



ORIGINAL ARTICLE

Identification and product optimization of amyolytic *Rhodococcus opacus* GAA 31.1 isolated from gut of *Gryllotalpa africana*



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Received 8 September 2015; revised 27 March 2016; accepted 21 May 2016
Available online 9 June 2016

KEYWORDS

Gryllotalpa;
Gut;
Rhodococcus;
Amylase;
Optimization;
Agro-waste

Abstract An attempt has been made to isolate potent amylase producing gut bacteria from *Gryllotalpa africana*. Out of 82 isolates, GAA 31.1 was selected as potent producer, having enzyme activity 9.6 ± 0.861 U/ml. The isolate GAA 31.1 was identified as *Rhodococcus opacus* following morphological, biochemical, physiological characterization and phylogenetic analysis through 16S rRNA gene sequencing. Fatty acid methyl ester profile of the isolate was also studied. The optimized physical cultural conditions for amylase production were found as incubation period 48 h, inoculum volume 2%, initial pH of the fermentation medium 7.0, temperature 38 °C and aeration at 150 rpm. Optimum nutrient conditions were determined as: supplementation of maltose 1.4% and sodium nitrate 1.4%. Surfactants SDS, EDTA, Tween 80 and Triton X-100 showed positive effect on enzyme production. Riboflavin (50 µg/ml) among the tested vitamins stimulated the production maximally. The isolate was also able to produce amylase using agro-industrial waste. This actinobacterium may be a potent candidate for amylase as it is capable of enhanced production (326.72 ± 6.081 U/ml) by utilizing agro-residues.

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1. Introduction

Amylases are among the most important industrial enzymes with great significance in biotechnological approach. Microbial amylases are extensively utilized in industries including food, paper, textiles, sweeteners, detergents, fuel ethanol,

alcoholic beverages, digestive aid, dry cleaning, medicinal and analytical chemistry [1]. Enzymes of microbial origin are widely used in industrial processes due to their low cost, large productivity, vast availability, chemical stability and flexibility [2,3]. Bacteria and fungi are the workhorses in the company of amylase producers. In spite of diverse biochemical properties, similar attention is yet to be paid to actinobacteria.

Insects harbor a rich and complex community of microorganisms in their guts as symbiont, depending on their feeding habits [4]. These symbionts can retain and multiply in

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Peer review under responsibility of National Research Center, Egypt.

<http://dx.doi.org/10.1016/j.jgeb.2016.05.005>

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the gut in consequence of degrading diet compounds, whereas, microbes that are inefficient, are washed out [5]. Insects have evolved symbiotic interactions with different microorganisms carrying out key hydrolytic activities [4] and play an indispensable role in the digestion of food that make the insect gut a 'hot spot' for gene transfer [6,7]. Less than 1% of insect gut symbionts are studied so far [8]. The taxon Orthoptera consists of a good number of economically important pest species of which *Grylotalpa africana*, a soil inhabitant pest, normally feeds upon a wide range of commercially important plant taxa including its starchy elements [9,10]. So, it can be assumed that the endosymbionts may be the good producer of digestive enzymes, particularly amylase.

Biosynthesis is a hereditary property that has developed in the course of evolution. Apart from its genetic makeup, efficiency of the producer depends greatly upon the conditions of cultivation, i.e. physical and physicochemical factors of cultivation. There is no common medium that might be used for the study of all phenomena or regularities concerning production of metabolites of the microorganisms. By varying the conditions of cultivation it is possible to stimulate production by controlling its property. Therefore, it is essential to pay special attention to the medium formulation and physical factors for cost effective production. Solid state fermentation (SSF) is getting momentum for amylase production over submerged fermentation (SmF) due to its higher productivity, minimum waste generation and lesser time consumption.

The present investigation was under taken for formulation of suitable medium condition for low cost amylase production by *Rhodococcus opacus* GAA 31.1, isolated from *G. africana*.

2. Materials and methods

2.1. Isolation and screening

The insects, *G. africana* (Order-Orthoptera), were collected from the crop fields and agro-waste dumps of Burdwan and Hooghly districts of West Bengal, India. Based on their morphological characterization the insects were identified as *G. africana* (Order-Orthoptera), by Prof. (Dr.) Abhijit Mazumdar, Entomology Laboratory, Department of Zoology, The University of Burdwan, India. The insects (eight) were kept in starvation for two days to eliminate allochthonous microbial community and used as a source of amylolytic microorganisms. Isolation of amylolytic microbes from different parts of insect gut was made as per Zhang and Jackson [11] and plated in Tryptone Starch (TS) agar medium [12]. Primarily, amylolytic capabilities of the insect gut isolates were tested using TS agar plates in replica by flooding with iodine solution and the positive isolates were collected from the replica plates [13]. Starch hydrolysis ratio (SHR) was calculated following Abd-Elhalem et al. [14]. Finally the promising isolates were screened through DNS method [15].

2.2. Identification of the selected isolate

Colony morphology was made through visual study. Micro morphological studies were done using phase contrast microscope (Leitz-Laborlux D, Germany) and scanning electron microscope (Hitachi-530, Japan) [16]. Physiological and biochemical characterizations of the selected isolate were made

according to the American Society for Microbiology [17] and Bergey's Manual of Systematic Bacteriology [18].

DNA was isolated according to Rainey et al. [19]. The 16S rRNA gene was amplified with primers 8-27f (5'-AGAGTTT GATCCTGGCTCAG-3') and 1500r (5'-AGAAAGGAGGT GATCCAGCCA-3'), separated on 1% agarose gel, eluted and purified using a QIA quick gel extraction kit (Qiagen). The purified PCR product was sequenced with four forward and three reverse primers, namely 8-27f, 357f (5'-CTCCTAC GGGAGGCAGCAG-3'), 704f (5'-TAGCGGTGAAATGCG TAGA-3'), 1114f (5'-GCAACGAGCGCAACC-3'), 685r (5'-TCTACGCATTTACCCGCTAC-3'), 1110r (5'-GGGTTG CGTCTGTTG-3') and 1500r (*Escherichia coli* numbering system). The 16S rRNA gene sequence was determined by the dideoxy chain-termination method with the Big-Dye terminator kit using an ABI 310 Genetic Analyzer (Applied Biosystems, USA).

A sequence similarity search was done using GenBank BLASTN [20]. Sequences of closely related taxa were retrieved and aligned using the program CLUSTAL_X [21] and the alignment was adjusted manually. For neighbor-joining analysis [22], distances between the sequences were calculated using Kimura's two-parameter model [23]. Bootstrap analysis was performed to assess the confidence limits of the branching [24].

Fatty acid composition of cell membrane was determined by fatty acid methyl ester analysis according to Sherlock version 6.1, Method SACTIN 6, Matches Library RTSBA6 6.10 [25].

2.3. Submerged fermentation

Two day old culture of *R. opacus* GAA 31.1, in TS medium [12] stored at 4 °C, was utilized for experiment. To determine the suitable medium for maximum amylase production M1 [26], M2 [12], M3 [27], M4 [28] and M5 [29] were tried.

Batch experiments were carried out in basal medium M2 (100 ml in 500 ml Erlenmeyer flask) at 35 °C for 7 days at shaking (150 rpm) using 4% inoculum (1.2×10^7 CFU/ml) in pH 7.0. Fermented broth after centrifugation at 8000 rpm for 10 min was used as crude enzyme. One parameter was optimized keeping other as constant at a time. The optimized parameter of an experiment was considered for the designing of subsequent experiments. Experiments were done in triplicate.

2.4. Solid state fermentation

Different agro-residual substrates (10 gm each) such as wheat bran (WB), rice bran (RB), rice husk (RH), gram husk (GH), potato waste (PW), coconut oil cake (COC) and mustard oil cake (MOC) were tested for amylase production. The substrates were moistened at 50% (w/v) with sodium phosphate buffer (0.2 M, pH 7.0). Fermentation was done using 2% (v/v) inoculum (1.2×10^7 CFU/ml) into 250 ml Erlenmeyer flask and incubated at 38 °C for 2 days.

2.5. Enzyme assay

Amylase activity was measured by the DNS method [15] and expressed as U/ml. One enzyme unit is defined as the amount

of enzyme releasing 1 mM of glucose from the substrate in one minute at 38 °C.

2.6. Statistical analysis

Standard error (\pm) of mean was calculated from using Microsoft Office Excel 2007.

3. Results and discussion

3.1. Isolation and screening

Initially 82 amylase producers were isolated. Among them ten isolates were screened on the basis of their SHR capability and enzyme production on TS medium (Fig. 1). Finally, the isolate GAA 31.1 was selected as potent amylase producer (9.6 ± 0.861 U/ml). Relevant amyolytic organisms,

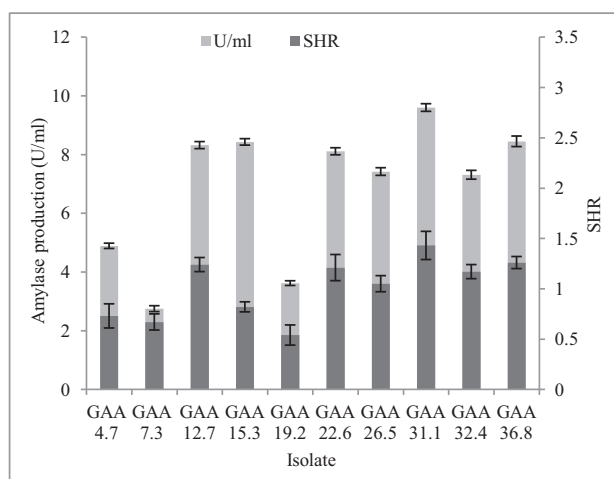


Figure 1 Screening of amyolytic isolates on the basis of SHR and amylase production.

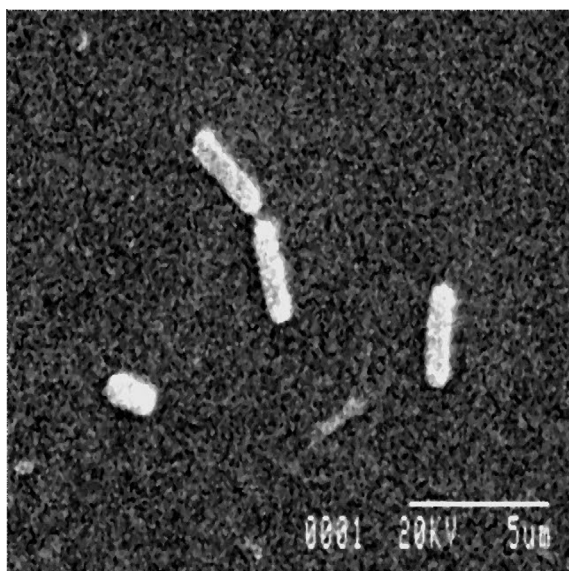


Figure 2 Scanning electron micrograph of GAA 31.1.

Halobacillus sp. MA-2 capable to produce 3.2 U/ml [30], 7.01 ± 0.21 U/ml by *Bacillus licheniformis* [28], 1.44 and 1.42 U/ml by *B. licheniformis* and *B. subtilis*, respectively [31] and 4 U/ml by *Bacillus* spp. [32] were reported.

3.2. Identification of the isolate GAA 31.1

Colonies of the isolate GAA 31.1 were irregular, valvate with umbonate elevation, showing lobate to erose margin and yellowish in maturity. The cells appeared singly or in aggregate, rod shaped $0.8-1 \times 4-5$ μm (Fig. 2). Gram positive, growth between 20 and 50 °C, pH tolerance up to 10.0 and NaCl tolerance < 7%, citrate and urea utilization negative but can produce acid from only glucose and nitrate reduction positive (Data not shown). 16S rDNA of GAA 31.1 contains 1372 bases (NCBI GenBank Acc No. JX993905) with G + C content 58.09%. The BLAST analysis using 16S rRNA sequence for phylogenetic tree revealed 98% homology (Fig. 3) with *R. opacus* DSM 43205^T (X80630).

Analysis of cell membrane fatty acids can be effectively applied for chemotaxonomy [33]. The unique pattern of fatty acids in bacteria is considered as signature sequence and is the basis of bacterial identification. From the FAME study (Fig. 4), saturated and unsaturated fatty acids of cell membrane of the isolate GAA 31.1 were found 96.78% and 3.93%, respectively (Table 1). Similarity index was close to *Staphylococcus schleiferi* (Index 0.241) and *Bacillus alcalophilus* (Index 0.185).

Members of the genus *Rhodococcus* are widely distributed in natural environments such as soil, water and marine sediments [34], and insect gut [35]. But amylase production by the member of *Rhodococcus* from insect gut is yet to be reported.

3.3. Optimization of amylase production

3.3.1. Effect of media

It is essential to select medium for optimum production because no specific medium has been established for amylase production [36]. The isolate GAA 31.1, performed best in M2 (9.6 ± 0.86 U/ml) followed by M4 (8.33 ± 0.32 U/ml), M3 (5.63 ± 0.28 U/ml), M1 (1.63 ± 0.16 U/ml) and M5 (0.41 ± 0.09 U/ml) (Fig. 5A). Probably, M2 persuades nutrient requirement for the isolate optimally (Fig. 5A).

3.3.2. Effect of inoculum size

Inoculum concentration is one of the important factors to consider while optimizing enzyme production. It was found that the isolate produced maximally at 2% level, further increase in inoculum volume inversely correlated with the production (Fig. 5B) that corroborates with the finding of Vishnu et al. [37]. Inoculum size beyond optimum causes higher growth and fast nutrient depletion results in accumulation of byproducts in the fermentation medium that tends to decline in production [38].

3.3.3. Effect of medium pH

Initial pH of the fermentation medium plays a marked role on the product of interest. Sometimes, it affects biosynthesis by stimulating enzymatic processes and transport of components

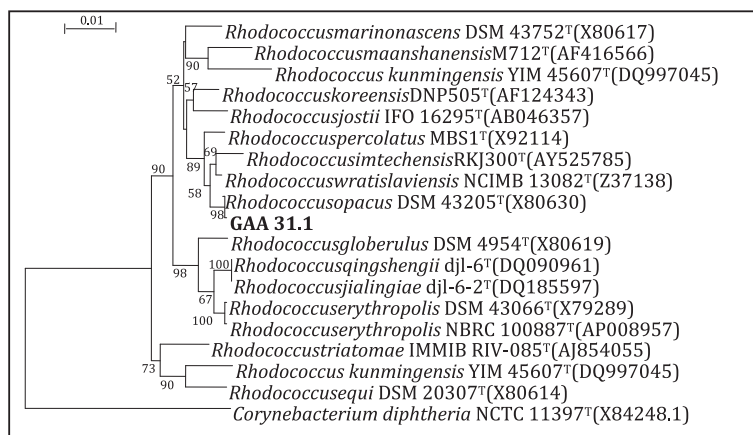


Figure 3 Phylogenetic tree showing relations of *R. opacus* GAA 31.1 with other closely related strains. Bar, 0.005 substitutions per nucleotide position.

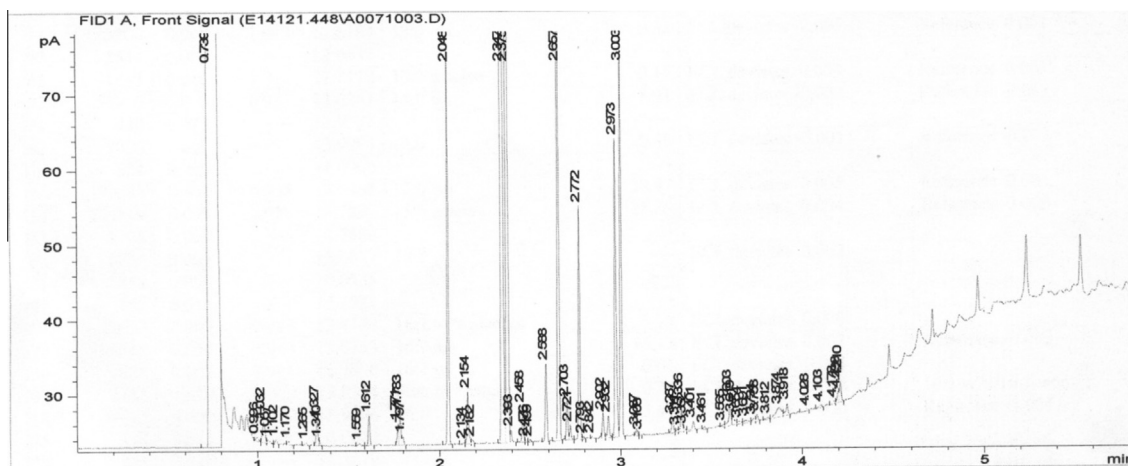


Figure 4 Chromatogram of isolate GAA 31.1 showing the fatty acid peaks.

Table 1 Fatty acid content of GAA 31.1.

Fatty acid	Content (%)	Fatty acid	Content (%)	Fatty acid	Content (%)	Fatty acid	Content (%)
Straight chain		Branched chain		Unsaturated		Hydroxyl	
C 12:0	0.50	ISO 13:0	0.32	16:1 ω 7C alcohol	1.15	ISO 17:13OH	0.05
C 14:0	0.88	ISO 14:0	5.91	16:1 ω 11C	0.61	Sum in feature	
C 15:0	T*	ISO 15:0	38.47	18:1 ω 9C	0.41	3	0.24
C 16:0	3.40	ISO 16:0	1013	Cyclo 19:0 ω 8C	0.12	4	0.38
C 17:0	0.12	ISO 17:0	4.69			8	0.07
C 18:0	0.22	ISO 17:1 ω 10C	0.38				
		ISO 18:0	0.28				
		ISO 19:0	0.44				
		Anteiso 11:0	0.08				
		Anteiso 13:0	0.15				
		Anteiso 15:0	25.35				
		Anteiso 17:0	5.67				

T* = Trace amount, sum in 3 feature comprises 16:1 ω 7C/16:1 ω 6C; Sum in 4 feature comprises 17:1 iso I; Sum in 8 feature comprises 18:1 ω 7C

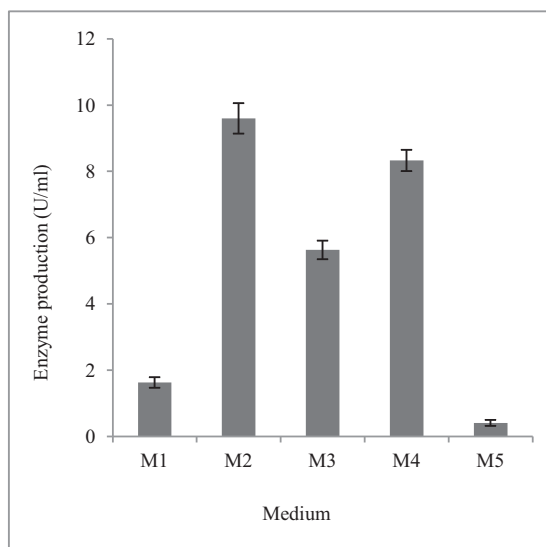


Figure 5A Selection of suitable medium on the amylase production.

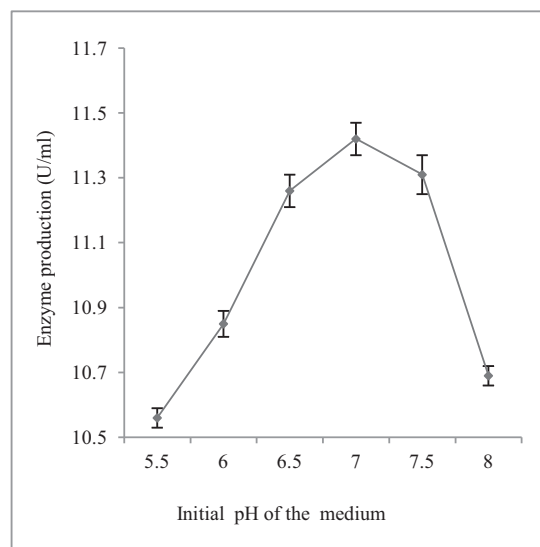


Figure 5C Optimization of initial pH of the medium on the amylase production.

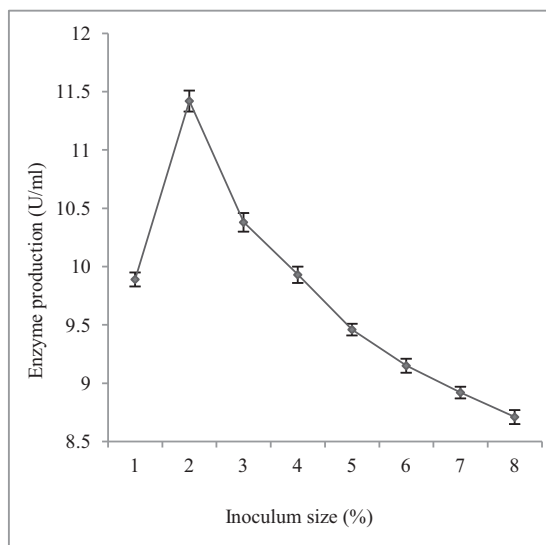


Figure 5B Suitability of inoculum volume on the amylase production.

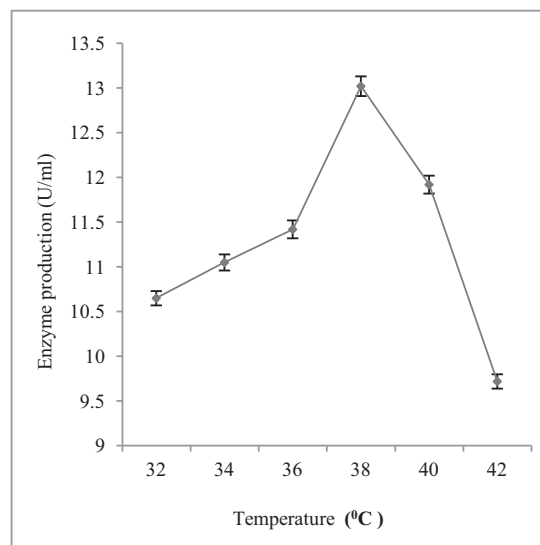


Figure 5D Optimization of incubation temperature on the amylase production.

across the cell membrane [39]. The experimental data (Fig. 5C) revealed elevated performance of the organism concerned up to pH 7.0 (11.42 ± 0.05 U/ml). A good number of amylase producers exhibit their performance optimally at neutral pH [12,26,38].

3.3.4. Effect of temperature

At lower incubation temperature, enzyme inactivation and suppression of substrate transport across the cells are affected that causes the lowering of yields, whereas, at temperatures beyond the optimum energy requirement for cellular growth is high due to thermal denaturation of enzymes involved in metabolic pathway that results in the inhibition of product formation [40]. The isolate GAA 31.1 was capable of continuing its enzyme production (13.02 ± 0.11 U/ml) up to 38 °C

(Fig. 5D). The mesophilic nature of the isolate shows a close harmony with actinobacteria like *Rhodococcus spp.* and *Streptomyces spp.* PDS1 [41], also with *Cronobacter sakazakii* [42] and *Bacillus spp.* [43].

3.3.5. Effect of incubation period

Incubation period is an important factor for fermentation. There was a gradual increase in amylase production (27.49 ± 0.41 U/ml) up to 48 h of incubation beyond which the product declined (Fig. 5E). Sankaralingam et al. [44] report optimum amylase production by *B. licheniformis* after 48 h of incubation. Similar results are also found by Alariya et al. [38] and Deb et al. [45]. At the early stage, the availability of nutrient and other growth factors was sufficient

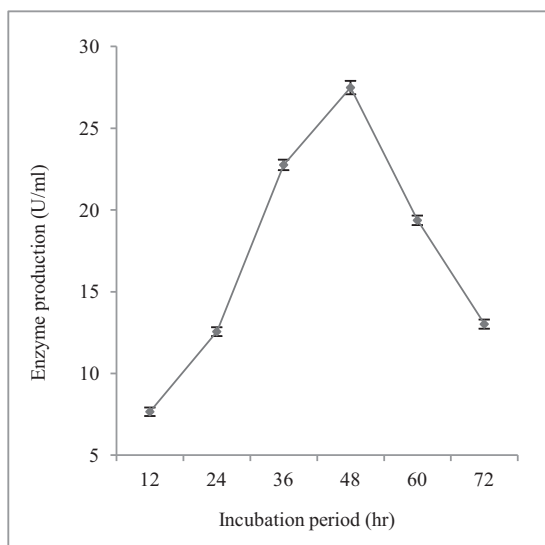


Figure 5E Suitability of incubation period on the amylase production.

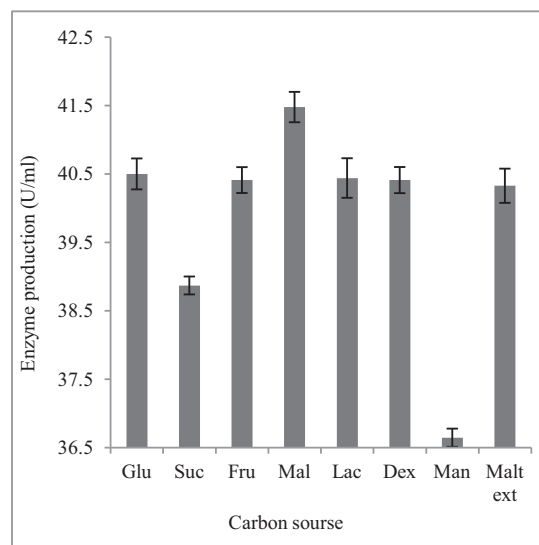


Figure 6A Effect of supplementary carbon sources on amylase production [Glu-Glucose, Suc-Sucrose, Fru-Fructose, Lac-Lactose, Dex-Dextrose, Man-Mannitol, Malt ext- Malt extract].

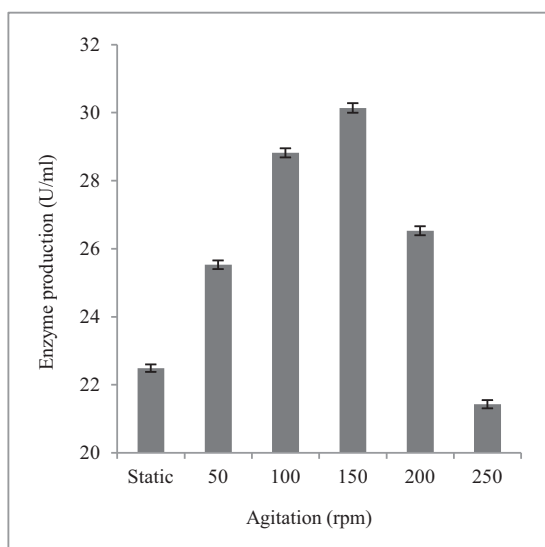


Figure 5F Effect of aeration on the amylase production.

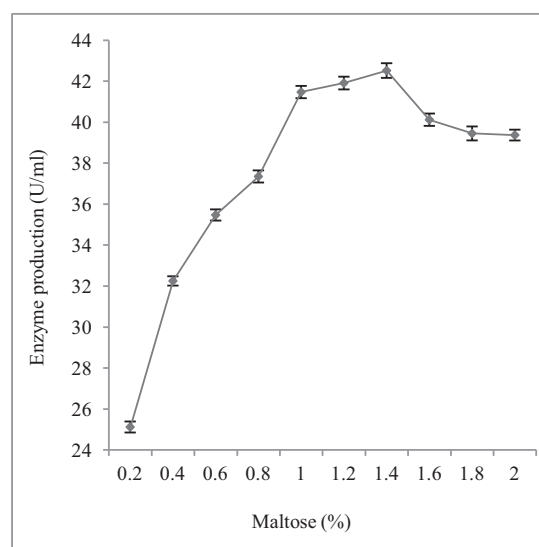


Figure 6B Effect of different concentrations of maltose on the amylase production.

but at the later stage depletion of nutrients and accumulation of toxic substances led to unfavorable conditions [46].

3.3.6. Effect of aeration

While studying the effect of aeration, it was found that at 150 rpm the enzyme production was $(30.14 \pm 0.14 \text{ U/ml})$ 9.6% increased than that of static (Fig. 5F). Normally oxygen of the air has limited solubility thus the fermentation broth must be agitated to ensure sufficient oxygen supply to the organism [47]. Optimum amylase production at 150 rpm was also reported [45]. At higher agitation, the catalyst particles may be thrown out outside the liquid phase, sticking to the wall of the reaction vessel, leading to oxygen tension. Higher agitation may also cause shearing of the enzyme molecule responsible for production diminution [48].

3.3.7. Effect of carbon sources

Amylase is an inducible enzyme, generally induced in the presence of starch or its hydrolytic product, maltose [49]. Among the tested supplementary carbon sources, maltose persuades best production $(41.47 \pm 0.22 \text{ U/ml})$ by isolate GAA 31.1 (Fig. 6A). The optimum concentration of maltose was found as 1.4% $(42.52 \pm 0.358 \text{ U/ml})$ (Fig. 6B). Reports in related to maltose as a best source of carbon for amylase production are also available [50,51].

3.3.8. Effect of nitrogen sources

There was marked increase in amylase production $(107.12 \pm 1.841 \text{ U/ml})$ when sodium nitrate was used as supplementary nitrogen sources (Fig. 6C). Further study

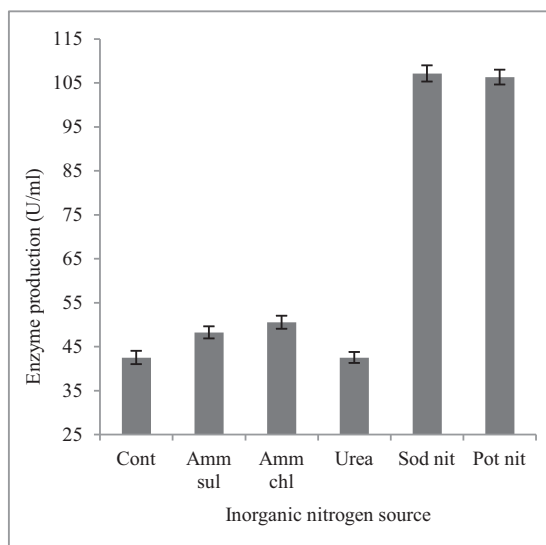


Figure 6C Effect of Inorganic nitrogen sources on the amylase production.

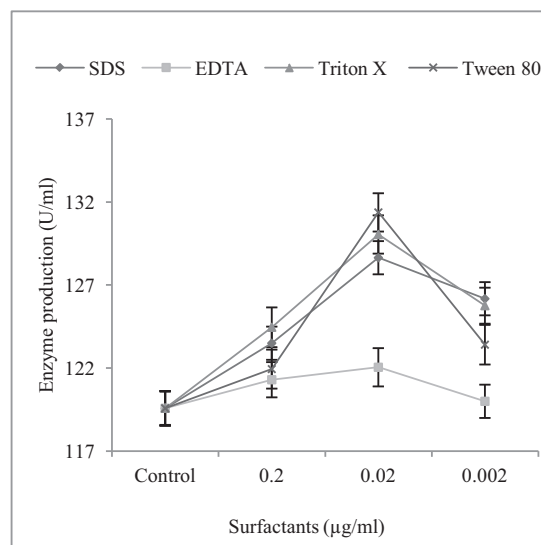


Figure 6E Effect of surfactants on the amylase production.

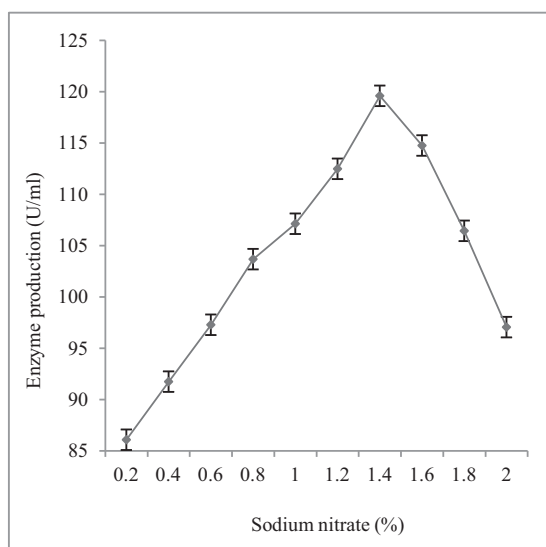


Figure 6D Effect of different concentration sodium nitrate on the amylase production.

revealed the optimum concentration of sodium nitrate at 1.4% level (Fig. 6D) for maximum amylase production (119.58 ± 2.043 U/ml). Earlier, comparable study was also reported [52]. Swain et al. [53] and Deb et al. [45] reported that, urea inhibited amylase activity similar to our findings (Fig. 6C). Decline in amylase production beyond 1.4% of sodium nitrate could be due to lowering of pH in production medium or the induction of protease, which suppress the amyolytic activity [54].

3.3.9. Effect of surfactants

Surfactants are known to alter the cell membrane permeability by disrupting lipid bilayer that facilitate nutrient uptake as well as leaching of metabolite into the culture environment. Among the tested surfactants, Tween 80 (0.02 µg/ml) support

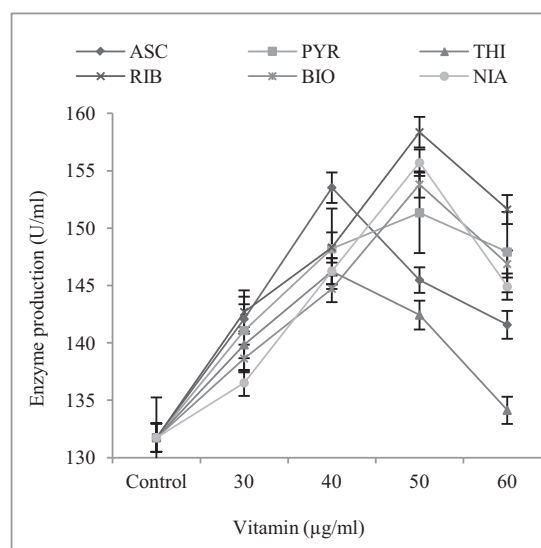


Figure 6F Effect of supplementation of vitamins on the amylase production.

production maximally (131.38 ± 2.153 U/ml). Both the anionic and non-ionic surfactants showed a positive role on production (Fig. 6E) but superiority of Tween 80 may be due to the effect on homogeneity of the broth leading to enhanced nutrient and oxygen supply to the organism [55]. The present study corroborates with the finding of Arnesen et al. [56].

3.3.10. Effect of vitamins

Any metabolic activity is much influenced by vitamins as it acts as the prosthetic group of many enzymes [57]. Supplementation of vitamins to the fermentation medium, particularly riboflavin (RIB) (50 µg/ml) promoted production (158.37 ± 1.331 U/ml). Ascorbic acid (ASC), pyridoxine (PYR), thiamine (THI), biotin (BIO) and niacin (NIA) were also found as positive for amylase production (Fig. 6F). The positive role of riboflavin on enzyme production may be due to its ability to regulate cellular metabolism.

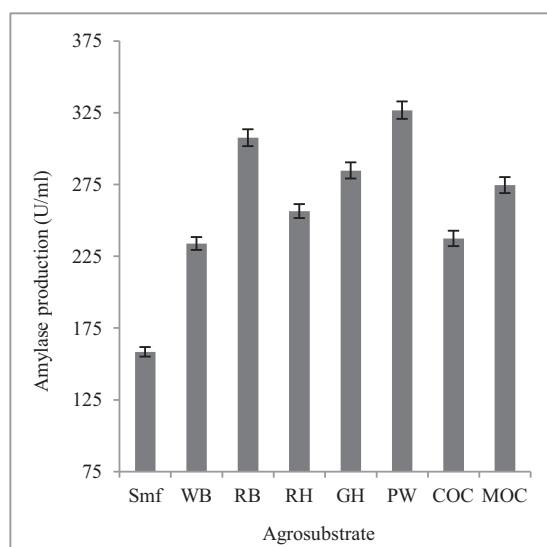


Figure 7 Effect of agro-substrates on the amylase production.

3.3.11. Effect of solid state fermentation

SSF agro-industrial substrates are considered best for cost effective enzyme production [58]. The isolate GAA 31.1 is competent to utilize preferably all the test substrates and found potato waste as the most ideal for amylase production (326.72 ± 6.081 U/ml), followed by rice bran and gram husk (Fig. 7). Similar substrate utilization for optimum amylase production was also reported by Abd-Elhalem et al. [14].

4. Conclusion

The potent extracellular amylase producing gut symbiont of *G. africana* was identified as *R. opacus*. It is the first report related to amylase production by the genus *Rhodococcus* isolated from insect gut. The cultural conditions and composition of medium for optimal amylase production by this actinobacterium have also been developed. After optimizing the fermentation parameters amylase production was enhanced more than 16 folds, amounting to 158.37 ± 1.331 U/ml. Further doubling of production occurred in SSF using potato waste was also noted. Thus, the isolate can be used as a potent biotechnological tool for industrial use, as well as environmental monitoring for agro-waste management.

Acknowledgement

S. Banerjee and R.N. Roy are grateful to the University Grants Commission (India) for financial support [File No. 39-391/2010 (SR)] and Prof (Dr). Abhijit Mazumdar, Entomology Laboratory, Department of Zoology, The University of Burdwan, India for authentication of the insect.

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