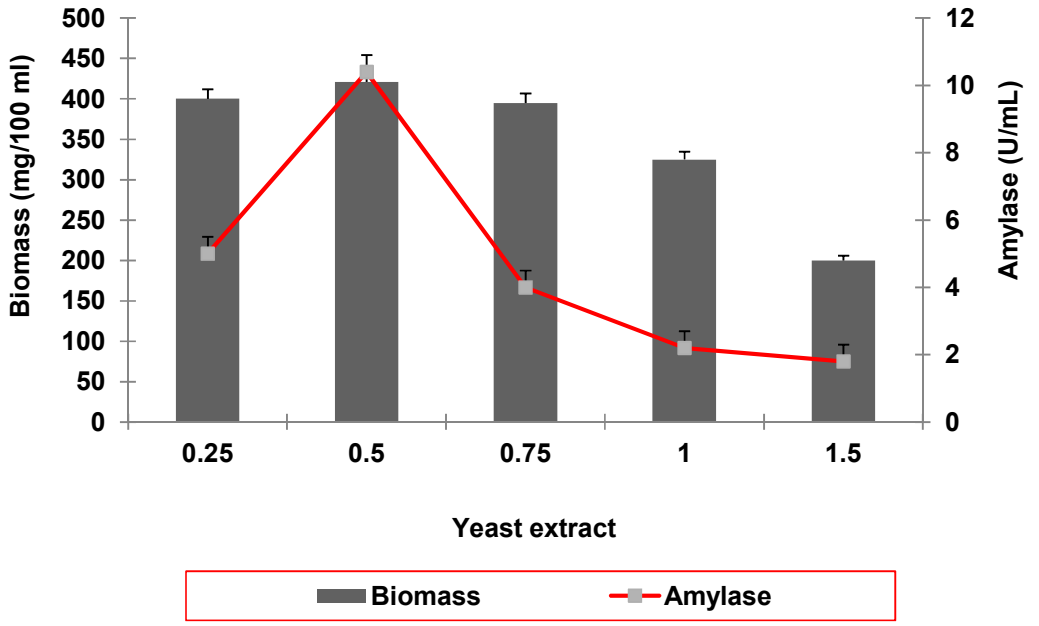


**Fig. 8. Impact of concentration of yeast extract on growth and amylase production by *Arthrobacter kerguelensis* VL-RK_09**
*(Values are the means of three replicates ±SD)*

## 4. CONCLUSION

The present study revealed enhanced amylase production of *Arthrobacter kerguelensis* VL-RK_09 isolated from Mango orchards in the medium (ISP-4) amended with sorghum flour (2%) and yeast extract (0.5%) with initial pH 7.0 at 30°C after 96 h of incubation. The enzyme yield was improved from initial 4.0 U to 10.4 U / mL under optimized conditions. This is the first report of amylase production by *A. kerguelensis* VL-RK_09 isolated from Mango orchards.

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## COMPETING INTERESTS

Authors have declared that no competing interests exist.

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