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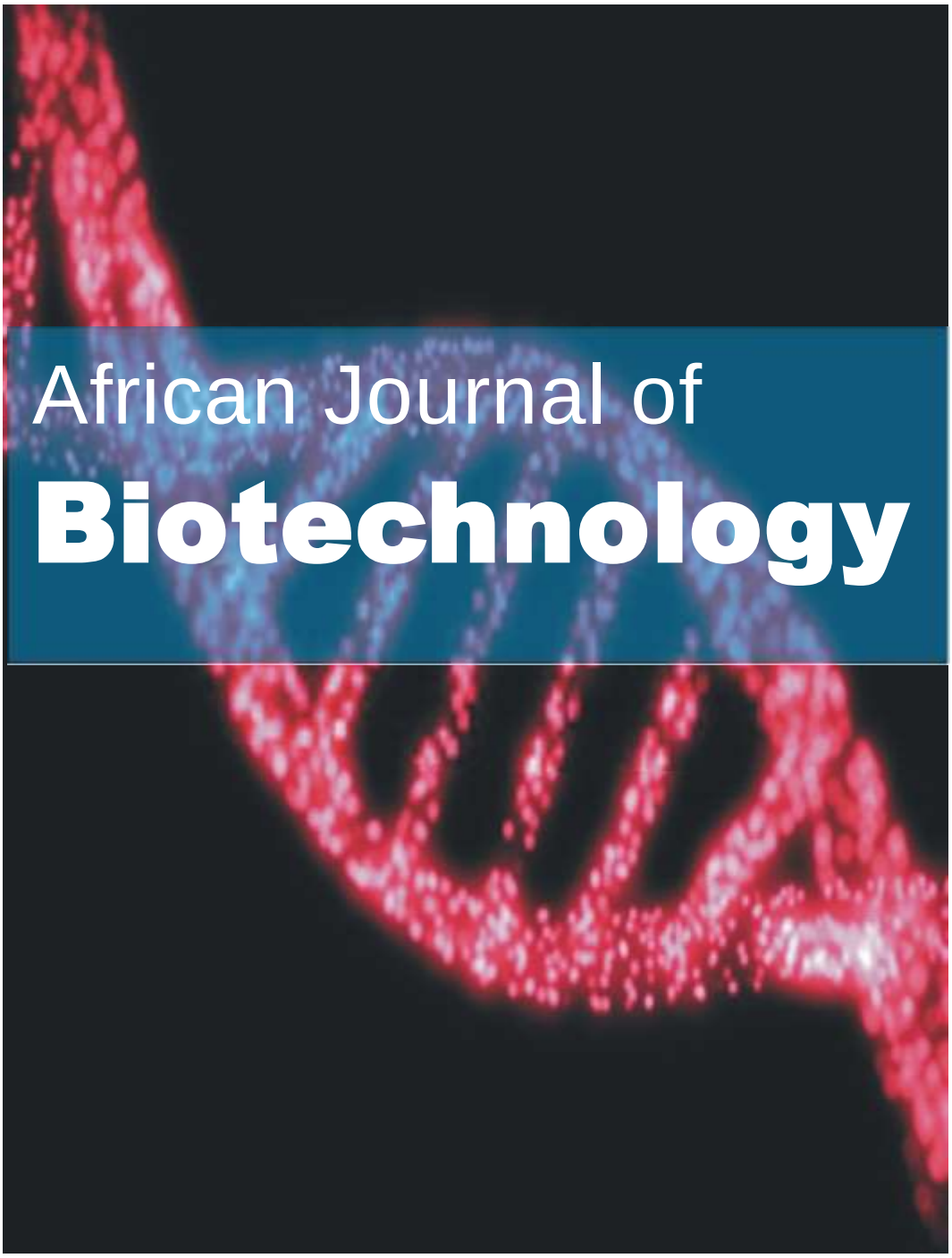

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Full Length Research Paper

Extraction of high quality RNA from hard tissues of adult coconut palms

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An experimental protocol originally designed to isolate plant DNA was modified to obtain high quality total RNA from organs of adult coconut palms collected *in situ*. With this protocol, high quality RNA was extracted from leaves, inflorescences, primary and secondary roots, zygotic embryos and solid endosperm, with no carbohydrate or protein contamination. Reverse transcription-polymerase chain reaction (RT-PCR) amplification of a 470 bp cDNA, corresponding to a highly conserved domain of the eukaryotic mitogen-activated protein kinases, demonstrated the integrity of the RNA samples. Isolation of intact RNA from coconut palms growing under wild conditions facilitates the study of gene regulation *ex vivo*.

Key words: Coconut palms, RNA extraction, secondary metabolites.

INTRODUCTION

Mangrove and coconut palms are key elements that protect the coastal zones and tropical wetlands against the environmental impacts. Cultivation of coconut represents a major income for the people in the tropical and subtropical zones of the world (Mathew, 1986; Hyman, 1990); however, plantations are continuously threatened by different pests and diseases that reduce crop productivity (Zizumbo-Villarreal et al., 2006; Magalhães et al., 2008). Breeding of the coconut palms to generate stress-resistant varieties by means of biotechnological methods has been delayed due to their recalcitrance to cultivation *in vitro* (McCown, 2000). Also, their organs contain secondary metabolites that potentially interfere with the RNA isolation. The leaves are rich in lignin and their surfaces have a dense coat of epicuticular waxes (Escalante-Erosa et al., 2007); the inflorescences contain high amounts of lignin fibers, which have been found to reinforce epoxy composite materials (Sapuan et al., 2005); the embryos are specialized in the accumulation of lipids and carbohydrates (Sugimura and Murakami, 1990; López-Villalobos et al., 2001); and the roots possess a complex

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structure and composition that allow the palm to struggle with the high saline environment found in the coast soils (Nainanayake et al., 2000). High polyphenol contents in adult tissues become evident because activated charcoal must be added to the culture media when adult tissues are used as explants for somatic embryogenesis *in vitro* (Gupta et al., 1984; Chan et al., 1998).

The isolation of coconut ribonucleic acid (RNA) from soft tissues, young seedlings and calli cultivated can be performed by the use of standard protocols that are based on the use of guanidine thiocyanate (Chomczynski and Sacchi, 1987) or cetyltrimethylammonium bromide (CTAB) detergent (Xiao et al., 2012; Gao et al., 2014; Liang et al., 2014; Yuan et al., 2015). Lizama et al. (2007) analyzed the molecular regulation of disease responses by comparing transcript populations isolated from chitosan-elicited *in vitro* coconut calli. In a different study, Pérez-Núñez et al. (2009) quantified transcript levels of a gene encoding a receptor-like kinase during the development of coconut embryogenic calli. Rajesh et al. (2015) isolated total RNA from embryogenic calli and characterized the global transcriptome of coconut palm (*Cocos nucifera* L.) during somatic embryogenesis. In this study, the isolation of high quality RNA was performed by the use of Trizol® reagent (Invitrogen). Other commercial protocols the like RNeasy™ Plant Kit (Qiagen) or the Plant Total RNA Miniprep Purification Kit™ (GMbiolab Co., Ltd.) have also been used to isolate RNA from embryonic tissues (Bandupriya et al., 2014) or seedling leaves (Huang et al., 2013), to determine the expression of a coconut homeotic gene in zygotic and somatic embryos and during germination, and to analyze the chloroplast genome of the coconut palm, respectively.

An effort had been made in the laboratory to isolate total RNA from different organs of adult palms growing in the coasts. However, neither of the protocols reported above nor other protocols designed to isolate RNA from woody or secondary metabolite-rich plants yielded RNA from several organs of adult palms (Jaakola et al., 2001; Valenzuela-Avendaño et al., 2005), with the minimum quality even visualized in agarose gels. Thus, a specific protocol for the isolation of high quality total RNA from adult coconut palms was established, by complementing reported protocols with modifications devoted to eliminate interfering contaminants during the isolation of RNA.

In the present work, the isolation of high quality total RNA of different organs from adult coconut palms collected *in situ* was reported, by the modification of a CTAB method designed to extract RNA from plant tissues with high phenolic compounds, polysaccharides and elevated levels of RNases (Jaakola et al., 2001). The purity and integrity of the RNA samples was evaluated spectrophotometrically and by electrophoretic fractionation in agarose gels. The integrity of the isolated RNAs samples was confirmed by the successful reverse transcription-polymerase chain reaction (RT-PCR) amplification of a complementary deoxyribonucleic acid (cDNA) fragment corresponding to a coconut mitogen-activated protein kinase (MAPK) transcript.

MATERIALS AND METHODS

Plant

The coconut immature inflorescences (physiological state (PS) = -4, numbered regressively from the last open inflorescence), the pine group of the last emitted leaf (flag leaf), and the meristematic zone of primary and secondary roots were collected from adult palms of the “Atlantic Tall” variety, cultivated in the San Crisanto town in the north coast of the Yucatan Peninsula, Mexico (21°, 21´ 00.74´´ N; 89°, 11´32.49´´ O). All tissues were immediately frozen in liquid nitrogen and transported to the laboratory, and then they were stored at -80°C until processed. Mature nuts were collected *in situ* and transported to the laboratory to dissect the zygotic embryos and the solid endosperm by the method reported by Chan et al. (1998).

RNA extraction

RNA extraction was attempted following different methodologies. Trizol® reagent, TRI Reagent® and Concert® were used accordingly to the manufacturer’s instructions. The CTAB method reported by Jaakola et al. (2001) and the method reported by Valenzuela-Avendaño et al. (2005) were followed as reported. The CTAB modified method presented here was performed as follows. 250 mg of each coconut tissue were ground to powder in liquid nitrogen. Then 1 mL of the CTAB solution (2% cethyl-trimethyl-ammonium bromide; 2 M NaCl; 20 mM EDTA, pH 8; 100 mM Tris-HCl, pH 8, with freshly added β-mercaptoethanol and polyvinylpyrrolidone-40 (PVP-40) to 2% final concentration each) was added and the solution was thoroughly homogenized. The crude extracts were halved into two 1.5 mL Eppendorf tubes and then they were mixed vigorously for 10 min at room temperature. The samples were centrifuged at 12,000 ×*g* for 15 min at room temperature. The supernatants were transferred to new tubes and then they were extracted twice with one volume of a chloroform: isoamyl alcohol solution (49:1) and centrifuged at 12,000 ×*g* for 10 min at room temperature. The last supernatants were transferred to clean tubes and mixed perfectly with 0.5 volumes of ice-cold isopropanol; then, 0.5 volumes were added of the saline solution (0.8 M sodium citrate/ 3 M NaCl) and the tubes were gently mixed for 10 min. The solution was centrifuged at 12000 ×*g* for 10 min at 4°C. The pellets were washed twice with 75% ethanol and centrifuged at 12000 ×*g* for 10 min at 4°C and they were air-dried for 10 min at room temperature. The pellets were dissolved in 100 µL of H_2O-DEPC, and then they were mixed with 264 µL of ice-cold 4 M LiCl (2.85 M final concentration) and stored for 1 h on ice. After a centrifugation at 12000 x*g* for 10 min at 4°C, the RNA pellets were washed twice with 75% ethanol and they were air-dried for 10 min at room temperature. The pellets were dissolved in 30 µL of H_2O-DEPC free (SIGMA). The integrity and purity of the RNA preparations were assessed by electrophoretic fractionation in agarose gels, and by measuring the optical density at 230, 260 and 280 nm, respectively.

Reverse transcription-polymerase chain reaction (RT-PCR)

To evaluate the functionality of the RNA preparations, 0.8 µg of

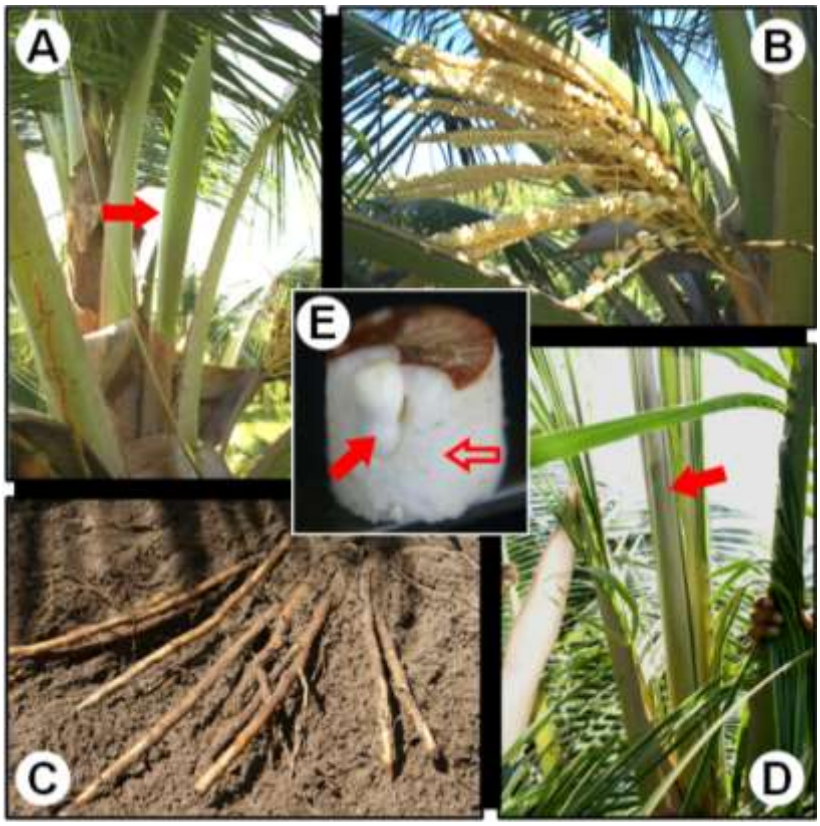

Figure 1. Organs of the coconut plant used as source of RNA. Organ samples were collected *in situ*, immersed immediately in liquid nitrogen and transported to the laboratory. A: Immature inflorescence (solid arrow); B: open immature inflorescence as reference; C: secondary roots; D: flag leaf (solid arrow); E: zygotic embryo (solid red arrow) and solid endosperm (open red arrow) were isolated from mature nuts in the laboratory.

total RNA isolated from each organ sample were used as template for the RT-PCR amplification of a 470 bp cDNA fragment, corresponding to the conserved domain of mitogen-activated protein kinases. The Superscript One-Step RT-PCR kit with Platinum *Taq* polymerase® (Invitrogen) was used as recommended by the manufacturer, using the degenerate primers 5'-GGNGCYTACGGHATYGTTTGYTCK-3' (forward) and 5'-GGNGCYTACGGHATYGTTTGYTCK -3' (reverse), under the following cycling conditions: one cycle at 42°C, 2 min; 48°C, 30 min; 94°C, 2 min; then, forty cycles at 94°C, 15 s; 50°C, 30 s; 72°C, 1 min, followed by a final extension step at 72°C for 10 min. 3 µL aliquots of each RT-PCR product were fractionated by agarose gel electrophoresis, and then the gel was stained with 1 µg ml^{-1} ethidium bromide and visualized under UV light.

RESULTS AND DISCUSSION

The isolation of high quality RNA is an essential step to carry out molecular studies in plants; however, the extraction of RNA is compromised in plant tissues rich in secondary metabolites and complex carbohydrates (Jaakola et al., 2001). This is the case of the coconut palm; indeed, the breeding of elite coconut varieties has been hampered because of several adverse factors inherent to the adult palm, including its size and long life cycle. In addition, the coconut palm is a perennial monocot woody plant with high recalcitrance to *in vitro* cultivation (McCown, 2000). Furthermore, the difficulty of isolating biological molecules from coconut can be evidenced by the existence of extremely few reports in this area.

In the laboratory, investigation of the regulation of the molecular responses of coconut cells to the presence of pathogenic signals in the environment was done. While, the use of coconut tissues growing *in vitro* has been suggested as an alternative to study the gene regulation (Chakraborty et al., 2009; Lizama et al., 2007), the analysis of gene function must be done in whole palms, requiring the extraction of RNA from the organs of interest.

Adult organs of coconut palms growing in the field was collected (Figure 1) and tested different commercial methods to isolate total RNA; however, their use yielded

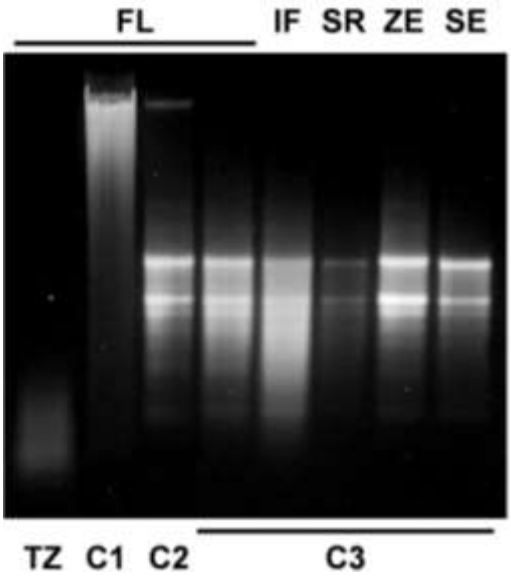

Figure 2. Integrity of the RNA samples isolated from coconut organs. 1 µg of total RNA isolated from different organs of the adult coconut palms were fractionated by gel electrophoresis in 1.3% agarose and stained with 1 $\mu g \cdot mL^{-1}$ ethidium bromide. RNA samples were extracted from the following organs: FL: flag leaf; IF: immature inflorescence; SR: secondary root; SE: solid endosperm; ZE: zygotic embryo. Different methods were applied: TZ: Trizol®; CN: Concert®; TR: Trireagent®; C1: CTAB original method; C2: CTAB modified method I; C3: CTAB modified method II.

RNA samples that could not be visualized [Trizol® (Invitrogen), TRI Reagent® (SIGMA-ALDRICH)] or appeared as smears in agarose gels [Concert® (Invitrogen)] (Figure 2, TZ, TR and CN, respectively). Conversely, the use of CTAB method as described by Jaakola et al. (2001) yielded only DNA (Figure 2, lane C1).

It has been proposed that the CTAB effect during the extraction of nucleic acids from plants resides in its capacity to bind anionic polymers (that is glucuronoarabinoxylans) (Kiefer et al., 2000). The authors decided to modify the CTAB protocol to increase its capacity to eliminate phenolic compounds and complex carbohydrates by the addition of polyvinylpyrrolidone and a further precipitation step in the presence of a high concentration of salts. The addition of PVP during the extract preparation, and the extraction of the cleared crude extract with a mixture of chloroform: isoamyl alcohol (49:1) followed by the precipitation of carbohydrates from the aqueous phase with a saline solution (0.8 M sodium citrate/3 M NaCl), and a final precipitation step with isopropanol were determinant to

Table 1. Purity and yield of the RNA samples isolated with the CTAB-II method.

Tissue	Absorbance ratio		RNA yield[1] ($ng \cdot mg^{-1}$)
	260/230	260/280	
Flower	2.22	2.04	235.4
Inflorescence	1.95	2.08	89.0
Secondary R	209	1.94	42.8
Zygotic Embryo	2.14	2.01	82.9
Solid Endosperm	2.06	2.04	28.3

RNA yield is expressed as nanograms of RNA per milligram of fresh weight of tissue.

precipitate integral RNA from all samples (Figure 2, lane C2). The soluble nature of PVP could extend its capacity to form complex with phenolic compounds, preventing their union and the further oxidation of the RNA samples (Bekesiova et al., 1999). In this protocol, it was not necessary to heat the CTAB extracts at 65°C. It has been reported that precipitation of aqueous extracts with high concentrations of salts (1 M NaCl) favours the elimination of polysaccharides from genomic DNA (Fang et al., 1992) and from RNA (Valenzuela et al., 2005). However, with these concentrations of salt, the spectrophotometric measurements gave 260/230 absorbance ratios ≤ 1.5 (data not shown). The increment of the NaCl concentration to 3 M (0.8 M sodium citrate/3 M NaCl) during the precipitation step produced RNA samples with 260/230 absorbance ratios ≅ 2. The contaminating DNA was eliminated from the salt-cleared RNA samples by a further precipitation with ice cold LiCl (2.85 M final) (Figure 2, lanes C3). Also, it was found that incubation of the LiCl-RNA mix at -20°C was not necessary because incubation on ice for 1 h produced high RNA yields. All preparations obtained with the new modifications yielded RNA with high integrity, as estimated by the band integrity of the major ribosomal RNAs (Figure 2).

The yield and purity of the RNA samples were evaluated by spectrophotometric absorbance at 230, 260 and 330 nm. Table 1 shows that both 260:230 and 260:280 absorbance ratios were around the value of 2.0, implicating no significant contamination of the RNA samples with carbohydrates and proteins, respectively. The modified CTAB method gave good RNA yields, especially from the palm flag leaf (235.4 ng mg^{-1}). The smaller yield was obtained from solid endosperm secondary roots (28.3 ng mg^{-1}).

The quality of the RNA populations isolated from *in situ* collected adult organs was assessed by their capacity to function as template for reverse transcription *in vitro*. As can be seen in Figure 3, a single band was obtained by reverse transcription coupled with the polymerase chain reaction, using a pair of DNA oligonucleotides flanking a

Table 2. Key modifications to the CTAB original method.

Method	Phenol extraction	T (°C)	Salt concentration (NaCl/NaCit)	LiCl[2]
CTAB[1]	+	65	--	--
CTAB-I	–	Room temperature	1 M/0.8 M	Overnight -20°C
CTAB-II	–	Room temperature	3 M/NaCit	1 h, Ice

The CTAB method as originally described by Jaakola et al. (2001). Ultracentrifugation in 2.85 M LiCl.

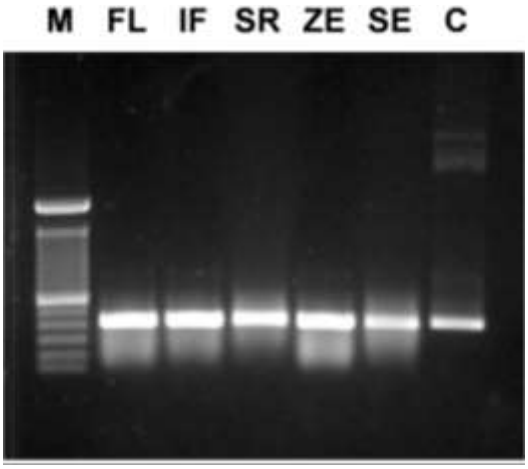

Figure 3. Quality of the RNA isolated from coconut organs. 2 µl aliquots of the RT-PCR products synthesized with degenerate primers from RNA samples isolated by different protocols were fractionated by gel electrophoresis in 1.3% agarose and stained with 1 µg ml^{-1} ethidium bromide. FL: flag leaf; IF: immature inflorescences; SR: secondary roots; SE: solid endosperm; ZE: zygotic embryos; M: 100 bp DNA ladder (Invitrogen); (−): RT-PCR assay with no reverse transcriptase as negative control; (+): PCR product from the coconut MAPK-2 cDNA cloned in the pGEM-T-Easy® vector (PROMEGA) as positive control; TZ: Trizol® method; CN: Concert® method; TR: TriReagent® method.

470 bp fragment of the ribonucleotide sequence encoding part of catalytic domain of the universally-conserved eukaryotic mitogen-activated protein kinases (MAPK). This result confirmed that the improved CTAB method yielded RNA extracts from adult palms cultivated in their natural ecosystems, with the quality required to perform molecular biology experiments required in modern biotechnological breeding programs. It is interesting to note that although the RNA samples obtained with the Concert® protocol served as templates to obtaining an RT-PCR amplicon of the expected size (Figure 3, lane CN), they appeared smeared after electrophoretic fractionation in agarose gels (Figure 2, lane CN), and their absorbance ratios 260/230 nm were below 1.0 units (data not shown). These results suggested contamination of the Concert® RNA sample with carbohydrates, which did not interfere with RT-PCR experiments, but affected electrophoretic mobility.

Recently, few protocols to isolate RNA populations from soft tissues of coconut palms have been reported (Xiao et al., 2012; Bandupriya et al., 2014; Gao et al., 2014; Liang et al., 2014; Rajesh et al., 2015; Yuan et al., 2015), however, they have not been applied to hard tissues of coconut palm, like inflorescences or roots. The protocol presented here utilizes economic and easy-to find chemical ingredients; it could be applied to small amounts of tissue, reducing the cost of transportation and the amount of liquid nitrogen required to preserve samples collected in the field. It does not employ toxic chemicals (guanidine isothyocianate) or organic solvents (phenol) (Table 2), and it could be applied, with simple modifications, to the isolation of both DNA and RNA from the same coconut tissue.

Conflict of interests

The authors have not declared any conflict of interest.

ACKNOWLEDGEMENTS

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Abbreviations

CTAB, Cethyl-trimethyl-ammonium bromide; **PVP,** polyvinylpyrrolidone; **FL,** flag leaf; **IF,** immature inflorescence; **SR,** secondary root; **ZE,** zygotic embryo; **SE,** solid endosperm, **MAPK,** mitogen-activated protein kinase.

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Full Length Research Paper

Shell morphology and the radula structures of two closely related bulinid snails intermediate host of *Schistosoma haematobium* in Nigeria

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Bulinid snails act as intermediate host of schistosomes and the presence of the snail gives schistosomiasis an expansive characteristics. Schistosomiasis is of medical and veterinary importance in the tropical and subtropical regions. The shell shape and structure of radula teeth of bulinids are often specific to a species or genus, and are widely used for gastropod species identification. Bulinid species collected from schistosome endemic areas of Ogun State, South-western Nigeria were used for this study. Shell morphometrics were recorded using vernier caliper while the buccal mass of each snail was removed and permanent slides of the radulae were made according to standard procedure. There was a significant difference in the shell height, width, aperture height and aperture width between *Bulinus globosus* and *Bulinus jousseaumei* ($p<0.05$). The average shell height measurement for *B. globosus* was 7.6±1.9 mm, while *B. jousseaumei* measured 5.1±1.6 mm. Each transverse row of *B. globosus* radula had a ratio of 26:8:1:8:26 while *B. jousseaumei* had a ratio of 25:8:1:8:25. The marginal teeth of *B. globosus* possessed five cusps while *B. jousseaumei* possessed six cusps. The differences observed in shell, radula ratio and cusps in both species could be used to differentiate both species.

Key words: Radula, shell, *Bulinus* species, identification, Nigeria.

INTRODUCTION

Digenetic trematodes of the genus *Schistosoma* are causative agents of schistosomiasis in human and have an indirect lifecycle with freshwater snails serving as the intermediate host. Urinary schistosomiasis is often transmitted by different species of the genus *Bulinus* (Brown, 1994).

The use of shell and internal anatomy of snails have been very useful in the past for identifying and separating *Bulinus* species, although, these characteristics are also thought to be problematic in their use in phylogenetic studies (Inaba, 1969; Brown, 1994; Stothard and Rollinson, 1996). The variability in internal anatomy tends to occur as a result of selective processes in snail species (Bargues et al., 2001; Remigio, 2002). However, some of these characteristics such as distal genitalia, prostate, shell and radula teeth have been useful in the identification of snail species (Walter, 1968; 1969, Mimpfoundi and Ndassa, 2005). Shell characteristics

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have been said to be of limited value for the identification of bulinid species (Stothard et al., 1997). However, early species descriptions were grouped into genera based on shell characteristics, especially while in the field before using other methods of identification.

The radula has been described as a chitinous ribbon-like series of nearly colourless transverse tooth rows resting on top of the radula membrane (Radwin and Wells, 1968). The way a radula function is often affected by size, form, and nutrient materials. The reactions between teeth and nutrient materials tend to determine the need of each species to special radula. The type, form, number, and arrangement of radular teeth in different species indicate its different functions, which include nutrition capabilities, quality of reaction between different species as well as differentiating the species.

Radula features such as number and structure of radula have been of higher importance in molluscan taxonomic relationships. It often shows general similarities or differences at the species level. These observable differences and similarities have been utilized in the classification of gastropods (Arularasan et al., 2011). Although many factors such as food, seasonal changes and sexual differences affect the structure of radula (Carlos and Helena, 2003; Matthews-Cascon et al., 2005), some radula characters are generally constant within the same species (Fretter and Graham, 1994). Moreso, radula morphology is frequently used as a taxonomic character for studies on molluscan systematics (Fretter and Graham, 1994; Padilla, 1998; deMaintenon, 2004). The shape and form of the radula teeth are typically unique to a species or genus and some features of the radula, such as tooth numbers have been used to investigate higher levels in molluscan phylogenetic studies (deMaintenon, 2004).

Apart from the above mentioned anatomical features of the snails which are used for speciation, other anatomical characters such as kidney, nervous system, pneumostome, tentacles and digestive system are equally useful in snail identification (Jackiewicz, 1990; Paraense, 1994; 1995; Ponder and Waterhouse, 1997; Jackiewicz and Buksalewicz, 1998; Samadi et al., 2000). Though anatomical characteristics have some short comings in species identification, some of the characters referred to above have proven to be more useful in discriminating snail species (Samadi et al., 2000). Therefore, the use of anatomical characters in understanding the systematics of the freshwater snails is equally important.

Although molecular biomarkers have played important role in species identification (Stothard, 1996; Hebert et al., 2003; Akinwale et al., 2015), morphological characteristics such as shell and radula structure continue to be the primary means of identification of freshwater snails (Schander and Willassen, 2005). *Bulinus globosus* and *Bulinus jousseaumei* are two closely related snails, both of which are intermediate hosts of the parasitic flatworm known to cause schistosomiasis (Salawu and Odaibo, 2012; Stothard et al., 2013; Akinwale et al., 2015; Mkize et al., 2016; Hassan et al., 2016; Chibwana and Nkwengulila, 2017). The study therefore describes the morphological differences in the shell and radula of two snail intermediate hosts of schistosome in South-western Nigeria.

MATERIALS AND METHODS

Snail species (expand methods)

A total of 917 *B. globosus* and 543 *B. jousseaumei* specimens were collected from water contact sites in Yewa North Local Government Area of Ogun State (latitude 7°15' N and longitude 3° 3' E). The snails were preserved in 70% ethanol for dissection.

Shell morphometrics

The following linear measurements were recorded using a vernier caliper: shell height (H), shell width (W), shell aperture height (AH), and shell aperture width (AW) according to Chiu et al. (2002).

From the values obtained for each linear measurement, the following ratios were calculated: shell height/shell width (H/W); shell height/aperture height (H/AH); shell height/aperture width (H/AW); shell width/aperture height (H/AW) and aperture height/aperture width (AH/AW).

Dissection and preparation of radula

The head region was opened by an incision from the mantle edge between the tentacles. The body walls were relaxed and fixed with pins, exposing the buccal mass and penial complexes. The buccal mass was macerated in 7.5% sodium hydroxide (NaOH) for 2 h at 60°C. The freed radula was washed in water and membranes were removed under a dissecting microscope. Radula was transferred to a drop of 10% glacial acetic acid on a slide and orientated with its teeth uppermost and straightened out. The acid was left to evaporate.

A drop of Mallory stain was placed on the radula for 2 to 3 mins after which the radula was rinsed in running water. The radula was then rinsed in 2% oxalic acid, 96% ethanol and xylene (Mandahl-Barth, 1962). A drop of Canada balsam was used to mount the radula at room temperature. These radulae were imaged using a light microscope.

Determination of radula teeth formula

Each row of radula teeth consists of one central tooth (C) which is always found in the middle, on each side of the central teeth are the lateral teeth (L) and then beyond the lateral teeth are the marginal teeth (M). Different species of snails have different numbers of lateral and marginal teeth.

Statistical analysis

The mean, standard deviation, minimum, maximum values, independent t-test and the general linear regression were performed on shell character, using statistical package SPSS version 22.0.

Table 1. Mean and range values of *Bulinus globosus* in Yewa North LGA.

Shell morphometrics	Minimum Value (mm)	Maximum Value (mm)	Mean ± SD (mm)
Height (H)	3.0	13.5	7.6 ± 1.9
Width (W)	2.0	9.5.0	5.5 ± 1.2
Aperture height (AH)	0.5	10.0	5.7 ± 1.4
Aperture width (AW)	0.5	6.5	3.5 ± 0.9
Height Width ratio (H/W)	0.2	2.1	1.4 ± 0.2
Height and Aperture Height ratio (H/AH)	0.8	17.0	1.4 ± 0.5
Height and Aperture Width ratio (H/AW)	1.3	7.0	2.2 ± 0.4
Width and Aperture Height ratio (W/AH)	0.7	12.0	1.0 ± 0.5
Aperture Height and Aperture Width ratio (AH/AW)	0.1	3.0	1.7 ± 0.3

Table 2. Mean and range values of *Bulinus jousseaumei* in Yewa North LGA.

Shell morphometrics	Minimum Value (mm)	Maximum Value (mm)	Mean ± SD (mm)
Height (H)	2.0	11.0	5.1 ± 1.6
Width (W)	1.2	8.0	3.9 ± 1.1
Aperture height (AH)	1.5	8.0	4.3 ± 1.3
Aperture width (AW)	1.0	6.0	2.3 ± 0.8
Height Width ratio (H/W)	0.7	3.0	1.3 ± 0.2
Height and Aperture Height ratio (H/AH)	0.6	2.3	1.2 ± 0.2
Height and Aperture Width ratio (H/AW)	1.3	6.0	2.4 ± 0.6
Width and Aperture Height ratio (W/AH)	0.5	1.4	0.9 ± 0.1
Aperture Height and Aperture Width ratio (AH/AW)	1.1	5.0	2.0 ± 0.5

RESULTS

Morphometrics

On average, *B. globosus* shells measured 7.6±1.9 mm in height, 5.5±2.3 mm in width, 5.7±1.4 mm in aperture height and 3.5±0.9 mm in aperture width (Table 1). The longest *B. jousseaumei* shell height recorded was 11.0 mm, although this species' mean shell height was 5.1±1.6 (Table 2).

Figures 1 and 2 show the adult *B. globosus* and *B. jousseaumei*. The relationship of bulinids shell morphometrics showed linearity (Figures 3, 4, 5 and 6). Analysis of shell height, width, aperture height and aperture width shows that *B. globosus* was significantly larger than *B. jousseaumei* ($p<0.05$).

Radula morphology

Bulinus jousseaumei

The general radula ratio is M:L:C:L:M. Generally, bulinid species have the same radula shape (Figures 7 and 9) however there is variation in different parts of the radula amongst different species. The radular teeth ratio of *B. jousseaumei* was 25:8:1:8:25 and it consist of a single row of central teeth found at the middle of the radula, eight pairs of lateral teeth (Figure 8a) and 25 pairs of marginal teeth (Figure 8b). Variation in the mesocone showed that the cusps of the central radula were intermediate while the lateral cusps were angular.

The cusps of the central teeth were small and reduced in size. The lateral teeth were broad and asymmetrically tricuspid. The endocone was short and fused with the mesocone while the mesocone was broader than the ectocone. The marginal teeth which is the last morphological tooth type, comprised the outermost group of the teeth on each side of a transverse row. *B. jousseaumei* teeth possessed six cusps. Variation in the mesocone of the lateral radula teeth was angular while the central teeth were intermediate.

Bulinus globosus

The radula teeth ratio of *B. globosus* was 26:8:1:8:26, which consist of a single row of central teeth found at the

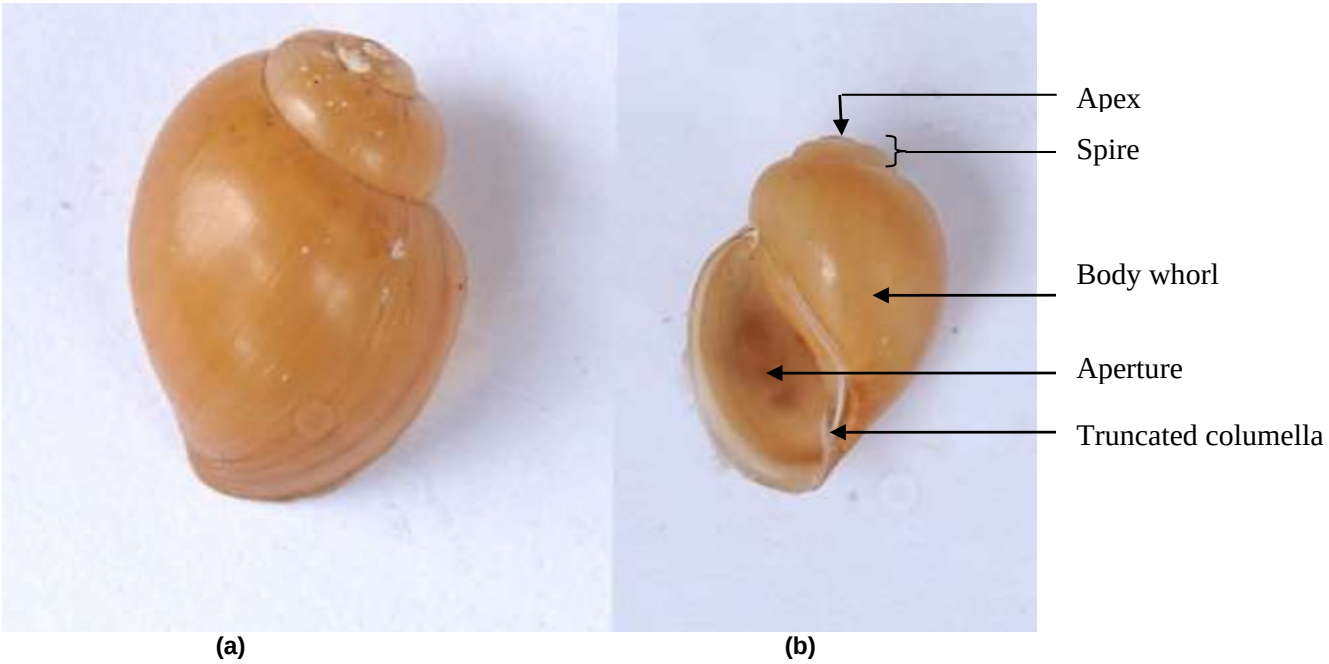

Figure 1. Abapertural (a) and Apertural (b) view of *Bulinus globosus* (x3). The shell is sinistral and the columella truncated.

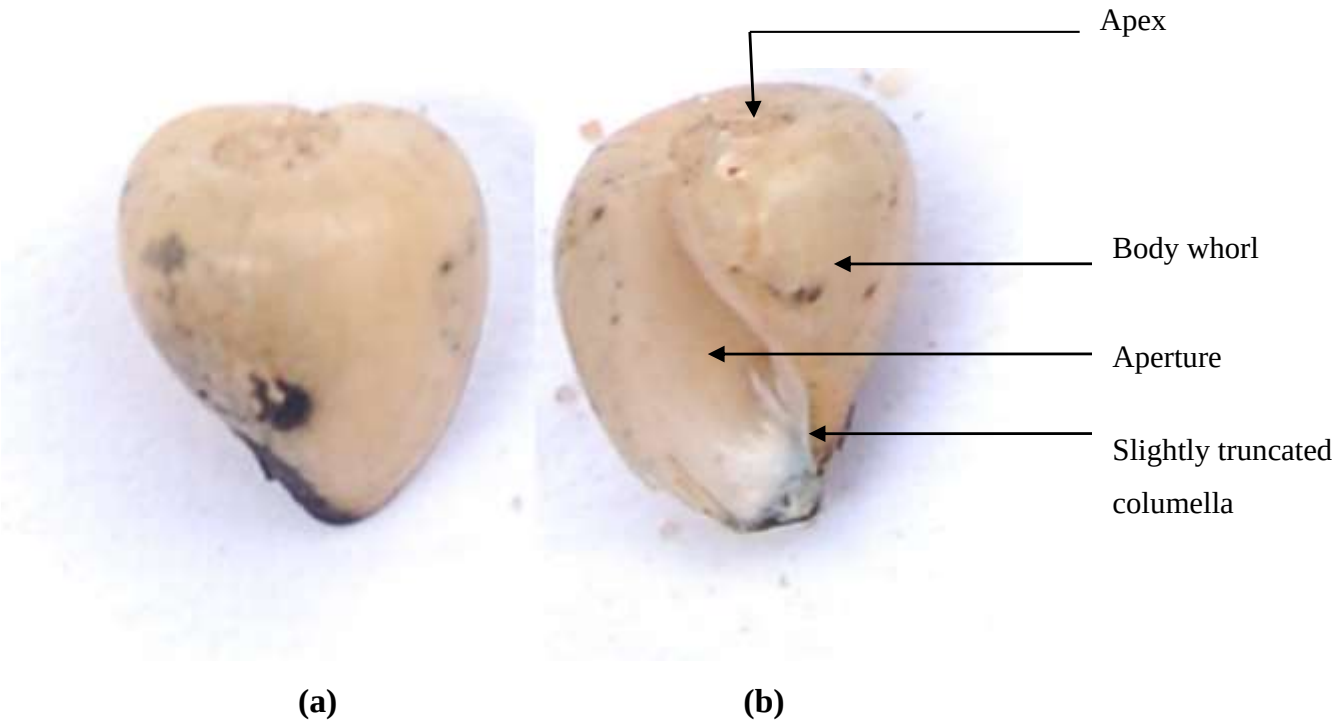

Figure 2. Abapertural (a) and apertural (b) view of *Bulinus jousseaumei* (x3). The shell has a depressed spire and it is sinistral.

middle of the radula, eight pairs of lateral teeth (Figure 10a) and twenty-six pairs of marginal teeth (Figure 10b). The cusps of the central teeth were small and reduced in size. The lateral teeth were broad and asymmetrically tricuspid.

The endocone was short and fused with the mesocone

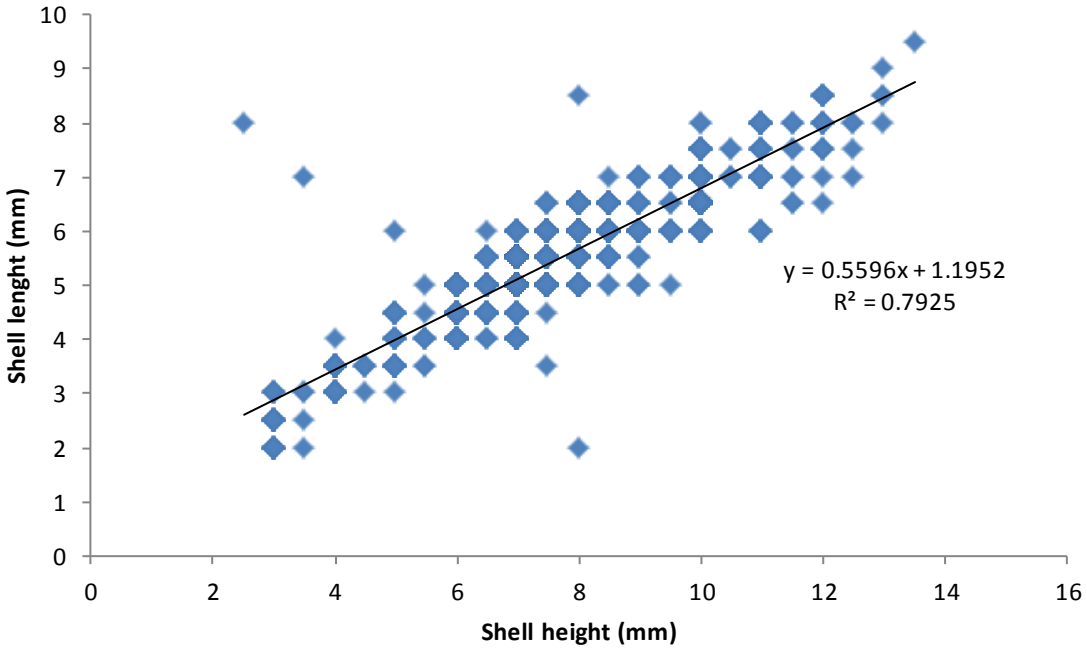

Figure 3. Relationship between Shell Height and Shell Width of *Bulinus globosus.*

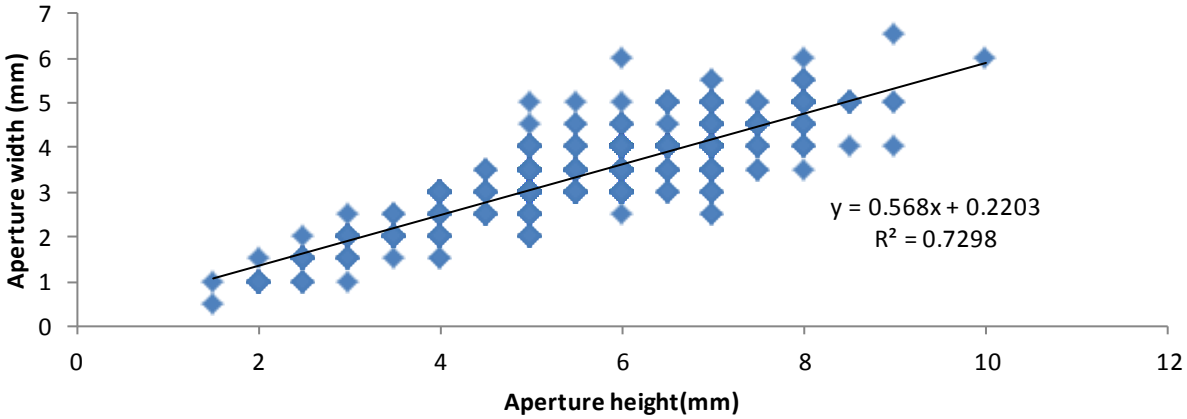

Figure 4. Relationship between aperture height and aperture width of *Bulinus globosus.*

while the mesocone was broader than the ectocone. The marginal teeth of *B. globosus* possessed five cusps.

DISCUSSION

Generally, shells of freshwater snails provide important taxonomic information that can be used to show differences in species as well as reveal evolutionary relationships in different taxa. Shell characters such as the spire height, and width of *B. globosus* have been widely reported from different schistosome endemic areas of Nigeria, however *B. jousseaumei* was only recently reported in Nigeria (Salawu and Odaibo, 2012).

The significant difference observed in all the shell characters (shell height, width, aperture length and aperture width) between *B. globosus* and *B. jousseaumei* showed that all these shell characteristics can be used to differentiate these species. The significant difference in the morphometrics of *B. globosus* and *B. jousseaumei* recorded in this study had been observed in other similar pulmonates (Monzon et al., 1993). The long-spired and short-spired form in *B. globosus* and *B. jousseaumei* respectively has been reported in previous observation.

The line of best-fit plot shows the positive trend in the measured characters. In most cases, shell height forms

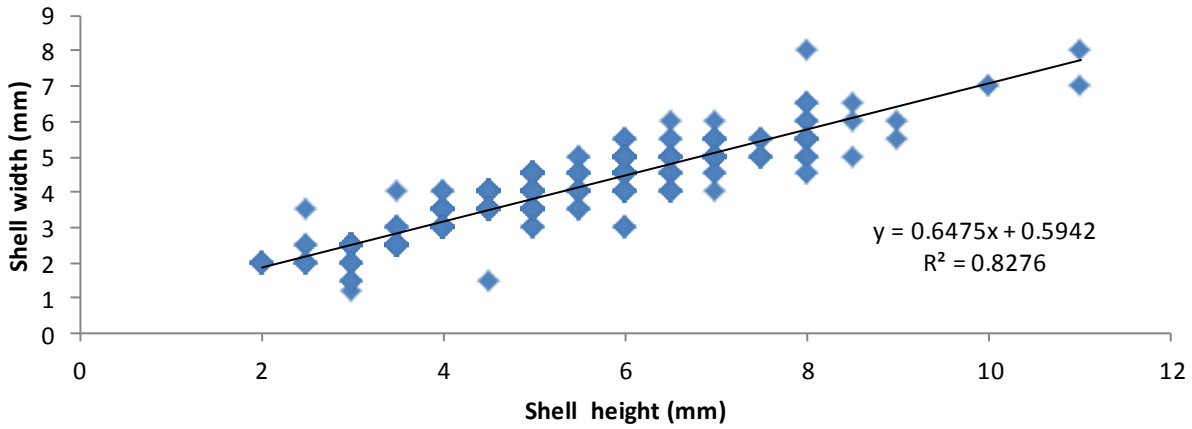

Figure 5. Relationship between shell height and shell width of *Bulinus jousseaumei.*

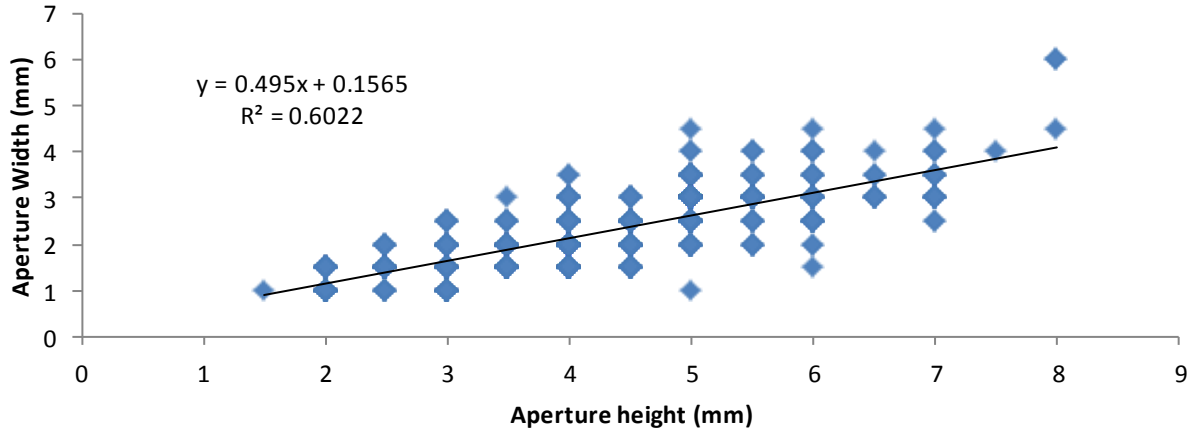

Figure 6. Relationship between Aperture Height and Aperture Width of *Bulinus jousseaumei.*

part of the taxonomic characters in differentiating snail species (Chiu et al., 2002). Environmental changes without genetic change can result in distinct non-genetic changes in shell morphology. Calcium contributes to shell formation in gastropods (Jokinen, 1982), although calcium level in the environment acts as a limiting factor and selective pressure on shell morphology (Rundel et al., 2004). The higher the calcium in the environment, the thicker the shell formed and there is a narrow aperture in the snail (Rundle et al., 2004).

Most time calcium correlated positively with water pH (Hunter, 1990) and in the presence of low calcium and low pH in the environment, snail shells are eroded easily (Glass and Darby, 2009). In this study, the short spire found in *B. jousseaumei* could be a result of underutilization of calcium from the environment. Water flow also affects shell morphology. This factor often affects snail shell in large lakes compared to shallow river bodies (Trussell, 1997), in our study area, snails were collected from shallow river bodies. Snails with thick shell are often associated with high water flow. The thick shell of the snail minimizes the shell damage, when the snails are dislodged by high water current (Trussell, 1997; Minton et al., 2008). Also, under the shallow areas, shells with very short spire are common and look alike (Shileyko, 1967).

In this study, each transverse row of radula of *B. globosus* had a radula formula of 26:8:1:8:26 while *B. jousseaumei* had formula of 25:8:1:8:25. The difference in the morphology in these two species focuses attention on the use of radula morphology for the species differentiation. Soft parts of snails have been proved useful for species identification; however, some studies argued that such anatomical characteristics are too variable and should be avoided for phylogenetic studies because these anatomical characteristics are prone to selective processes which could hamper the normal formation of these characters (Bargues et al., 2001; Remigio, 2002).

However, distal genitalia, prostate and radula teeth

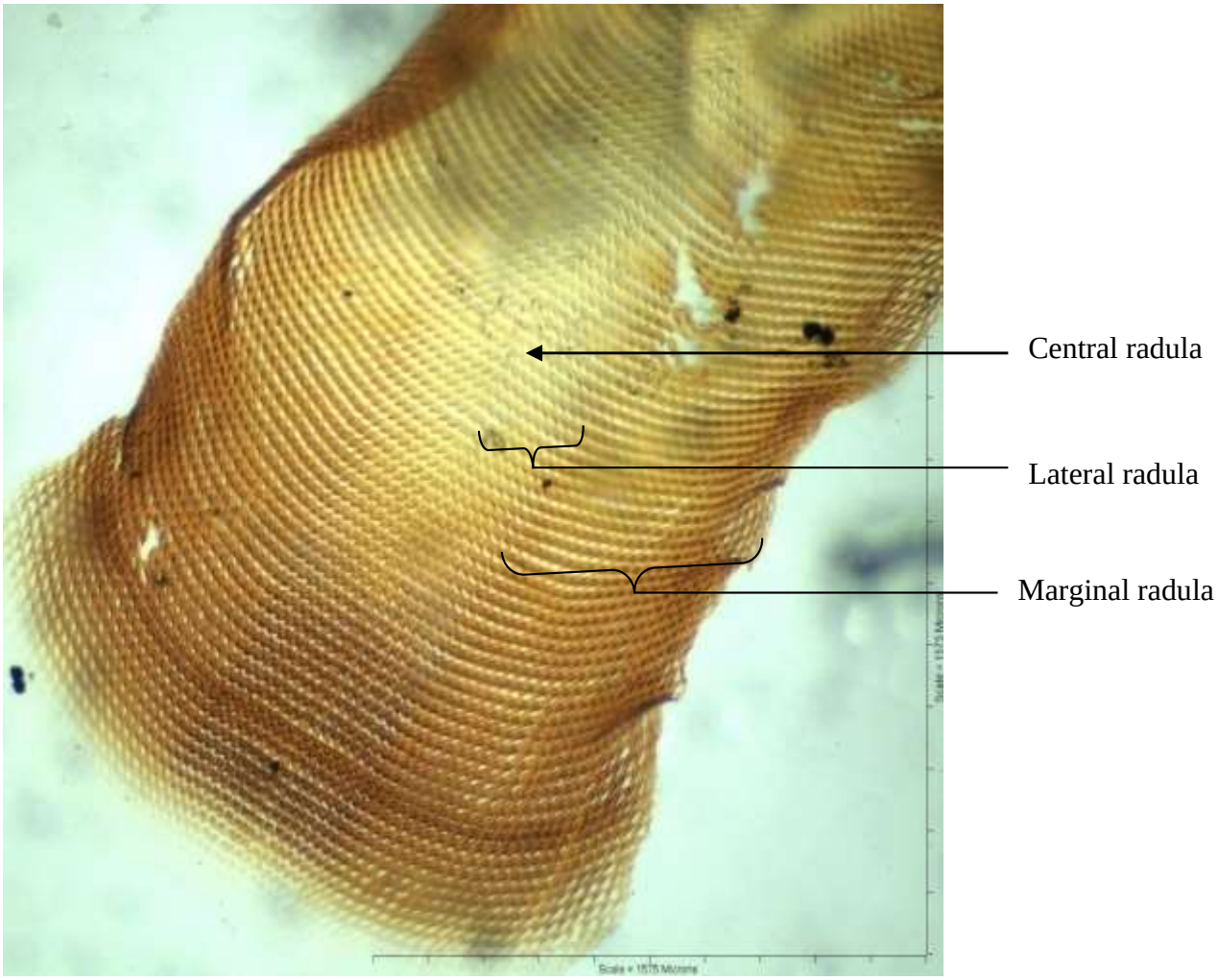

Figure 7. Whole radula of *B. jousseaumei*. Scale bar: 575 microns.

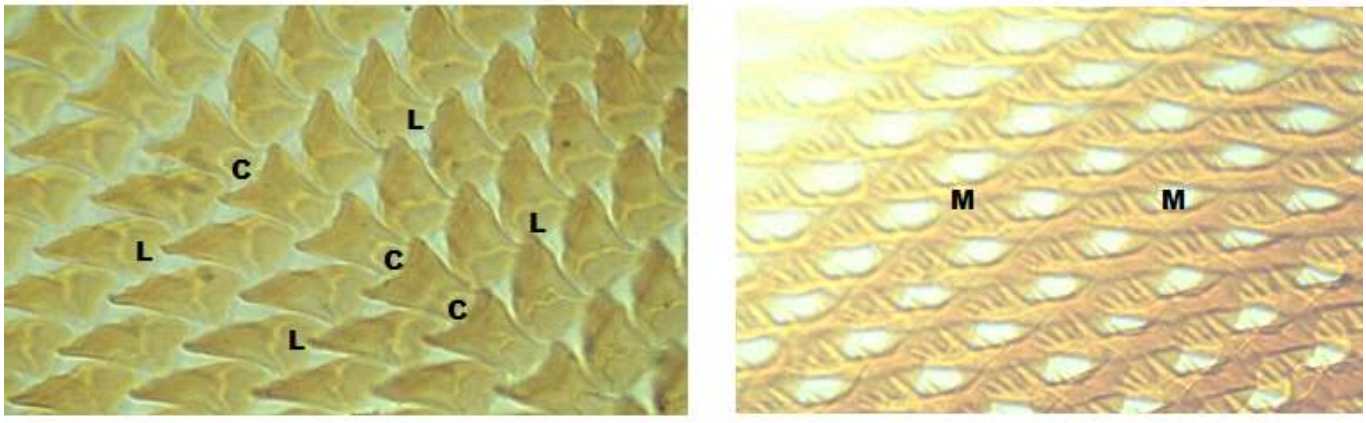

Figure 8. (a) *Bulinus jousseaumei* (Lateral (L) and central (C) teeth). Scale bar: 1575 microns. The mesocone cusp of the central radular tooth is intermediate while the lateral radular cusp is angular. The endocone of the lateral radula is fused with the mesocone. (b) *Bulinus jousseaumei* (marginal (M) teeth). Scale bar: 1575 microns. The marginal radula of *B. jousseaumei* possessed six cusps each.

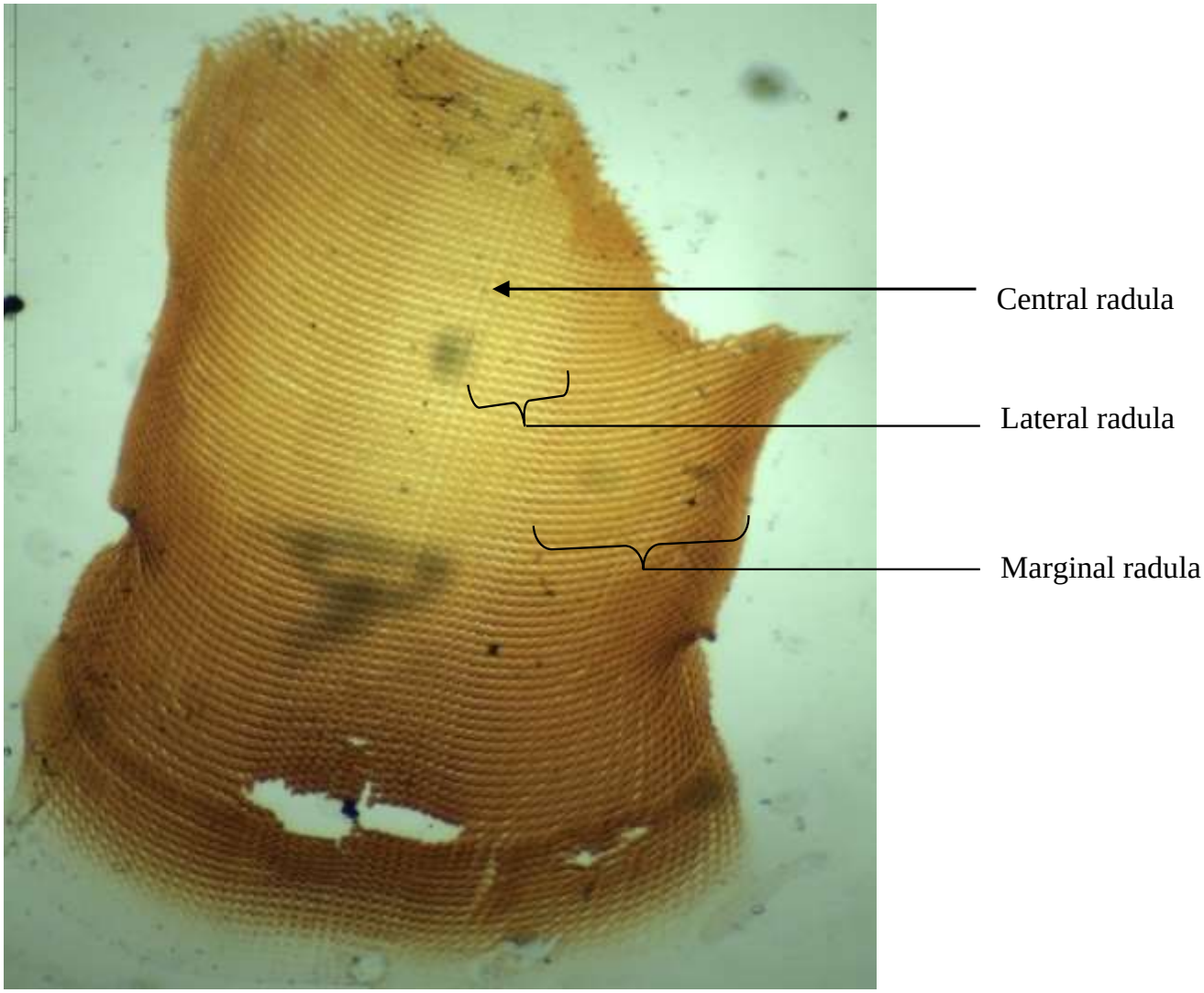

Figure 9. Whole radula of *B. globosus.* Scale bar: 575 microns.

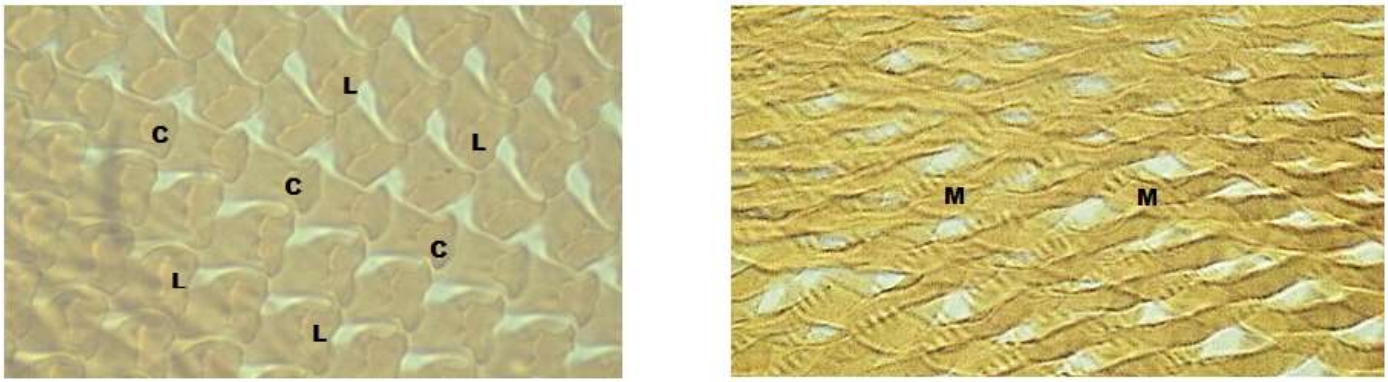

Figure 10. (a) *Bulinus globosus* (Lateral (L) and central (C) teeth). Scale bar: 1575 microns. The mesocone of central radula is intermediate while the lateral radula cusp is angular. The endocone of the lateral radula is fused with the mesocone. **(b)** *Bulinus globosus* (marginal (M) teeth). Scale bar: 1575 microns. The marginal radula of *B. globosus* possessed five cusps each.

were useful characters that can be used for successful species identification (Hubendick, 1951; Walker, 1968). Besides, the formula of radula (Ponder and Lindberg, 1996), shape, size and structure of the cusps (Kilburn, 1988; Monzon et al., 1993) of radula, has provided the needed information for the taxonomy of different snail species. The unicuspid characteristic of the central tooth of bulinids in this study was similar to observations in Mozambique (de Azevedo et al., 1961).

Conclusion

Shell and radula morphology of bulinid species could be adopted as part of the characteristics used for the classification of these species, as clear differences occurred between the shell and radulae of *B. globosus* and *B. jousseaumei*. These observable differences in these bulinid species can ensure correct species identification with the aim of controlling schistosome in the area. Moreso, the similarity between these two intermediate hosts of schistosomes need quick and cost effective means of differentiation.

Thus, this study observed differences in conchological measurements and radula morphology of these species.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Full Length Research Paper

Effect of water deficit at different stages of development on the yield components of cowpea (*Vigna unguiculata* L. Walp) genotypes

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Cowpea cultivation is widespread in West Africa where it is an important source of protein. This study is aimed at determining the effects of water deficit applied at different stages of cowpea development on yield and its components (pod number, seed number, seed yield, aerial biomass yield, harvest index and root biomass of the plant). The experiments were carried out in pots during the rainy season of 2016 under natural conditions of illumination, temperature and relative humidity. Three water regimes were applied to plants at different stages of cowpea development: total suspension of watering at flowering phase (43 days after sowing) (S1); suspension of watering at the beginning of pod formation on the 46th day after sowing (S2); and normal watering as control until harvest (S0). At the water regime level, yield components had higher values in S0 followed by S2. The lowest values were obtained at S1 level. The root to aerial biomass ratios was higher under water deficit than in the control. In conditions of water deficiency, Suvita2, IT96D-610, and ISV128 genotypes gave the highest seed yields and Tiligré the lowest yield. The harvest index showed a genotypic variation according to the water regime. Suvita2 and ISV128 gave the best harvest index in all water regimes. This study may have contributed to the selection of genotypes adapted to drought.

Key words: Cowpea, harvest index, water deficit, yields, Niger.

INTRODUCTION

The frequency of periods of water deficit of variable intensities makes agricultural production very uncertain in Niger, and for proper management of production systems in these areas it is necessary to have a thorough knowledge of the different resistance strategies adopted by the plants under these limiting conditions of water supply.

Cowpea is the main legume crop grown in Niger where

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it plays an important nutritional role for its richness in protein and economic role for the income it generates to producers. Although adapted to semi-arid conditions, drought pockets observed during its development cycle have a negative impact on it production. The choice of varieties adapted to water deficit is important to improve yields in these areas where drought occurs at different stages of plant development. The local varieties which are widely used are late maturing and photoperiod sensitive (Singh et al., 1997) with low yields. According to Singh (1987, 1994), early maturing varieties can escape terminal drought by reducing the length of their development cycle, but when they are expose to intermittent stress, their performance decreases. Varieties that have an average development cycle can be adapted to the climatic conditions of these areas and contribute to increasing agricultural production.

Several physiological and biochemical criteria have been identified in order to distinguish sensitive cowpea varieties from water-stress resistant varieties (Blum, 2011). The effect of water deficit results in morphological (to increase the absorption of water and decreased sweating) and physiological changes (decreased tissue water content, increase in canopy temperature, decreased chlorophyll content and consequent photosynthesis) (Hamidou et al., 2005, 2007). The impact and intensity of water deficit on plants depend on the phenological stage during which this deficit occurs and vary according to the plant. According to Turk et al. (1980), cowpea is more sensitive to water stress during flowering and pod filling. Water stress in the vegetative phase followed by re-irrigation has little influence on the final yield of cowpea seeds (Faisal and Abdel-Shakoor, 2010; Hall, 2012). Although cowpea has the capacity to resist drought more than any legume grown in tropical regions (Hall, 2004; Dadson et al., 2005), a difference between genotypes has been recorded for adaptation to drought (Watanabe et al., 1997; Mai-kodomi et al., 1999).

The physiological and biochemical processes determining the harvesting quality of cowpea under water stress during flowering and pod filling have been widely described by Hamidou (2006) and Halilou et al. (2015). However, there are still shady areas in the choice of yield parameters relevant for the selection of cowpea genotypes at terminal water stress. This study was conducted to evaluate the effect of water deficit at flowering and pod-forming stages on yield components of cowpea.

MATERIALS AND METHODS

Experimental materials

The study involved 5 genotypes whose origin and maturity are presented in Table 1. All genotypes have an intermediate development cycle (90 days).

Method of culture

The trials were conducted at the ICRISAT Sahelian center station (Sadoré, Niger, 13°15′N, 2°18′E) during the rainy season 2016 (August-October) under natural conditions. The plants were grown in 16-L pots pierced at the base. In each pot, 500 g of gravel was deposited at the base to allow for good drainage of water. Each pot was then filled with 17 kg of soil collected at a depth of 20 cm at the station's field. This soil was mixed with organic fertilizer at a ratio of 25 g/kg of soil. The pots were placed on a tarpaulin to prevent the roots from being in contact with the soil.

Before sowing, the pots were saturated and allowed to drain for 24 h to reach the field capacity. Field capacity is the amount of soil moisture or water content held in the soil after excess water has drained away and the rate of downward movement has decreased. This usually takes place 2 to 3 days after rain or irrigation in pervious soils of uniform structure and texture. The seedlings started out at the rate of 4 seeds of cowpea in pots followed by a two plants seedling on the 14th day after sowing (DAS) and one plant at 23 DAS.

The experimental design (Figure 1) is of a split plot completely randomized with the water regime as the main factor and genotypes grown as a sub-factor and randomized within each 4-repeat subblock. The water regimes are: (1) Regime 0 (S0): Well watering as control, the well watering is to bring each day a quantity of water of 500 ml to the plants to maintain pots at field capacity until harvesting; (2) Regime 1 (S1): Permanent irrigation suspension at 43 DAS corresponding to the stage of 50% flowering; (3) Regime 2 (S2): Permanent irrigation suspension at 46 DAS corresponding to the beginning of pod formation.

The control plants (regime 0) were maintained at field capacity. During subjection to stress, the plants were protected from rainwater by a mobile shed with a translucent roof. Climate data (temperature and humidity) were recorded daily using a thermo hygrometer (Tiny tag Ultra 2 TGU-4500 Gemini Data Loggers Ltd., Chichester, UK) installed next to the test. During the test, mean temperature was 29°C and the relative humidity was 75% (Figure 2).

Data collection

Phenological stages

The following phenological stages were noted per pot: emergence, early flowering date, early date of pod filling and maturity. The stage is noted when 50% of the plants of the same genotype in each subblock have reached the stage.

Yield components and root dry biomass

The harvest consisted of cutting the plant close to the surface of the soil, leaving the roots in the soil. For normally watered plants, as soon as the plant reaches maturity, it is immediately harvested. The date of harvest is mentioned. For stressed plants, the plant is harvested when it shows obvious signs of stress such as dryness, leaf drop and stopped growth. The number of days of stress was noted.

After each harvest, the above-ground biomass (stems + leaves) and the pods were separated, pods were counted and dried in an oven for 48 h at 80°C. Dry samples were weighed using a 0.01 g precision balance to determine dry aerial biomass and pod weight. After decorticating the pods, the total number and weight of seeds/plant were determined.

The cowpea seed harvest index was calculated using the following formula:

Table 1. Origin and earliness of genotypes studied.

Name	Origin	Response to drought
ISV128	ISC Niger[1]	Tolerant
IT93K-503-1	IITA Nigeria[2]	Tolerant
IT96D610	IITA Nigeria[2]	Tolerant
Suvita2	INERA Burkina[3]	Tolerant
Tiligré	INERA Burkina[3]	Susceptible

[1]ISC, ICRISAT Sahelian Centre; [2]IITA, International Institute for Tropical Agriculture; [3]INERA, Institut de l'Environnement et des Recherches Agricoles.

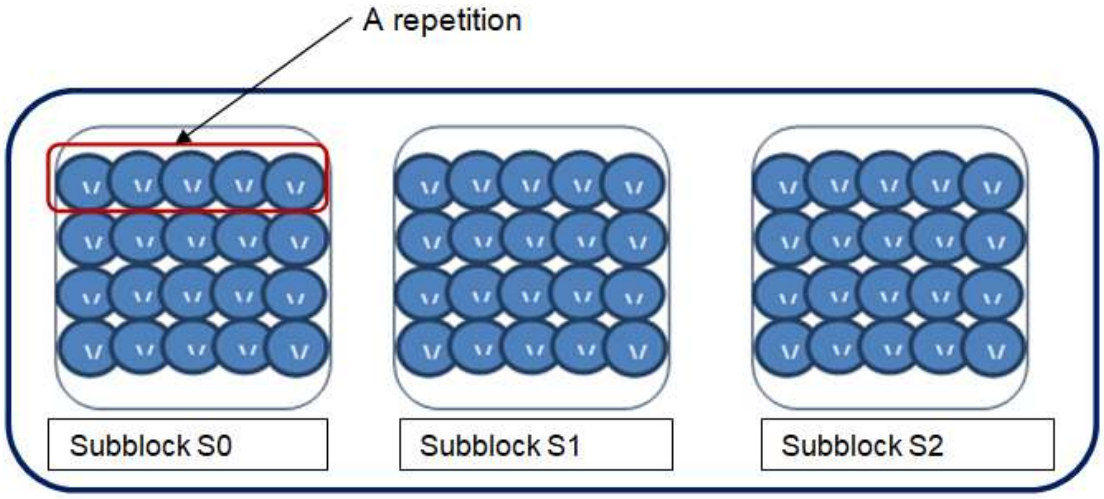

Figure 1. Experimental design. S0, Regime 0; S1, regime 1; S2, regime.

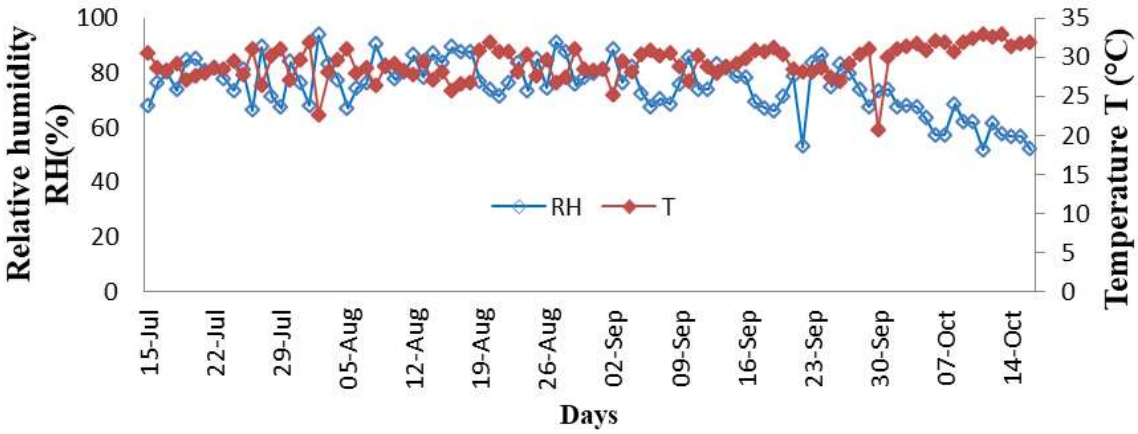

Figure 2. Change in temperature and relative humidity during the test period at Sadoré ICRISAT Sahelian Center.

IR (%) = Dry matter of seeds × 100 / total dry matter.

Total dry matter = Weight pod + Aerial biomass

To determine the root biomass, the soils of each pot were delicately removed through a low water pressure. A fine sieve was placed under the pot to recover any broken roots during the

Table 2. Phenological stages (in number of days after sowing: DAS) of the genotypes studied.

Genotype	Emergence	50% flowering	Maturity
ISV128	3.50±0.58	40.00±1.63	62.00±3.16
IT93K-503-1	3.50±0.58	41.75±1.26	64.50±3.00
IT96D-610	3.00±0.00	40.50±0.58	63.75±2.87
Suvita2	3.75±0.96	40.00±1.16	59.75±2.50
Tiligré	4.00±1.15	42.75±0.96	60.25±2.06
P value	0.46	0.06	0.11

operation. When the total amount of soil is removed, the roots were collected, dried in an oven for 48 h at 80°C and weighed to determine the dry root biomass.

Data analysis

The variance analysis was carried out using the JMP.009 version software. Separation of means for the various parameters measured was carried out by the Student Newman Keuls test at the threshold of α = 5%. To evaluate the effect of genotype, treatment and genotype × treatment interaction, an analysis of variance (ANOVA) by the generalized linear model procedure was performed. Microsoft Office Excel 2007 software was used to perform linear regressions, determine the R^2 and the regression equation. Minitab16 was used to test the significance of linear regression using the Pearson's correlation test.

RESULTS

Phenology

Table 2 shows that there were no significant differences in physiological stages among the genotypes studied. Emergence occurred for all genotypes after 3 to 4 days after sowing (DAS). The stage of 50% flowering was reached between 40 and 42 days after sowing and maturity, between 60 and 64 DAS for all genotypes.

Impact of water deficit on root biomass and root biomass ratio to aerial biomass of cowpea according to stage of development

When plants were normally irrigated, there were significant differences in aerial and root biomass between genotypes (Table 3). The highest aerial biomass was recorded for Tiligre (32.41 g/plant), followed by IT93K-503-1 (28.14 g/plant). IT96D-610 and Suvita2 had the lowest biomass of 24.57 and 21.35 g/plant, respectively. ISV128 genotype recorded an aerial biomass which is intermediate (26.50 g/plant). IT93K-503-1 and Tiligre genotypes had the highest root biomass yields whereas IT96D-610 and Suvita2 had the lowest yields (Table 3). When the aerial biomass/root biomass ratio was considered, IT93K-503-1 gave the highest value (0.37) and Suvita2 the lowest (0.18).

When water stress was applied at flowering stage (S1), no significant differences were observed among genotypes for root biomass (Table 3). However, significant differences exist for aerial biomass. Tiligre obtained the highest aerial biomass (23.56 g/plant), followed by ISV128 and IT96D-610 (21.19 and 20.19 g/plant, respectively). The other genotypes had lower aerial biomass. At root biomass/aerial biomass ratio, IT93K-503-1 and Suvita2 were the highest with 0.47 and 0.40, respectively (Table 3). Tiligre was the lowest position with a ratio of 0.31.

When water stress was applied at the beginning of pod formation, the yield of aerial biomass of IT93K-503-1 was significantly higher than the other genotypes (25.14 g/plant) (Table 3). Suvita2 had the lowest yield (21.21 g/plant). The other genotypes are intermediate. However, there were no significant differences among genotypes for root biomass yield and root biomass/aerial biomass ratio.

The impact of water deficit on yield components and crop index of cowpea

Table 4 shows yield components and harvest index (HI) of different genotypes for the water regimes applied at flowering and beginning of pod formation. When plants were irrigated normally, the results showed significant differences among the genotypes for the different parameters measured. For example, according to the Newman Keuls test, Tiligré gave the lowest yield (15.73 g/plant) compared to other genotypes with higher yields (19.86 to 21.97 g/plant). IT93K-503-1, IT96D-610 and Suvita2 had the highest number of pods/plant (≥19.50 pods/plant), while ISV128 and Tiligre had the lowest (<16 pods/plant).

IT93K-503-1, IT96D-610, and Suvita2 gave the highest seed number (100 seeds/plant) and Tiligre the lowest (54 seeds/plant). The best seed yield was recorded for ISV128 (17.29 g/plant) and lowest for Tiligre (11.57 g/plant). Suvita2 has the best seed harvest index

Table 3. Effect of water deficit on aerial and root biomass and on the ratio of the root to the aerial portion.

Treatment	Genotype	AB (g)	RB (g)	RB/AB
Normal watering	ISV128	26.50±2.86^{bc}	6.72±1.07^{bc}	0.25±0.03^{bc}
	IT93K-503-1	28.14±3.37^{b}	10.25±0.96^{a}	0.37±0.02^{a}
	IT96D-610	24.57±1.26^{cd}	4.92±0.21^{cd}	0.2±0.01^{cd}
	Suvita2	21.35±0.80^{d}	3.75±0.30^{d}	0.18±0.01^{d}
	Tiligré	32.41±2.31^{a}	8.96±3.21^{ab}	0.27±0.08^{bc}
	Significance	***	***	***
Stress at flowering stage	ISV128	21.19±1.43^{b}	8.10±0.88	0.38±0.05^{bc}
	IT93K-503-1	17.86±0.58^{c}	8.76±3.26	0.47±0.05^{a}
	IT96D-610	20.19±0.74^{b}	7.72±1.34	0.35±0.02^{bc}
	Suvita2	17.66±1.29^{c}	6.88±0.78	0.40±0.05^{ab}
	Tiligré	23.56±1.38^{a}	8.36±0.84	0.31±0.05^{c}
	Significance	***	ns	*
Stress at pod formation	ISV128	23.32±1.46^{ab}	8.14±1.24	0.3±0.04
	IT93K-503-1	25.14±1.46^{a}	8.34±0.84	0.36±0.03
	IT96D-610	22.23±0.99^{bc}	7.26±0.66	0.38±0.09
	Suvita2	21.21±1.56^{c}	7.02±0.72	0.29±0.04
	Tiligré	23.71±1.09^{ab}	7.51±1.21	0.35±0.04
	significance	**	ns	ns

*, **, ***Significant at the probability threshold of 0.05, 0.01 and 0.005, respectively; ns: Not significant (p> 0.05). Numbers with the same letter(s) in the same column are not significantly different from the p<0.05 threshold. AB, Aerial biomass; RB, root biomass; RB/AB, ratio of root biomass to aboveground biomass.

(41.26%) and Tiligre had the lowest (24.04%). When stress was applied at flowering (S1), there were also significant differences between the genotypes for the different parameters. The best yield in pods was recorded for Suvita2 (5.36 g/plant) and lowest for Tiligre (1.77 g/plant). Yield for other genotypes (IT96D-610, IT93K-03-1 and ISV128) was between 3.27 and 3.85 g/plant.

When water stress was applied at the beginning of pod formation, genotypes also differed significantly for all measured parameters except seed/plant yield. Genotypes IT96D-610 and Suvita2 have the highest yields of pods, seed/plant number, and harvest index. When water stress was applied to flowering, there was a significant decrease in the aerial biomass of all genotypes relative to the control (Figure 3). This decrease, however, was greater for IT93K-503-1 and Tiligre, 36 and 31%, respectively. When water stress was applied at the beginning of pod formation, there were no significant differences in aerial biomass for the Suvita2, IT93K-503-1 and ISV128 genotypes as compared to the control (Figure 3). However, this difference is very significant for Tiligré and IT96D-610.

Water stress applied at both flowering and early pod formation drastically reduced seed yield as compared to control for all genotypes studied (Figure 4). Tiligre genotype was the most sensitive with a seed yield reduction of 92 and 71% when stress was applied to flowering stage and early pod filling, respectively.

Relationship between seeds weight and harvest index

Correlation analysis of seed weight and harvest index showed a significant positive correlation for the three treatments (Figure 5). The correlation was more significant in the water stress treatments R^2 = 0.99 ($p<0.0001$) for S1 and R^2 = 0.98 ($p <0.001$) for S2 as compared to control R^2 = 0.85 ($p<0.023$).

DISCUSSION

Root weights varied from one genotype to another. When water stress was applied during the flowering phase and the beginning of pod formation, dry root mass was reduced in IT93K-503-1 and Tiligre and increased in Suvita2, ISV128 and IT96D-610. Results similar to those for IT93K-503-1 and Tiligre were obtained by Meftah (2012) on two populations of cowpea Tizi Ouzou and Djanet. Hamidou et al. (2005), studying the effect of water stress on pod formation of two varieties of cowpea

Table 4. Effect of water stress on flowering (S1) and onset of pod formation (S2), yield and its components in cowpea.

Treatment	Genotype	AB (g/plant)	Pod N/plant	Pod weight (g/plant)	Seed N/plant	Seed weight (g/plant)	HI (%)
Normal watering	ISV128	26.50±2.86^{bc}	15.25±2.63^{c}	21.97±1.54^{a}	90.00±3.92^{b}	17.29±1.22^{a}	35.71±2.94^{ab}
	IT93K-503-1	28.14±3.37^{b}	22.75±0.96^{c}	20.71±2.72^{a}	100.50±11.12^{a}	15.11±2.54^{ab}	30.9±3.56^{b}
	IT96D-610	24.57±1.26^{cd}	19.75±1.50^{ab}	19.86±2.67^{a}	99.75±2.87^{ab}	14.43±2.10^{b}	32.71±5.67^{b}
	Suvita2	21.35±0.80^{d}	19.50±3.11^{b}	20.36±1.80^{a}	105.50±7.85^{a}	17.24±1.84^{ab}	41.26±2.97^{a}
	Tiligré	32.41±2.31^{a}	13.75±1.71^{c}	15.73±1.62^{b}	54.00±3.37^{c}	11.57±1.42^{c}	24.04±2.98^{c}
Significance		***	**	*	***	**	**
Watering flowering stage	Mean	26.60±4.31	18.20±3.85	19.72±2.88	89.95±20.04	15.12±2.74	32.93±6.70
	ISV128	21.19±1.43^{b}	6.75±0.96^{ab}	3.85±0.56^{b}	43.50±2.12^{a}	2.92±0.58^{ab}	11.52±1.44^{ab}
	IT93K-503-1	17.86±0.58^{c}	3.33±1.53^{b}	3.44±1.07^{bc}	15.50±3.54^{b}	1.79±0.22^{bc}	8.61±1.72^{b}
	IT96D-610	20.19±0.74^{b}	6.00±2.65^{ab}	3.27±0.49^{bc}	28.33±16.56^{ab}	2.70±1.86^{bc}	11.37±7.71^{ab}
	Suvita2	17.66±1.29^{c}	8.75±3.77^{a}	5.36±1.59^{a}	46.50±7.78^{a}	5.17±1.01^{a}	20.66±4.15^{ab}
	Tiligré	23.56±1.38^{a}	4.00±0.82^{b}	1.77±0.63^{c}	10.50±8.23^{b}	0.88±0.10^{c}	4.54±3.15^{b}
Significance		***	*	**	*	*	**
Watering pod formation	Mean	20.09±2.53	5.79±2.78	3.56±1.54	27.50±16.86	2.44±1.70	10.83±6.37
	ISV128	23.32±1.46^{ab}	12.25±1.26^{b}	5.89±0.97^{bc}	47.5±6.86^{ab}	4.91±2.20	16.56±6.31^{ab}
	IT93K-503-1	25.14±1.46^{a}	7.67±0.58^{c}	5.03±0.49^{c}	36.33±5.51^{bc}	3.83±0.33	12.72±1.19^{b}
	IT96D-610	22.23±0.99^{bc}	10.50±1.29^{b}	8.16±0.48^{a}	55.75±6.65^{a}	6.49±1.00	21.33±3.10^{a}
	Suvita2	21.21±1.56^{c}	15.75±0.96^{a}	7.06±2.12^{ab}	53.50±12.58^{a}	5.66±1.81	19.67±4.19^{a}
	Tiligré	23.71±1.09^{ab}	7.25±1.71^{c}	4.32±0.72^{c}	27.00±8.52^{c}	3.34±0.79	11.91±2.74^{b}
Significance		*	***	**	**	ns	*
	Mean	23.01±1.76	10.84±3.40	6.19±1.80	44.72±13.9	4.95±1.79	16.63±5.20^{b}
	Genotypes	***	***	***	***	***	***
	Treatment	***	***	***	***	***	***
	Geno*Treat	**	*	***	**	ns	ns

*, **, ***Significant at the probability threshold of 0.05, 0.01 and 0.005, respectively; ns: Not significant (p> 0.05). Numbers with the same letter(s) in the same column are not significantly different from the p<0.05 threshold. BA, Aerial biomass; Pod N, number of pods; seed N, number of seeds; HI, harvest index.

(Gorom and KN1), found an increase in the dry matter of the root (13.62% For Gorom and 29.74% for KN1).

This study shows that the root biomass/aerial biomass ratios are higher under stress conditions for all genotypes. These ratios were also higher when water stress was applied at the beginning of flowering. The root system was less affected by water stress than aerial biomass. According to Monneveux (1997), the sustained growth of the root system in conditions of water stress is a factor of resistance to water stress. This is due to the fact that when the soil dries on the surface, the roots tend to sink deeper into the soil in search of water (Aziadekey et al., 2014). The growth of the root front would not have been able to discriminate genotypes resistant to those sensitive especially in the condition of terminal stress, but the pattern of water extraction clearly discriminated them (Zaman-allah et al., 2011).

The application of water stress during flowering and at the beginning of pod formation led to a significant decrease in seed yields and its components. Reduced yield were more severe when stress was applied to flowering stage than early pod formation. The number of pods in a non-limiting water condition is higher than stressed condition. Water stress therefore affected flowering and also increased the rate of abortion of flowers and pods. In addition to the yield reduction, a difference in size between the seeds of the control and stress plants was observed. The results are in agreement with those of Turk et al. (1980) who reported that the intervention of water stress during the flowering phase and the pod filling phase reduces the number of pods per plant and the size of the seeds. This reduction in pod numbers and seed size can be explained by the acceleration of foliar senescence and the shortening of

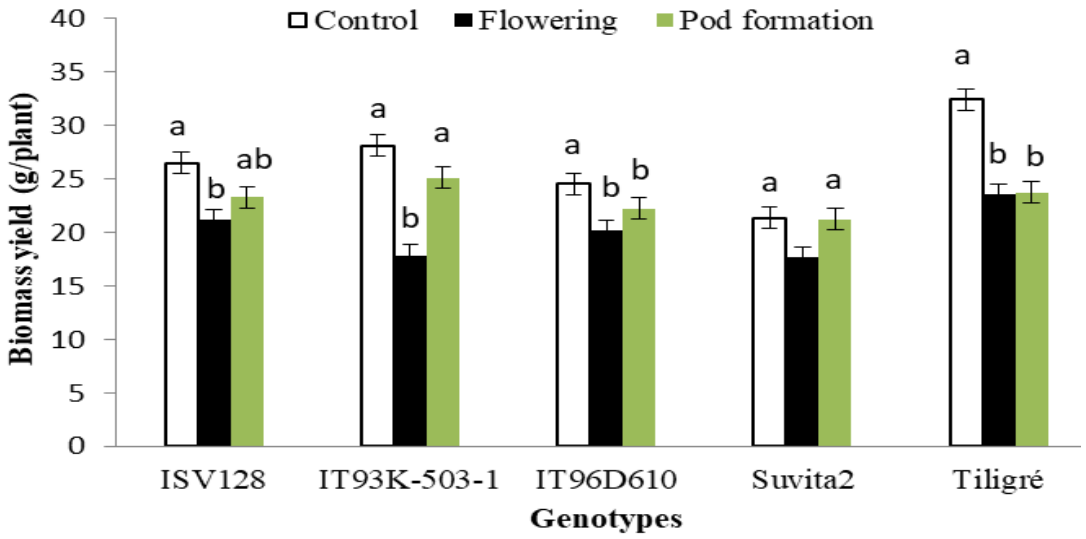

Figure 3. Comparison of aerial biomass yields of genotypes according to the period of application of stress: beginning of flowering (S1), beginning of pod formation (S2) and control (S0).

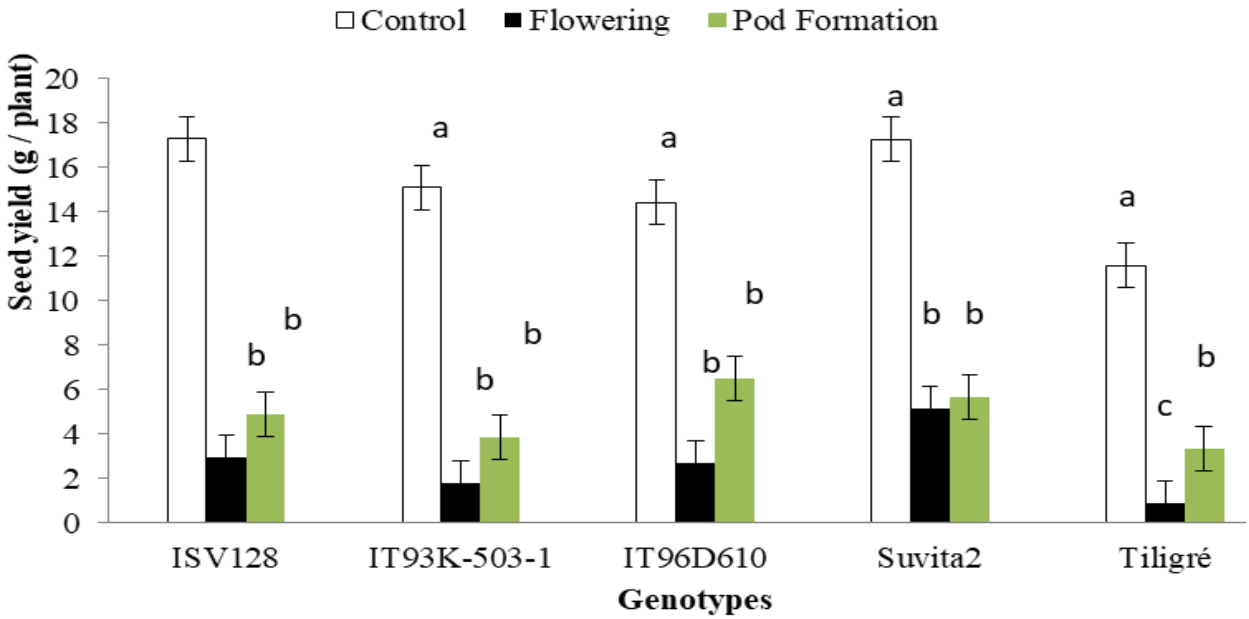

Figure 4. Comparison of seed yields of genotypes according to the period of application of stress: beginning of flowering (S1), beginning of pod formation (S2) and control (S0).

the seed filling period under the effect of water stress (De Souza and Da Silva, 1987). The terminal stress thus reduced the transfer of leaf assimilates to the seeds. The size of the seed is therefore directly related to the duration and/or filling rate (Sofield et al., 1977). A long filling time is often indicative of optimal photosynthetic activity as is the case with the control plants; whereas a high filling rate is indicative of the effects of water stress (Bahlouli et al., 2008).

The seed yield/harvest index relationship was much higher for water stress than non-limiting water condition. This strong correlation is explained according Jose et al. (2008) by the fact that some varieties of cowpea under water stress show a high harvest index following a large

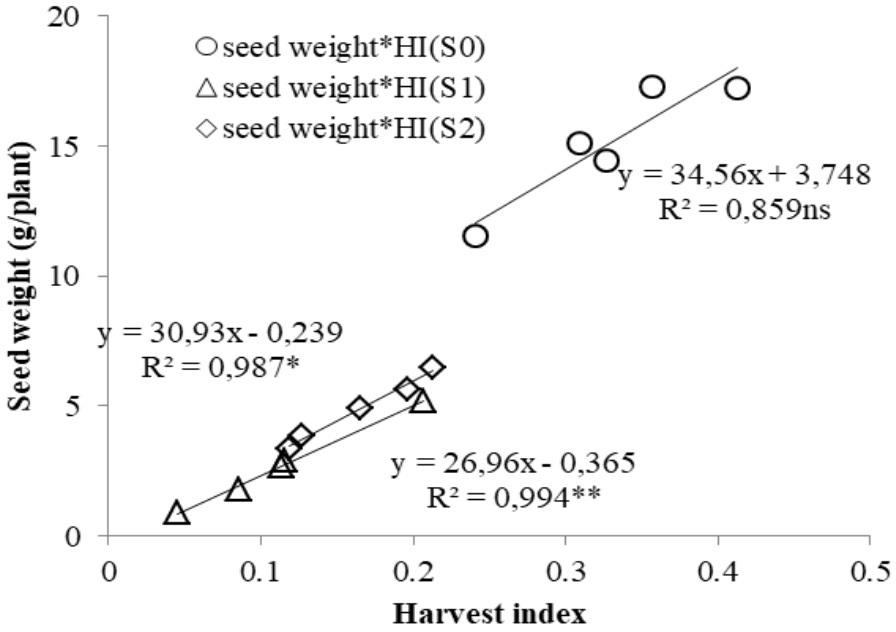

Figure 5. Seed weight relationship with harvest index for the three treatments.

mobilization of photosynthetic assimilates for the production and filling of seeds. Results show that the diminution of aerial biomass was followed by decrease pod production and filling under water stress. This low production of aerial biomass increases the relation of the harvest index and the yield of seeds under water stress conditions. This result is in agreement with those of Halilou (2016) who reported that in non-limiting water conditions, some varieties tend to favor a high production of aerial parts disproportionate to seed production, which reduces the relationship between harvest index and seed yield. Suvita2, ISV128 and IT96D-610 gave the highest harvest index for all the treatments which shows that this genotype assures better management of the assimilates on water stress condition.

Conclusion

This study did not allow the discrimination of genotypes on the basis of yield of seeds in conditions of water stress on pot experiment. The results show that yields decrease as conditions become constraining. Water stress was more severe when applied at flowering stage. Ideal genotype is the one that gave a higher harvest index under water stress. Suvita2, ISV128 and IT9D-610 genotypes recorded the highest seed yield and harvest index under water stress conditions would be more suitable and could contribute to combat food insecurity in Niger where climatic conditions are unfavorable for agriculture.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Maximizing L-glutaminase production from marine *Bacillus subtilis* JK-79 under solid state fermentation

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L-Glutaminase is majorly produced by microorganism including bacteria, yeast and fungi. It mainly catalyzes the hydrolysis of gamma-amido bond of L-glutamine. In the present investigation, the potent marine isolate, *Bacillus subtilis* JK-79 producing L-glutaminase was evaluated for the maximum L-glutaminase production by solid state fermentation (SSF). In this context, different agro-industrial residues reported in literature were tested and among them, wheat bran gave maximum L-glutaminase production (236.67 U/ml) and protein concentration (6.89 mg/ml). Statistical optimization of media components and culture conditions were successfully employed to markedly enhance the L-glutaminase production under SSF by marine *B. subtilis* JK-79. Optimization was sequentially performed from one factor at a time (OFAT), followed by Plackett and Burman Design (PBD) and response surface methodology (RSM). With the help of PB design, three significant factors such as moisture content, pH and L-glutamine were identified to significantly affect the L-glutaminase production. These three independent variables were then optimized by central composite design (CCD) of RSM. Maximum L-glutaminase production of 672.28 U/ml under flask condition was obtained at the predicted optimal values of moisture content of 62.5% (w/w), pH of 7.1 and glutamine of 2.44% (w/v). The maximum experimental L-glutaminase production was 680.8 U/ml, whereas the predicted value for L-glutaminase production was 672.28 U/ml, indicating a strong agreement between them. Statistical optimization has enhanced the production of the enzyme up to 2.88 fold as compared to the basal wheat bran medium. Thus, application of PBD and RSM for optimization studies proves to be an effective method for improving the L-glutaminase production and also understanding the interaction effects between the factors with minimum number of experiments.

Key words: *Bacillus subtilis* JK-79, L-glutaminase, Plackett-Burman design, response surface methodology, wheat bran.

INTRODUCTION

Solid state fermentation (SSF) has gained importance in the biotechnology industry due to its potential application in the production of enzymes. SSF has greater advantage as compared to submerged fermentation (SmF) due to the lower capital and operating costs as agro-industrial residues are preferably used as

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substrates. The low water volume used in SSF also has a large impact on the economy of the process mainly because of the smaller fermenter size, the reduced downstream processing, the reduced stirring and lower sterilization costs (Hölker and Lenz, 2005; Nigam, 2009).

SSF is a good alternate to the traditional chemical processes as it has several characteristics that make it eco-friendly, such as lower energy consumption, less waste water generation, use of agro-industrial residues as substrates thus avoiding environmental problems while disposing. The use of agro-industrial residues in SSF processes is of particular interest due to their availability and low cost, besides being an environment friendly alternative for their disposal (Castro and Sato, 2015).

Agro-industrial residues are derived from agricultural activities and these residues are generated in large amounts throughout the year, and are the most abundant renewable resources on earth. They are mainly composed of sugars, fibres, proteins and minerals, which are compounds of industrial interest. The presence of sugars, proteins, minerals and water make the agro-industrial residues suitable substrates for the growth of bacterial strains. If the cultivation conditions are controlled, different products of industrial interest may be produced, avoiding the loss of potential energy sources (Pandey, 2000).

Marine bacteria are the best suited for SSF processes due to their unique property to adsorb onto solid particles and can survive in different range of pH and temperature. Hence, there is an increasing interest in marine microorganisms for the production of L-glutaminase under SSF (Unissa et al., 2014). Only scanty reports are available on L-glutaminase production under SSF using agro-industrial residues as substrate. SSF was found to be more suitable than submerged fermentation for L-glutaminase production as 25-fold enhancement was obtained using *Pseudomonas flourescens* ACMR 171 when wheat bran was used as substrate (Renu, 1991). L-Glutaminase was produced by yeast *Zygosacharomyces rouxii* using wheat bran and sesame oil as substrate under SSF. Addition of 10% (w/v) NaCl and seawater to wheat bran and sesame oil enhanced the enzyme production (Kashyap et al., 2002). *Beauveria* sp., an alkalophilic and salt-tolerant fungus isolated from marine sediment, was used for L-glutaminase production using seawater-based medium supplemented with L-glutamine as substrate (Sabu et al., 2000, 2001). Seawater being a natural reserve for marine organisms can provide them sufficient nutrients when used as supplement in SSF for production of industrially important enzymes. Recent studies by Sayed (2009) showed that wheat bran was the best solid substrate for the production of L-glutaminase by *Trichoderma koningii*.

In the present investigation, the potent marine isolate, *Bacillus subtilis* JK-79 producing L-glutaminase was evaluated for the maximum production of the enzyme under SSF.

MATERIALS AND METHODS

Microorganism and culture maintenance

The isolate, *B. subtilis* JK-79 (KC492745) used in this study was isolated from marine soil collected from Parangipettai costal area (Lat. 11°.29' N; Long. 79°.46' E). The strain was maintained in Zobell's marine agar slant (Himedia, India) at 4°C and was periodically sub-cultured.

L-Glutaminase production by SSF using agro-industrial residues

Preparation of solid substrate

Different agro-industrial residues (black gram husk, red gram husk, wheat bran, rice husk, green gram husk, coconut oil cake, groundnut oil cake, palm seed powder and sawdust) were collected from local market. Wheat bran and rice husk are widely accepted solid substrate due to their large surface area, high nutrition content and support the growth of different microorganisms. Black gram husk, red gram husk and green gram husk also have high nutrient content and are used in SSF for the production of enzymes. Coconut oil cake and groundnut oil cake were selected for SSF based on their availability, water retention capacity and requirement of reduced pre-treatment procedure.

All these substrates were powdered and dried in a hot air oven overnight at 60°C. The solid particles were sieved using standard sieves and the particles 1.4 mm were stored in air-tight containers for further use (Prabhu and Chandrasekaran, 1996). Five grams of the solid substrates were moistened with seawater containing L-glutamine at 1% (w/v) level to obtain 50% moisture content, autoclaved and cooled to room temperature before inoculation.

Inoculation and incubation

The sterilized solid substrate media was inoculated with 2% (v/w) inoculum size of the strain marine, *B. subtilis* JK-79. The contents were mixed thoroughly and incubated in a slanting position at 37°C for 24 h under 80% relative humidity (Prabhu and Chandrasekaran, 1997).

Enzyme recovery

L-Glutaminase from the fermented solid substrates was extracted with phosphate buffer (0.1 M, pH 7.0) by simple contact method (Renu, 1991; Prabhu and Chandradekaran, 1997).

L-Glutaminase assay and protein estimation

L-Glutaminase was assayed by Imada et al. (1973) method. Protein content in the crude enzyme source was estimated by Lowry et al. (1951) method using bovine serum albumin as the standard and the values were expressed as mg/ml.

Optimization of process parameters by OFAT approach

Among the various agro-industrial residues evaluated, wheat bran gave the maximum L-glutaminase production. Hence, wheat bran was used as the solid substrate for L-glutaminase production. Further, the various process parameters were optimized for

Table 1. High and low levels of variables for L-glutaminase production under SSF.

S/N	Code	Variables	Low level (-)	High level (+1)
1	A	Particle size	0.6 mm	1.4 mm
2	B	Moisture content	50% (w/w)	70% (w/w)
3	C	pH	6	8
4	D	Temperture	30°C	40°C
5	E	Inoculum size	1% (v/w)	5% (v/w)
6	F	Incubation time	18 h	30 h
7	G	Carbon source	1% (w/v)	3% (w/v)
8	H	Nitrogen source	1% (w/v)	3% (w/v)
9	J	Glutamine	1% (w/v)	5% (w/v)

maximal L-glutaminase production. The parameters studied include moisture content of the medium, initial pH, particle size of the substrate, incubation temperature, inoculum size, incubation time, amino acid, additional carbon source and nitrogen source in seawater. All the experiments were carried out in triplicates and the mean values were considered.

Effect of particle size of the solid substrate on L-glutaminase production

Impact of substrate particle size on the production of L-glutaminase by the bacteria was evaluated using substrate of different particle size. The solid substrate purchased from the market were of different particle size, hence they were sieved using standard sieves of known mesh size to obtain uniform particle size in the range 0.6, 1.0 and 1.4 mm.

Effect of initial moisture content on L-glutaminase production

The initial moisture content was varied (30 to 80% w/w) to understand the effect of moisture content on the production of L-glutaminase. This was achieved by altering the amount of sea water used for moistening.

Effect of inoculum size on L-glutaminase production

The effect of inoculum size on L-glutaminase yield by bacteria during SSF were determined by using 24 h old culture ($O.D_{600nm}$ = 0.8) of increasing size in the range of 1 to 6% v/w

Effect of incubation time on L-glutaminase production

The effect of incubation time on L-glutaminase production by marine *B. subtilis* JK-79 was analyzed by carrying out the SSF for 48 h.

Effect of initial pH of the medium on L-glutaminase production

The effect of initial pH of the solid substrate medium on L-glutaminase production was determined by adjusting pH of the seawater using 1 N HCl or 1 N NaOH.

Effect of incubation temperature on L-glutaminase production

The effect of incubation temperature on L-glutaminase production by marine *B. subtilis* JK-79 was analyzed by carrying out the SSF at different temperature from 25 to 45°C.

Effect of additional carbon source on L-glutaminase production

The effect of additional carbon sources (sucrose, starch, maltose, fructose, lactose and glucose) on the production of L-glutaminase was evaluated by adding them at 1% (w/v) level, in the SSF medium.

Effect of additional nitrogen source on L-glutaminase production

The effect of additional nitrogen sources (peptone, casein, gelatin, beef extract, tryptone, yeast extract, ammonium chloride, ammonium sulphate, potassium nitrate and sodium nitrate) on the production of L-glutaminase was evaluated by adding them at 1% (w/v) level in the SSF medium.

Effect of amino acids on L-glutaminase production

The effect of amino acids (L-glutamine, phenyl alanine, cysteine, glycine, histidine, methionine and L-aspargine) on the production of L-glutminase was evaluated by adding them at 1% (w/v) level in the SSF medium. Further, the optimum concentration of the amino acid was also determined by incorporating the selected amino acid in the range (1 to 5% w/v). The additional carbon and nitrogen sources did not increase the glutaminase production substantially. Therefore, the optimum fermentation conditions were found to be pH 7, 37°C, incubation period of 24 h, 3% (v/w) of inoculum size, particle size of 1 mm and moisture content of 60%.

Optimization by statistical design

Identifying significant variables by Plackett-Burman design (PBD)

After identifying the variables affecting L-glutaminase production by OFAT approach, the PBD (Plackett and Burman, 1946) was applied to screen the significant factors with respect to their main effects on enzyme production. A total of nine factors such as particle size, moisture content, pH, temperature, inoculum size, incubation time, carbon source, nitrogen source and glutamine concentration were considered for the experimental design. The high and low levels of the different factors are listed in Table 1. The main effect was calculated as the difference between the average of measurements made at the high level (+1) and the

Table 2. PB experimental design for evaluating factors influencing L-glutaminase production by *Bacillus subtilis* JK-79 under SSF.

Run order	A	B	C	D	E	F	G	H	J
1	+1	+1	-1	+1	+1	-1	+1	-1	-1
2	-1	-1	-1	-1	-1	-1	-1	-1	-1
3	-1	-1	+1	+1	+1	-1	+1	+1	-1
4	+1	+1	-1	+1	-1	-1	-1	+1	+1
5	+1	-1	-1	-1	+1	+1	+1	-1	+1
6	+1	-1	+1	-1	-1	-1	+1	+1	+1
7	+1	+1	+1	-1	+1	+1	-1	+1	-1
8	-1	+1	+1	-1	+1	-1	-1	-1	+1
9	-1	+1	+1	+1	-1	+1	+1	-1	+1
10	+1	-1	+1	+1	-1	+1	-1	-1	-1
11	-1	-1	-1	+1	+1	+1	-1	+1	+1
12	-1	+1	-1	-1	-1	+1	+1	+1	-1

Table 3. Actual and coded values of the factors employed in CCD for L-glutaminase production under SSF.

Factors	Range of levels					
	Codes	-α	-1	0	1	α
Moisture content (%)	A	51.591	55	60	65	68.409
pH	B	5.318	6	7	8	8.682
Glutamine (% w/v)	C	1.159	1.5	2	2.5	2.841

average of measurements observed at low level (-1) of each factor (Usha et al., 2011). The factors that have confidence level above 95% were considered the most significant factors that affect the L-glutaminase production.

The details of experimental design for screening the different factors are shown in Table 2. The medium was formulated as per the design and the flask culture experiments were performed. All the experiments were performed in triplicates and the average of L-glutaminase production was considered as the response. The PBD is based on the first-order polynomial model:

$$Y = \beta_o + \sum \beta_i X_i$$

Where, Y denotes the response (L-glutaminase activity U/ml), β_o is model intercept, β_i is the factor co-efficient and X_i is the level of independent variable. From the regression analysis, the variables showing p-value below 5% ($p<0.05$) were considered to have greater impact on L-glutaminase production and used further for CCD analysis.

Response surface methodology

RSM uses statistical experimental designs such as central composite design (CCD), Box-Behnken design etc. to develop empirical models that relate a response and mathematically describes the relationships between the independent and dependent variables of the process under consideration. The RSM has been used to obtain a predicted model for optimizing the fermentation media and/or process parameters, to carry out simulation with model equation and for better understanding of the fermentation process.

In the present study, RSM using CCD was adopted for improving the L-glutaminase production under SSF by strain *B. subtilis* JK-79 using the software Design Expert Release 9 (Stat-Ease INC. Minneapolis MN, U.S.A).

A full factorial central composite design was performed. The quadratic regression model can be illustrated as

$$Y = \beta_o + \sum \beta_i X_i + \sum \beta_{ii} X_i^2 + \sum \beta_{ij} X_i X_j$$

Where, Y is the response (L-glutaminase activity U/ml), β_o is the intercept term, β_i is the slope or linear effect of input variable X_i, β_{ii} is the quadratic effect of input variable X_i and β_{ij} is the linear by linear interaction effect between the input variable X_i and X_j. The statistical model was validated with respect to L-glutaminase production under the conditions predicted by the model in shake flask conditions. Samples were withdrawn at the desired intervals and L-glutaminase assay was performed as mentioned earlier.

RESULTS AND DISCUSSION

Production of L-glutaminase using different agro-industrial residues

Selection of suitable agro-industrial residue as solid substrate becomes a pre-requisite for optimizing the process parameters of the SSF fermentation medium. In this context, different agro-industrial residues (black gram husk, green gram husk, red gram husk, wheat bran, rice husk, coconut oil cake, groundnut oil cake, palm seed fibre and saw dust) were evaluated for the production of L-glutaminase by marine *Bacillus subtilis* JK-79 under SSF.

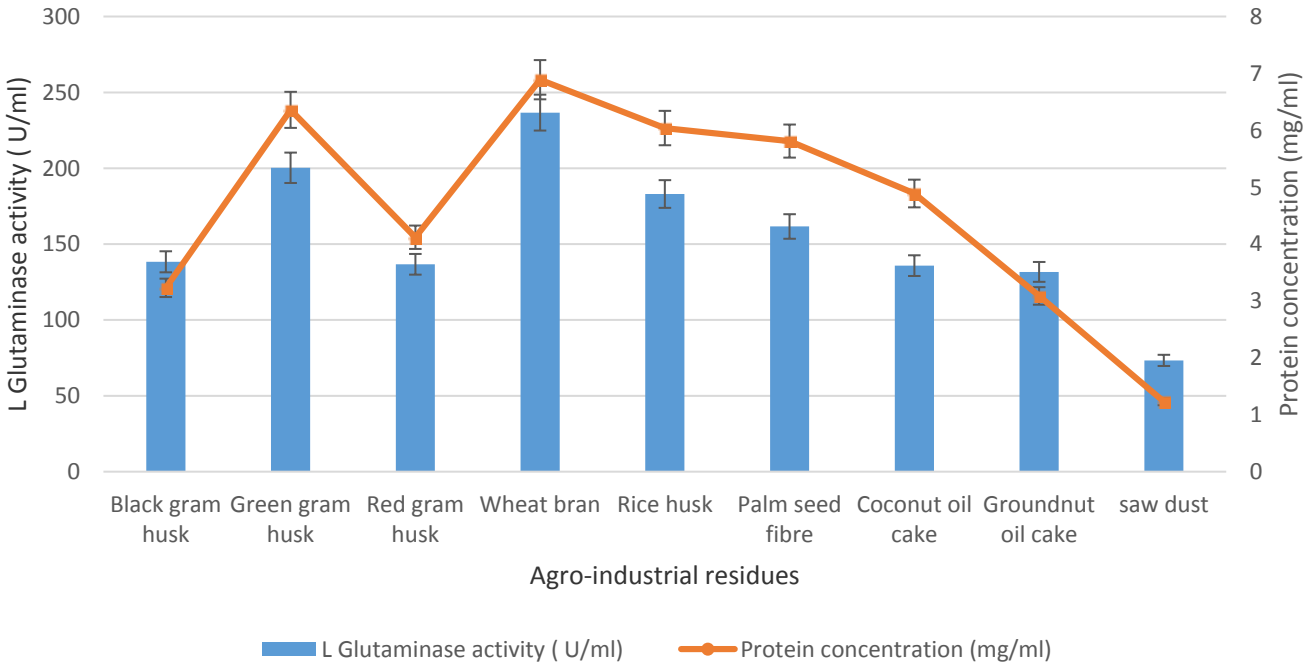

Figure 1. Production of L-glutaminase by *Bacillus subtilis* JK-79 on various agro-industrial residues under SSF.

From Figure 1, it is evident that among the various agro-industrial residues, wheat bran gave maximum L-glutaminase production (236.67 U/ml) and protein concentration (6.89 mg/ml). Many authors have reported that wheat bran was found to be most the preferable substrate for L-glutaminase production due to their excellent mechanical properties such as structure retention and lack of particle agglomeration in addition to their good nutritional value (Renu, 1991; Prabhu and Chandrasekaran, 1996; Kashyap et al., 2002; Sayed, 2009; Iyer and Singhal, 2010a,b; Athira et al., 2014). Following wheat bran, green gram husks (200.33 U/ml) and rice husk (183 U/ml) showed maximal L-glutaminase production. The observed variations in L-glutaminase production reveal that the composition of the substrate plays a pivot role in the L-glutaminase production.

Sathish et al. (2008) has reported that bengal gram husk supported maximal L-glutaminase production (2731 U/gds), followed by the palm seed fibre (1953 U/gds) and wheat bran (1036 U/gds), suggesting the role of agro-industrial residues in the production of L-glutaminase. Similarly, Han et al. (2003) produced L-glutaminase on sufi (soybean cheese) by *Actinomucor* and *Rhizopus oligosporus*, while Sabu et al. (2000, 2001) produced L-glutaminase with marine *Beauveria* sp. BTMFS 10.

From the literature reports, it is evident that *Bacillus* sp. in general has the tendency to utilize wheat bran as the preferred substrate for the production of various types of enzymes. Wheat bran was used as the sole substrate for the production of alkaline protease and α-amylase by *Bacillus* sp. P-2 (Kaur et al., 2001) and *Bacillus* sp. AS-1 (Soni et al., 2003), respectively. Baysal et al. (2003) also used wheat bran as substrate for the production of α-amylase from *B. subtilis*. Kashyap et al. (2003) produced pectinase from *Bacillus* sp. by using wheat bran along with polygalacturonic acid as the solid substrate. Similarly, Sodhi et al. (2005) has also produced α-amylase from *Bacillus* sp. PS-7 on a medium containing wheat bran, glycerol and soybean meal. Apart from wheat bran, *Bacillus* sp. also has tendency to utilize green gram husk as solid substrate which is evident from the report where Prakasham et al. (2006) has produced alkaline protease from *Bacillus* sp. by using green gram husk as the sole substrate. Since wheat bran showed the maximal L-glutaminase production by marine *Bacillus subtilis* JK-79, wheat bran was used as substrate for L-glutaminase production under SSF and further studies were carried out.

Optimization of process parameters and media components by OFAT approach

Effect of particle size on L-glutaminase production

Particle size of substrates is one of the critical factors that influence the fermentation process. Particle size of the substrate is an important factor which affects SSF, as it determines the heat and mass transfer during the process (Pandey et al., 2000).

From Figure 2, it is evident that particle size of the range 1.0 mm gave maximal L-glutaminase production

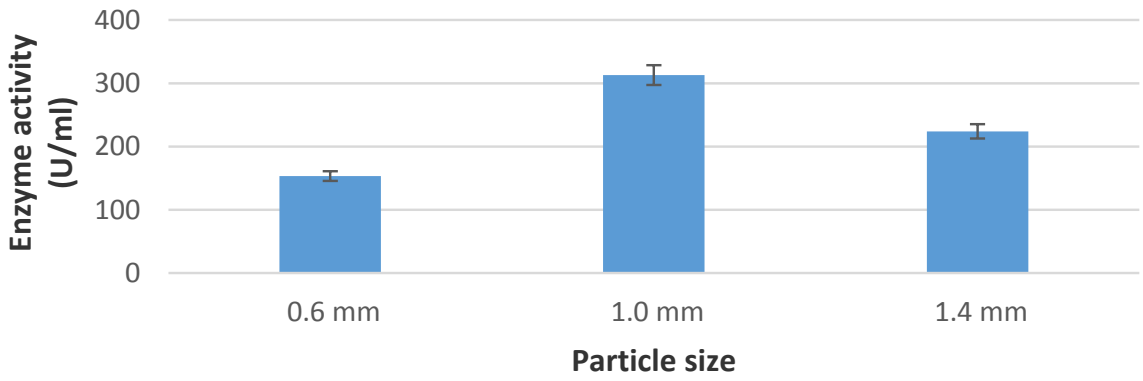

Figure 2. Effect of particle size on L-glutaminase production by marine *B. subtilis* JK-79 under SSF.

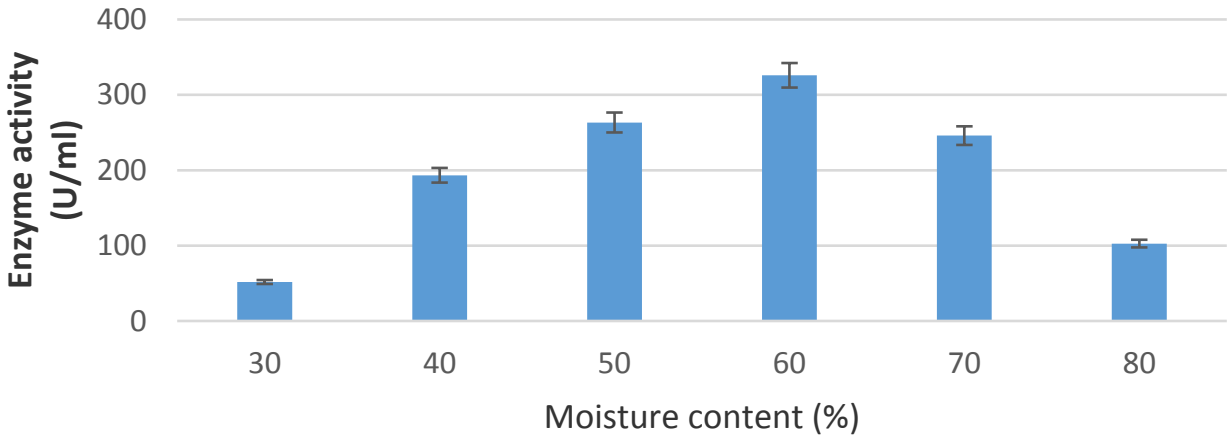

Figure 3. Effect of moisture content on L-glutaminase production by marine *B. subtilis* JK-79 under SSF.

(312.89 U/ml) by marine *B. subtilis* JK-79. The result is in accordance with the literature report of Prabhu and Chandrasekaran (1996). The authors showed maximal L-glutaminase production by *Vibrio costicola* at 2% (w/v) L-glutamine concentration, initial pH 7.0, 35°C, 60% moisture content, 0.6 to 1.0 mm particle size, and 24 h incubation time using wheat bran and rice husk.

Effect of moisture content of the medium on L-glutaminase production

In SSF, moisture content of the solid medium plays critical role as the microbiological activity on a substrate will eventually increase as the water content increases to the optimal level. Figure 3 reveals that moisture content of the wheat bran plays a significant role in the production of L-glutaminase. It could be seen that maximal L-glutaminase production (326.07 U/ml) was noticed at 60% (w/w) moisture content. Kashyap et al. (2002) and Sabu et al. (2000) has reported L-glutaminase production by *Z. rouxii* and *Beauveria* sp. at 64 and 60% initial moisture content, respectively.

Effect of inoculum size on L-glutaminase production

Optimal inoculum size is an essential requirement for maximal L-glutaminase production by the bacteria. From the Figure 4, it is clear that maximal L-glutaminase production by marine *Bacillus subtilis* JK-79 was obtained at 3% (v/w) inoculum size (367 U/ml). Increase in inoculum size beyond 3% (v/w) resulted in decrease in L-glutaminase production and this may be attributed to nutrient depreciation or accumulation of some toxic substance. However, with lower inoculum size, the decrease in L-glutaminase production could be due to lesser number of cells and hence requires longer time to grow and form the desired product (Sayed, 2009). The result is in accordance with the inoculum size reported by Sayed (2009), whereas Kashyap et al. (2002) reported maximal L-glutaminase production at 2% (v/w) inoculum size with wheat bran and sesamum oil cake.

Effect of incubation time on L-glutaminase production

24 h of cultivation on wheat bran resulted in L-

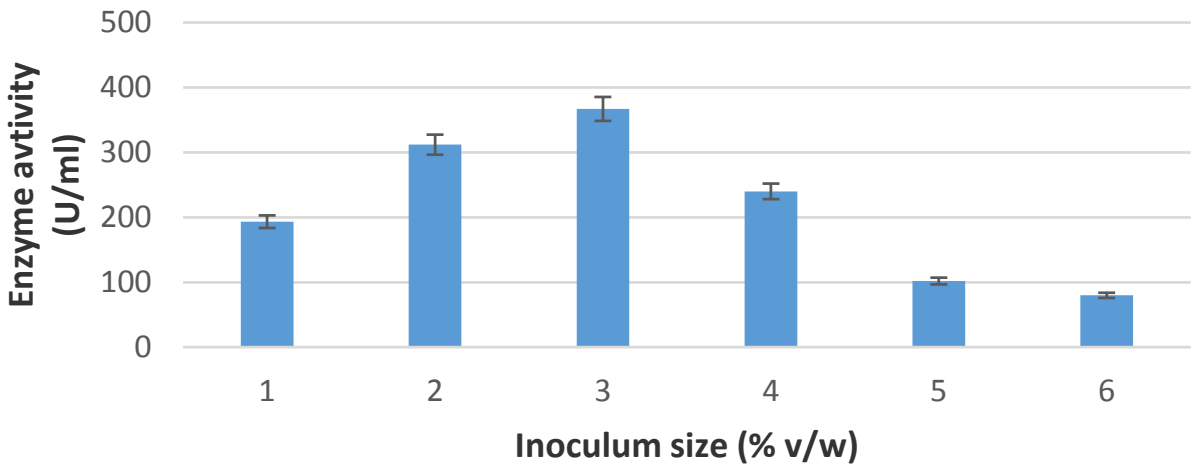

Figure 4. Effect of inoculum size on L-glutaminase production by marine *B.subtilis* JK-79 under SSF.

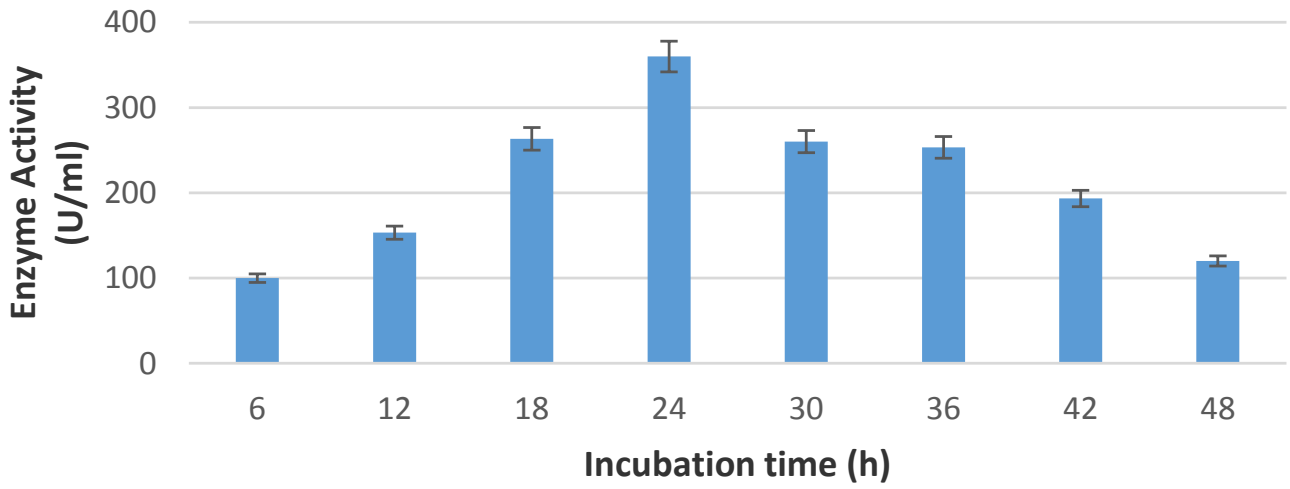

Figure 5. Effect of incubation time on L-glutaminase production by marine *Bacillus subtilis* JK-79 under SSF.

glutaminase synthesis of 360 U/ml (Figure 5) with *B. subtilis* JK-79. Further increase in incubation time, resulted in a gradual decline in the enzyme production. This is may be because of the inactivation of the enzyme. The result is in accordance with literature reports (Prabhu and Chandrasekaran, 1997; Sabu et al., 2000; Kashyap et al., 2002).

Effect of pH of the medium on L-glutaminase production

Experiments were conducted to optimize the pH of the SSF medium with wheat bran for maximum L-glutaminase yield. The medium with pH 7 supported maximal L-glutaminase production (356.33 U/ml) by *B. subtilis* JK-79 (Figure 6). This observation is in agreement with the results obtained by marine *V. costicola* (Prabhu and Chandrasekaran, 1997) and *T. koningii* (Sayed, 2009).

Effect of incubation temperature on L-glutaminase production

Maximum L-glutaminase production (394 U/ml) was obtained when SSF was carried out at 37°C (Figure 7). The enzyme production decreased above and below 37°C. The result is in good agreement with the optimal conditions reported by Sathish et al. (2008). The author showed 37°C as the optimal temperature for maximal L-glutaminase production by *Bacillus* sp. Renu (1991) showed that 35°C is the optimal temperature for maximal L-glutaminase production by *P. fluorescens* ACMR 171 and *Vibrio cholera* ACMR 347.

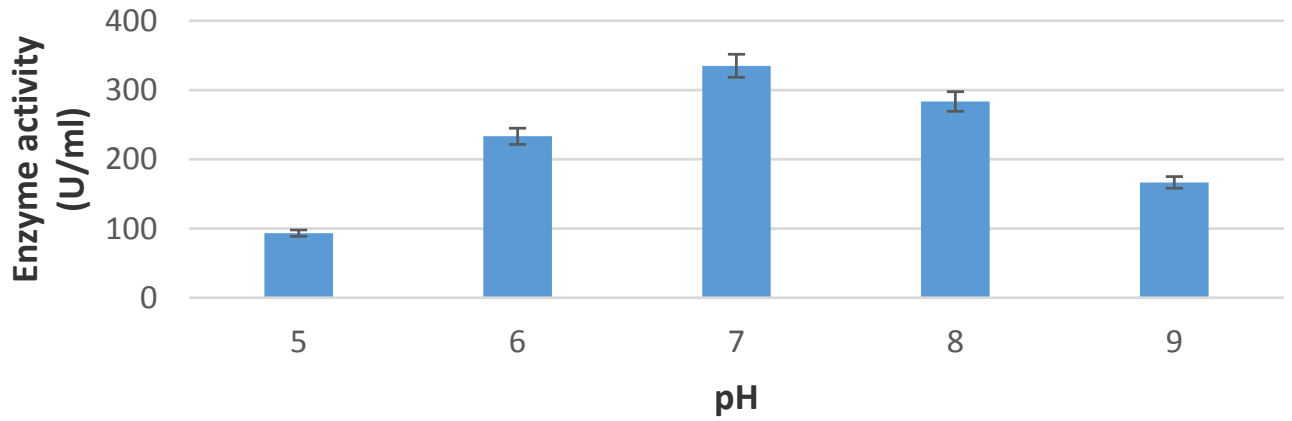

Figure 6. Effect of pH on L-glutaminase production by marine *B. subtilis* JK-79 under SSF.

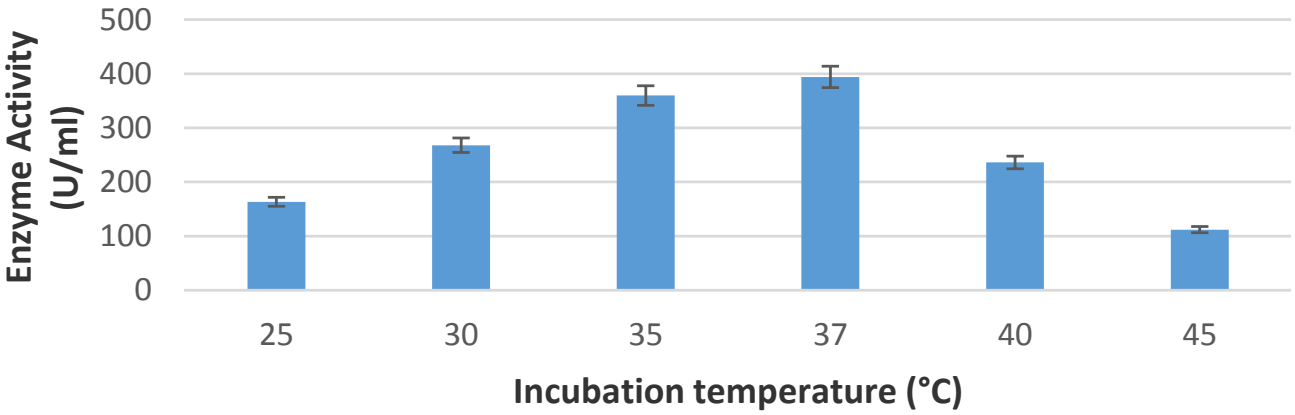

Figure 7. Effect of temperature on L-glutaminase production by marine *B. subtilis* JK-79 under SSF.

Effect of additional carbon source on L-glutaminase production

Incorporation of additional carbon sources at 1% (w/v) level did not show any improvement in the production of L-glutaminase by marine *B. subtilis* JK-79. This may be attributed to the chemical composition of wheat bran. Among the carbon sources, fructose at 1% (w/v) showed L-glutaminase production of 365 U/ml (Figure 8). However, Sayed (2009), Prabhu and Chandrasekaran (1997) and Kashyap et al. (2002) reported increase in L-glutaminase production by incorporating additional carbon source in wheat bran such as 1% (w/v) glucose, 1% (w/v) maltose and 1% (w/v) glucose, respectively.

Effect of additional nitrogen sources on L-glutaminase production

In the present work, different organic and inorganic nitrogen sources were evaluated (Figure 9). The results show that none of the inorganic nitrogen sources supported significant improvement in the production of L-glutaminase and among the organic nitrogen sources, yeast extract showed marginal increase in the L-glutaminase production. Kashyap et al. (2002), Prabhu and Chandrasekarn (1997) and Sayed (2009) has reported negative impact of additional nitrogen sources on L-glutaminase production by *Z. rouxii*, *V. costicola* and *T. koningii* respectively.

Effect of amino-acids on L-glutaminase production

The effect of different amino acids on the production of L-glutaminase by marine *B. subtilis* JK-79 was studied by incorporating amino acids in the medium. Among the different amino acids, L-glutamine which is the actual substrate for L-glutaminase showed a significant increase in the production of the enzyme (Figure 10). Thus, L-glutamine serves as an inducer for the production of L-glutaminase. The control medium does not contain glutamine but yet has shown L-glutaminase production. This may be attributed to the presence of L-

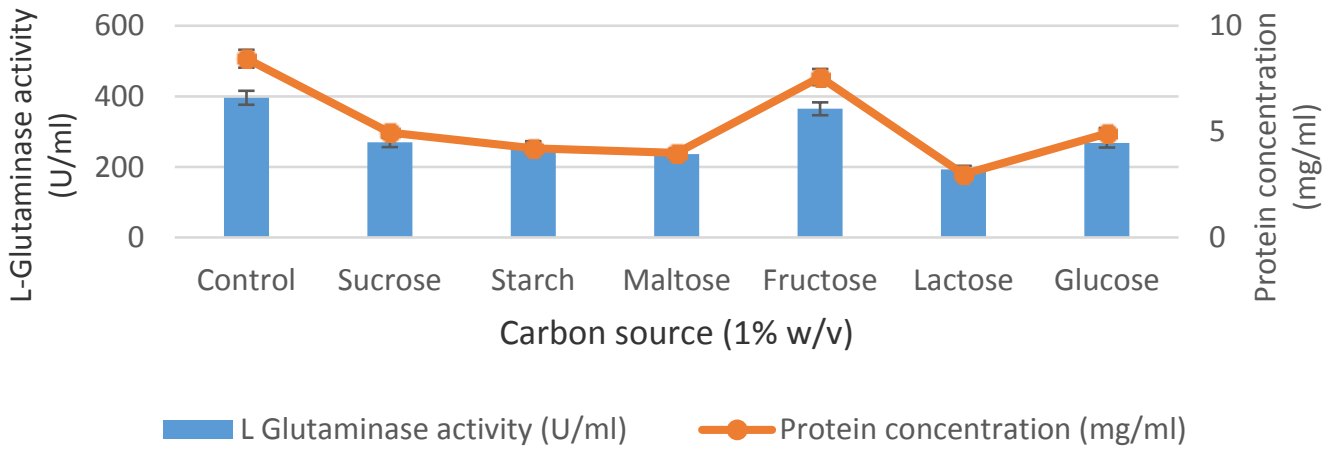

Figure 8. Effect of carbon sources on L-glutaminase production by marine *B. subtilis* JK-79 under SSF.

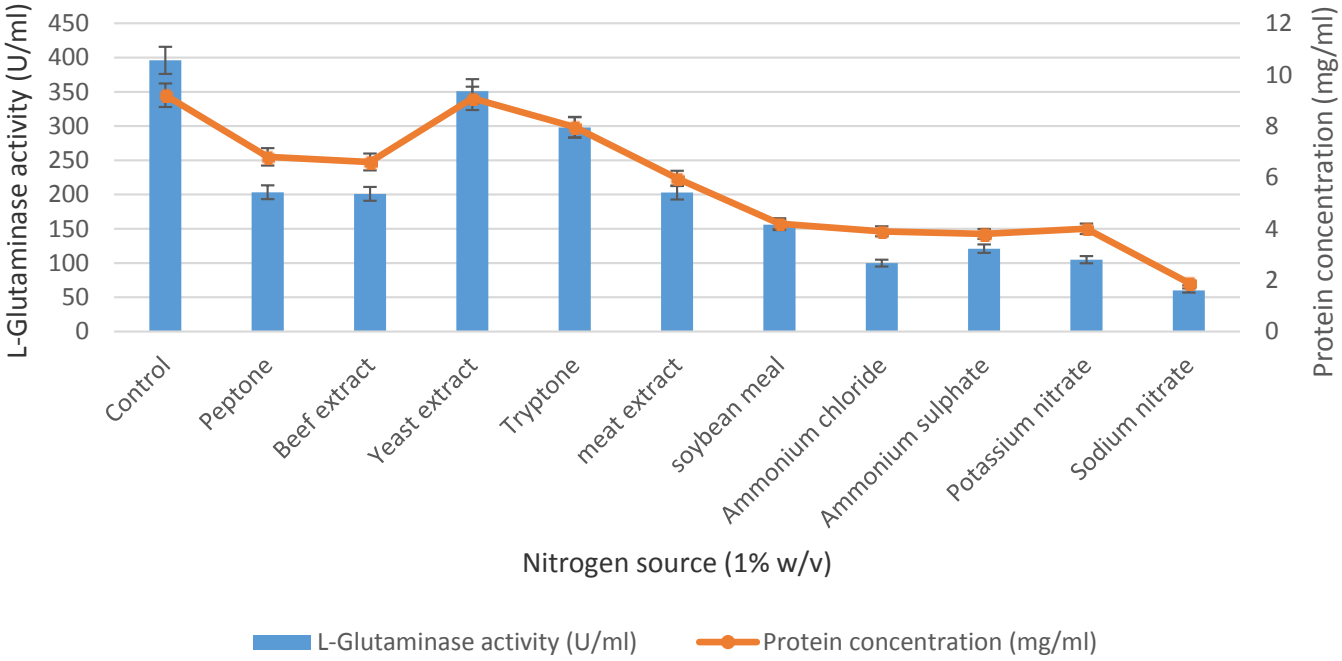

Figure 9. Effect of nitrogen sources on L-glutaminase production by marine *Bacillus subtilis* JK-79 under SSF.

glutamine amino acid in wheat bran. Further, the optimal concentration of glutamine was evaluated by adding different concentrations of L-glutamine in the range of 1 to 5% w/v. The results indicate that 2% (w/v) of L-glutamine showed maximal production (412 U/ml) of L-glutaminase (Figure 11). The result is in good correlation with the literature reports where Sayed (2009) reported maximal L-glutaminase production at 2% L-glutamine in wheat bran. However, Renu (1991) has reported apart from L-glutamine other amino acids like L-glutamic acid and lysine at 1% (w/v) which was found to induce L-glutaminase production under SSF with *V. cholera* ACMR 347 and *P. fluorescens* ACMR 171.

Thus, the optimal conditions for the maximal L-glutaminase production by marine *B. subtilis* JK-9 under SSF was found to be pH 7.0, temperature 37°C, incubation period 24 h with wheat bran as the solid substrate, supplemented with 2% (w/v) glutamine by OFAT approach.

Identification of significant factors using PBD

The influence of nine variables namely pH, temperature, moisture content, particle size, inoculum size, incubation time, carbon source, nitrogen source and glutamine on

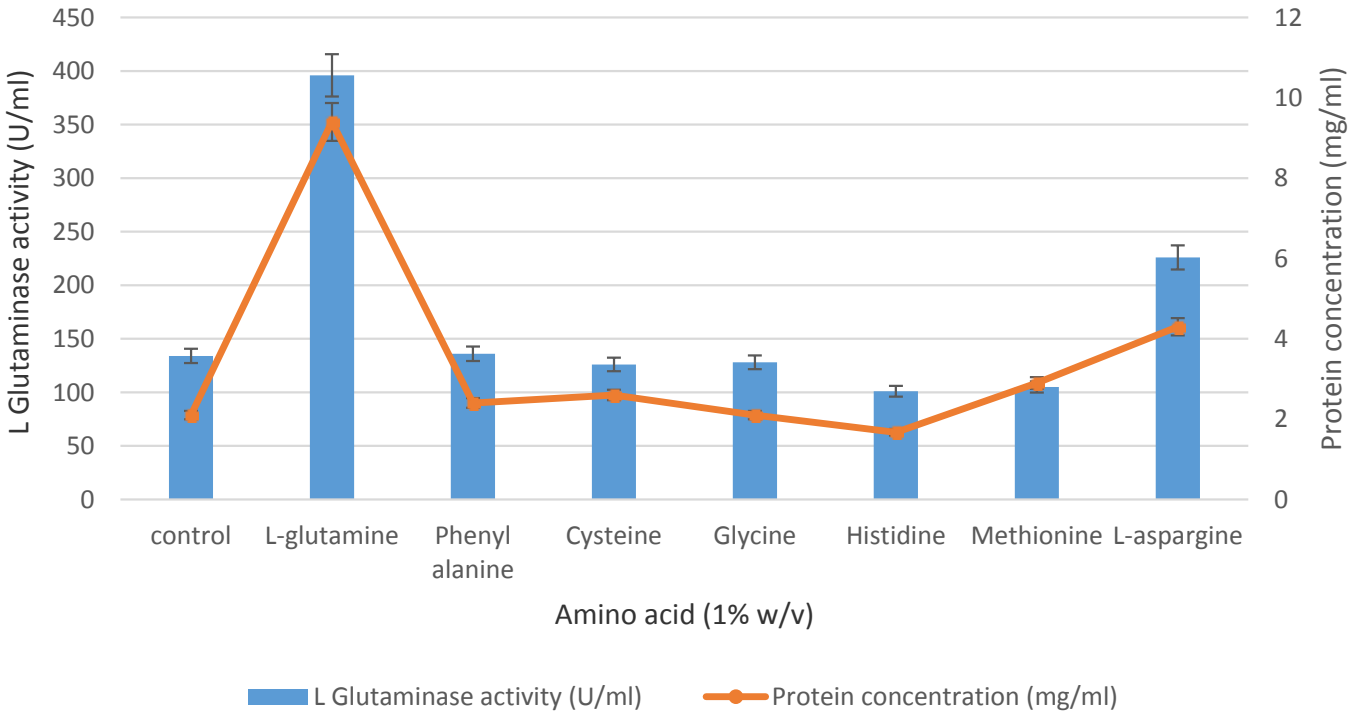

Figure 10. Effect of amino acids on L-glutaminase production by marine *Bacillus subtilis* JK-79 under SSF.

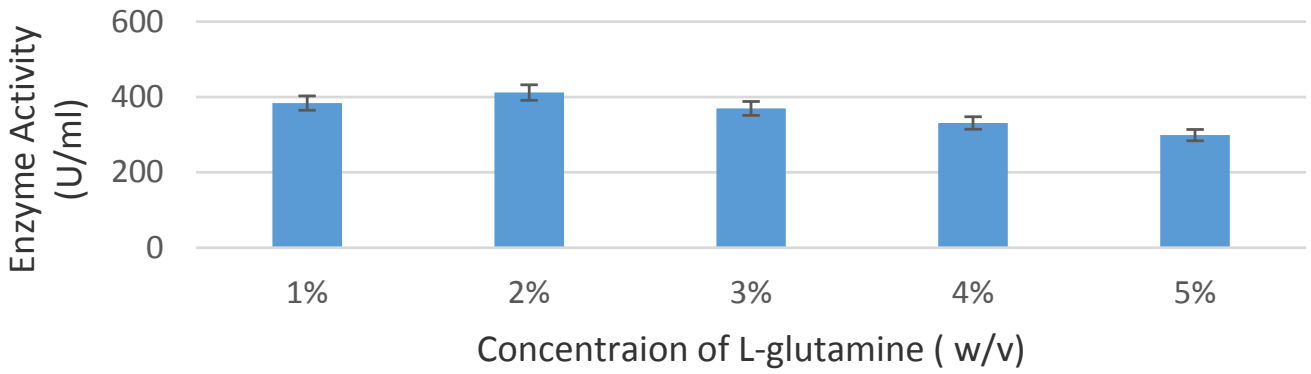

Figure 11. Effect of L-glutamine on L-glutaminase production by marine *Bacillus subtilis* JK-79 under SSF.

the production of L-glutaminase by the strain, *B. subtilis* JK-79 was investigated in 12 runs using PBD. Table 4 represents the PBD for the nine variables and the corresponding response for L-glutaminase production (U/ml). Variations ranging from 198.67 to 595.00 U/ml in the production of L-glutaminase was observed by PBD. The Pareto chart (Figure 12) illustrates the order of the significance of the variables affecting L-glutaminase production. On the basis of analysis of variance (ANOVA), and values of coefficient for significance ($P<0.05$), the factors with high significance were in the order: moisture content, glutamine and pH. Neglecting the insignificant variables, the first order polynomial equation was derived representing L-glutaminase production as a function of independent variables.

Y = -140 + 7.25 Moisture content – 37.0 pH + 67.90 L-glutamine

Where, Y is the response (L-glutaminase production U/ml).

The statistical analysis and the ANOVA of the experimental design are shown in Tables 5 and 6, respectively. The significance of each variable was evaluated based on its main effect. The main effect of the factors is shown in Figure 13.

Statistical analysis of PBD demonstrated that the model F value of 20.5 is significant and among the

Table 4. PB experimental design for evaluating factors influencing L-glutaminase production by *Bacillus subtilis* JK79 under SSF.

Run order	A	B	C	D	E	F	G	H	J	L-glutaminase activity (U/ml)	Protein content (mg/ml)
1	+1	+1	-1	+1	+1	-1	+1	-1	-1	483.58	9.85
2	-1	-1	-1	-1	-1	-1	-1	-1	-1	250.00	3.0
3	-1	-1	+1	+1	+1	-1	+1	+1	-1	198.67	2.65
4	+1	+1	-1	+1	-1	-1	-1	+1	+1	595.00	12.60
5	+1	-1	-1	-1	+1	+1	+1	-1	+1	458.88	9.30
6	+1	-1	+1	-1	-1	-1	+1	+1	+1	300.00	5.60
7	+1	+1	+1	-1	+1	+1	-1	+1	-1	342.42	7.70
8	-1	+1	+1	-1	+1	-1	-1	-1	+1	492.28	10.20
9	-1	+1	+1	+1	-1	+1	+1	-1	+1	523.33	11.80
10	+1	-1	+1	+1	-1	+1	-1	-1	-1	322.27	7.20
11	-1	-1	-1	+1	+1	+1	-1	+1	+1	438.87	9.10
12	-1	+1	-1	-1	-1	+1	+1	+1	-1	396.67	8.60

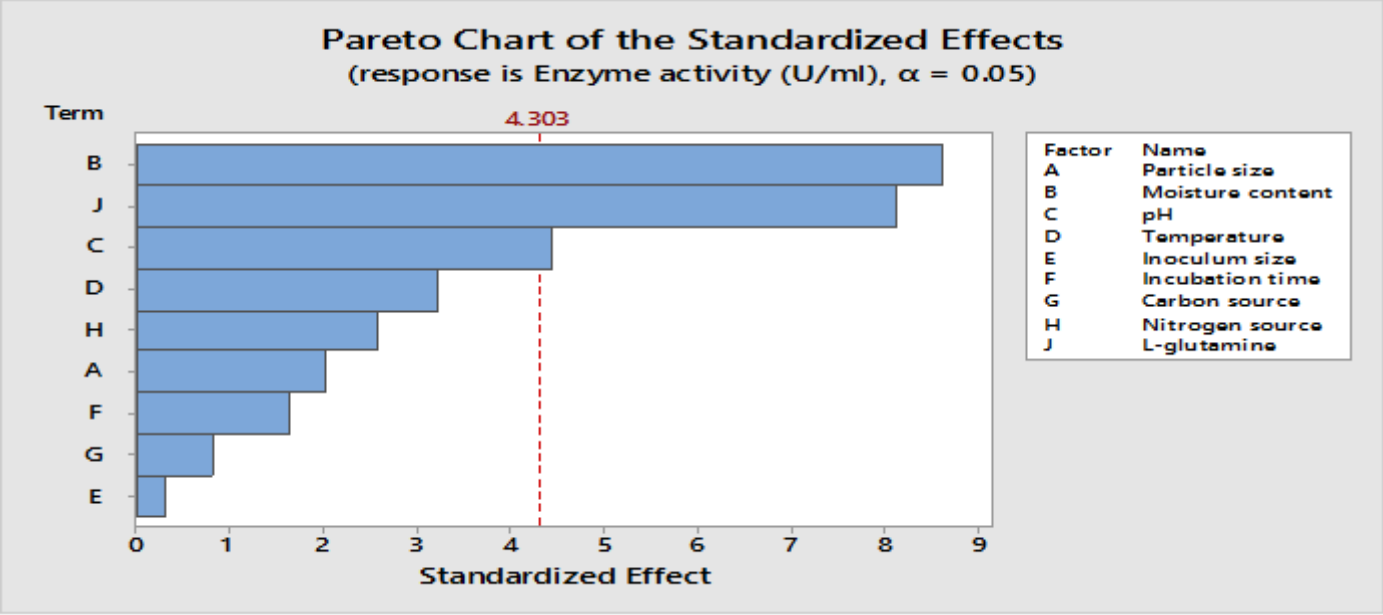

Figure 12. Pareto chart for Plackett Burman design for nine variables on L-glutaminase production by *Bacillus subtilis* JK-79 under SSF.

significant factors, moisture content and glutamine showed a remarkable effect on the production of L-glutaminase by *B. subtilis* JK-79. The goodness of fit of the regression model was represented by co-efficient of determination (R^2). In the present model, R^2 was 98.93%, which indicated that upto 98.93% of the total variability in the response could be explained by the model and only 1.07% variability was not explained. The value of the adjusted determination coefficient (Adj R^2 = 0.9412) confirmed the significance of the model as well. L-Glutaminase production, obtained from PBD showed a wide range of variation and this revealed the necessity for further optimization. Therefore, the entire set of insignificant variables was left and further optimization was carried out only with the significant variables.

Optimization using central composite design

Following the identification of significant variables by PBD, the optimal concentration level of these variables *viz* glutamine, moisture content and pH were identified by RSM. RSM using CCD, was adopted to understand the interactive effects of these four significant variables. Table 3 illustrates the details of actual and coded values employed in the CCD. The experimental trials were performed based on the CCD (Table 7) and the results obtained were fitted to a second order polynomial

Table 5. ANOVA for PBD for L-glutaminase production by *Bacillus subtilis* JK-79 under SSF.

Source	DF	Adj SS	Adj MS	F-Value	p-Value
Model	9	154446	17161	20.55	0.047*
Linear	9	154446	17161	20.55	0.047*
Particle size	1	3411	3411.5	4.09	0.181
Moisture content	1	62293	62293.0	74.60	0.013*
pH	1	16430	16430.2	19.68	0.047*
Temperature	1	8612	8611.9	10.31	0.085
Inoculum size	1	63	62.7	0.08	0.810
Incubation time	1	2212	2211.6	2.65	0.245
Carbon source	1	529	529.5	0.63	0.509
Nitrogen source	1	5578	5577.6	6.68	0.123
L-glutamine	1	55318	55318.1	66.25	0.015*
Error	2	1670	835.0		
Total	11	156116			

*Significant.

Table 6. Statistical analysis through PBD showing coded coefficients and effects for each factor on L-glutaminase production under SSF.

Term	Effect	Coefficient	SE coefficient	t-value	p-value
Constant		400.16	8.34	47.97	0.000
Particle size	33.72	16.86	8.34	2.02	0.181
Moisture content	144.10	72.05	8.34	8.64	0.013*
pH	-74.01	-37.00	8.34	-4.44	0.047*
Temperature	53.58	26.79	8.34	3.21	0.085
Incubation time	4.57	2.29	8.34	0.27	0.810
Inoculum size	27.15	13.58	8.34	1.63	0.245
Carbon source(Fructose)	-13.28	-6.64	8.34	-0.80	0.509
Nitrogen source (Yeast extract)	-43.12	-21.56	8.34	-2.58	0.123
L- Glutamine	135.79	67.90	8.34	8.14	0.015*

*Significant; R^2 = 98.93%, Adj. R^2 = 94.12%, Pred. R^2 = 61.49%; Significant at 95% confidence level ($p<0.05$); Insignificant at 95% confidence level ($p>0.05$).

equation to explain the dependence of L-glutaminase production with the independent variables.

$$Y = +661.62 + 45.74 * A + 21.24 * B + 41.31 * C - 4.86 * AB + 36.20 * AC + 4.04 * BC – 93.60 * A^2 -157.73 * B^2 – 85.36* C^2$$

Where, Y is the response of L-glutaminase production, A, B and C are the coded values of moisture content, pH and glutamine, respectively.

The analysis of variance of the quadratic regression model (Table 9) suggested that the model is very significant which was evident from the Fisher's F-test (F_{model} = 136.55) and a low probability value (P_{model} <0.0001). The p value for "lack of fit" (0.0578) also indicated that the quadratic model adequately fitted the data. Table 8 gives the model coefficients estimated by regression analysis for each variable and the p values were used a tool to check the significance of each variable. The smaller the magnitude ($p< 0.05$), the more significant the corresponding co-efficient, while those greater than 0.1000 indicates the model terms were insignificant. In this model, A, B, C, AC, A^2, B^2 and C^2 are significant model terms. The model's goodness of fit was checked by determination of co-efficient (R^2).

The R^2 was found to be 0.9919, indicating that the model could explain 99.19% variability with the response. The "Adeq Precision" measures the signal (response) to noise (deviation) ratio and for this model, it was found to be 31.546 which indicates an adequate signal. This model can be used to navigate the design space for the response Y. The "Pred R-Squared" of 0.9476 was in reasonable agreement with the "Adj R-Squared" of 0.9847. From Figure 14, it is evident that the actual response was in good agreement with the predicted values.

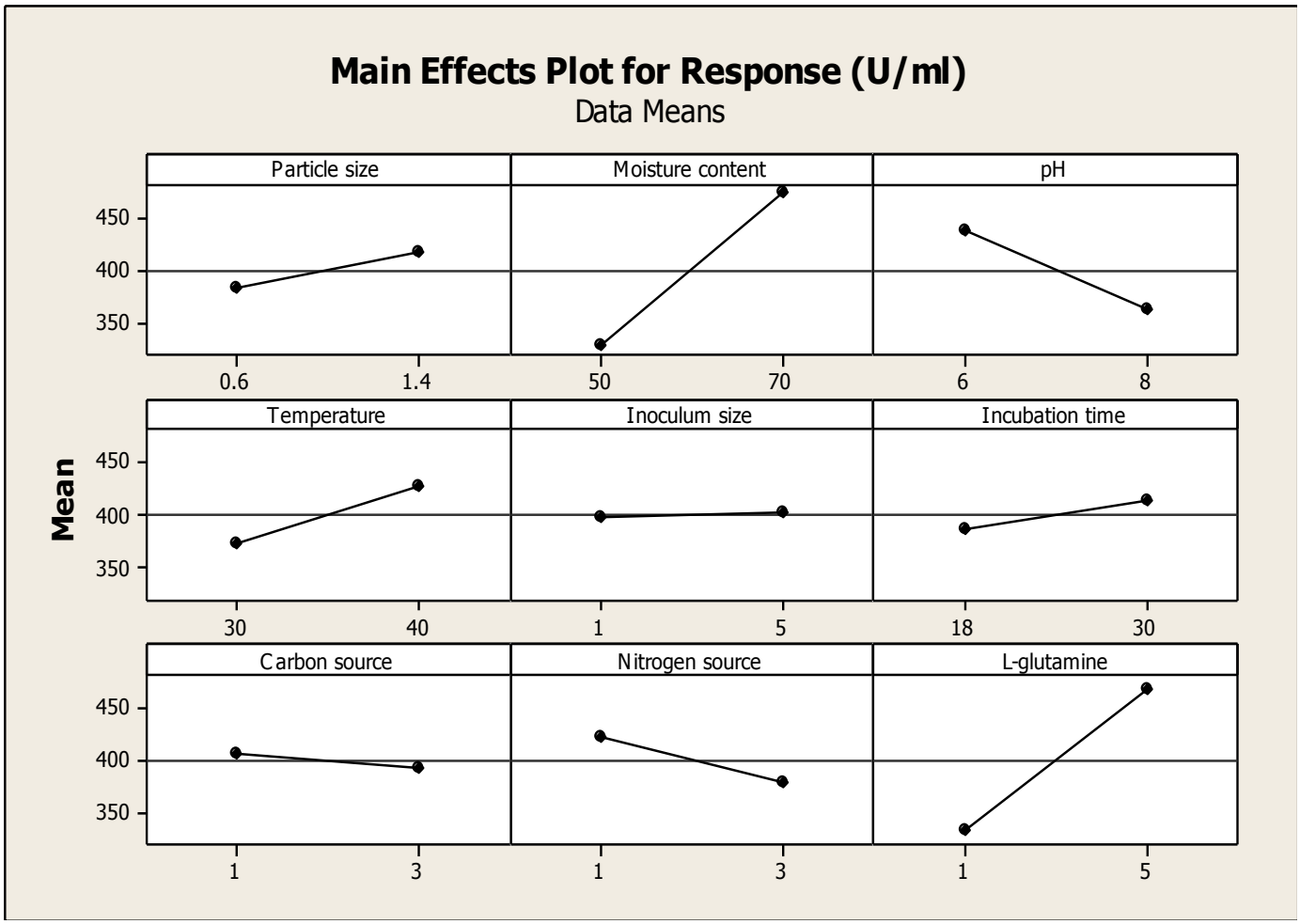

Figure 13. Main effects of the nine variables on L-glutaminase production by *Bacillus subtilis* JK-79 under SSF.

The relationship between the independent variables was assessed by examining the response surfaces. Three dimensional response surfaces were generated by holding one factor constant at a time and plotting the response obtained for varying levels of the other two factors. Figure 15a to c shows the response surface and contour plots generated for the variation in the yields of glutaminase as a function of concentrations of two variables with the other one variable at their central value.

From the Figure 15a, it was evident that when the concentration of pH was held at their middle values, the moisture content showed a parabolic response at the different concentrations of glutamine with the highest yield of L-glutaminase obtained in the range of 60 to 65%. Very low and high moisture content was not favourable for L-glutaminase production. Variations in glutamine concentration have also followed a parabolic curve and optimum yield was obtained in the range of 2.0 to 2.25%. In Figure 15b, the response of moisture content was parabolic at different levels of pH and the highest yield of L-glutaminase was obtained in the range of 60 to 65% and similarly, the response of pH was parabolic at different levels of moisture with the highest yield obtained at pH 7. In Figure 15c, the response of pH and glutamine was also parabolic at different levels of glutamine and pH, respectively. The highest yield of L-glutaminase was obtained in the range of 2 to 2.25% at pH 7.

Response optimization

The point prediction tool of design of expert software was used to predict the optimum values of the independent variables. Maximum L-glutaminase production of 672.28 U/ml under flask condition was obtained at the predicted optimal values of moisture content- 62.5% (w/w), pH 7.1 and glutamine 2.44% (w/v). The maximum experimental L-glutaminase production was 680.8 U/ml whereas the predicted value for L-glutaminase production was 672.28 U/ml, indicating a strong agreement between them.

Table 7. Results of CCD using three independent variables and six centre points.

Std	Run	Factor 1	Factor 2	Factor 3	Response	Protein concentration (mg/ml)
		A:Moisture content(%)	B:pH	C:glutamine (% w/v)	L-glutaminase activity (U/ml)	
1	1	-1	-1	-1	231.67	3.6
3	2	-1	1	-1	282	4.2
10	3	1.68179	0	0	492.23	9.8
4	4	1	1	-1	286.65	4.7
6	5	1	-1	1	415.58	9.4
9	6	-1.68179	0	0	338.83	8.36
5	7	-1	-1	1	246.67	4.1
11	8	0	-1.68179	0	199.98	2.7
2	9	1	-1	-1	265.59	4.5
20	10	0	0	0	654.47	14
15	11	0	0	0	666.67	14.3
19	12	0	0	0	638.87	13.5
14	13	0	0	1.68179	498.87	10.4
8	14	1	1	1	462.59	9.89
16	15	0	0	0	672.28	14.78
18	16	0	0	0	658.83	13.9
17	17	0	0	0	672.23	14.45
12	18	0	1.68179	0	268.33	4.9
13	19	0	0	-1.68179	378.82	7.9
7	20	-1	1	1	303.33	7.2

Table 8. Model coefficients estimated by multiple linear regressions.

Factor	Coefficient Estimate	df	Standard Error	95%CI Low	95%CI High
Intercept	661.62	1	8.31	643.51	679.74
A-Moisture content	45.74	1	5.52	33.73	57.76
B-pH	21.24	1	5.52	9.22	33.25
C-glutamine	41.31	1	5.52	29.29	53.33
AB	-4.86	1	7.64	-21.88	12.15
AC	36.20	1	7.21	20.50	51.90
BC	4.04	1	7.64	-12.98	21.05
A^2	-93.60	1	5.37	-105.30	-81.90
B^2	-157.73	1	5.37	-169.42	-146.03
C^2	-85.36	1	5.37	-97.05	-73.66

Comparison of L-glutaminase production in basal and optimized medium

The L-glutaminase production under submerged fermentation by marine *B. subtilis* JK-79 was determined in the unoptimized base medium, that is, wheat bran medium and the optimized OFAT and RSM medium. This was carried out to understand the fold increase in L-glutaminase production in the optimized medium as compared to the unoptimized medium. From the Figure 16, it is evident that about 2.88 fold increase in glutaminase production was obtained by sequentially optimizing the various components of the medium.

Several authors have reported an increased fold of L-glutaminase production under SSF by the application of RSM. Sathish and Prakasham (2010) found that a mixed substrate (66:34) of bengalgram husk and wheat bran optimized by simplex centroid design resulted in a significant improvement in the L-glutaminase yield by *B. subtilis* RSP-GLU.

L-glutaminase production by *Zygosaccharomyces rouxii* under SSF was studied by Iyer and Singhal (2010a, b). The authors employed a CCD to investigate the effect of variables such as moisture content, glucose,

Table 9. ANOVA for the quadratic model.

Source	Sum of squares	df	Mean square	F Value	p-value Prob > F	Significance
Model	5.731E+005	7	63708.49	136.55	< 0.0001	significant
A-Moisture content	28577.87	1	28577.87	61.25	< 0.0001	
B-pH	6158.53	1	6158.53	13.20	0.0046	
C-glutamine	23305.19	1	23305.19	49.95	< 0.0001	
AB	189.35	3	189.35	0.41	0.5384	
AC	10483.52	1	10483.52	22.47	0.0008	
BC	130.25	1	130.25	0.28	0.6008	
A^2	1.263E+005	1	1.263E+005	270.61	< 0.0001	
B^2	3.585E+005	1	3.585E+005	768.41	< 0.0001	
C^2	1.050E+005	1	1.050E+005	225.04	< 0.0001	
Residual	4665.66	10	466.57			
Lack of Fit	3844.25	5	768.85	4.68	0.0578	Not significant
Pure Error	821.42	5	164.28			
Cor Total	5.780E+005	19				

Std. Dev., 20.38; mean, 431.72; C.V. %, 5.00; PRESS, 30287.14; R-squared, 0.9919; Adj R-squared, 0.9847; Pred R-squared, 0.9476; Adeq precision, 31.546.

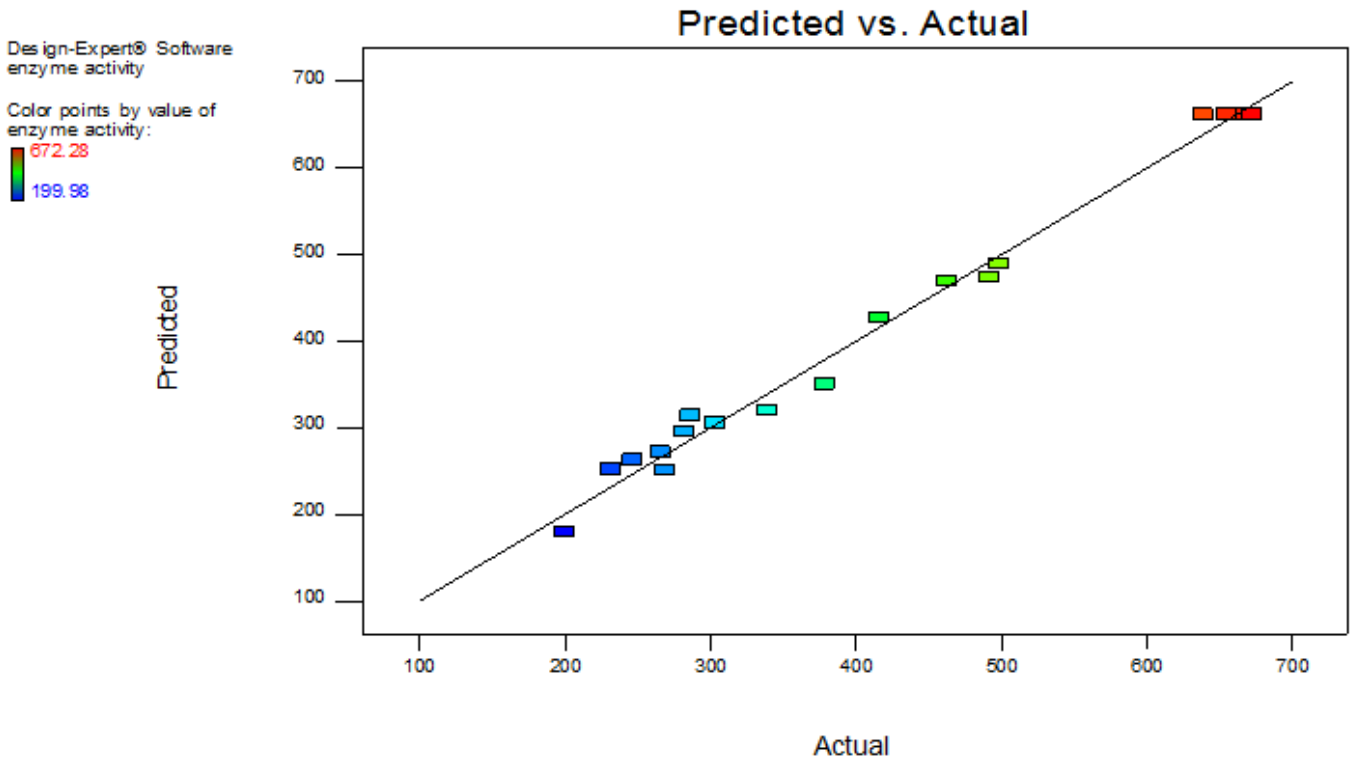

Figure 14. Plot between predicted and actual response of L-glutaminase production under SSF.

corn steep liquor and glutamine on glutaminase production and found a fourfold increase in L-glutaminase production. Nathiya et al. (2011) adopted CCD to obtain the best possible combinations for enhanced production of L-glutaminase by *Pencillium brevicompactum*. Under the optimal conditions, the experimental yield of L-glutaminase was 136.33 U/mg which is in close agreement with the value predicted by the model. However, Sayed (2009) and Sameera and Raju (2015) employed OFAT alone to attain a 2.2 and 4

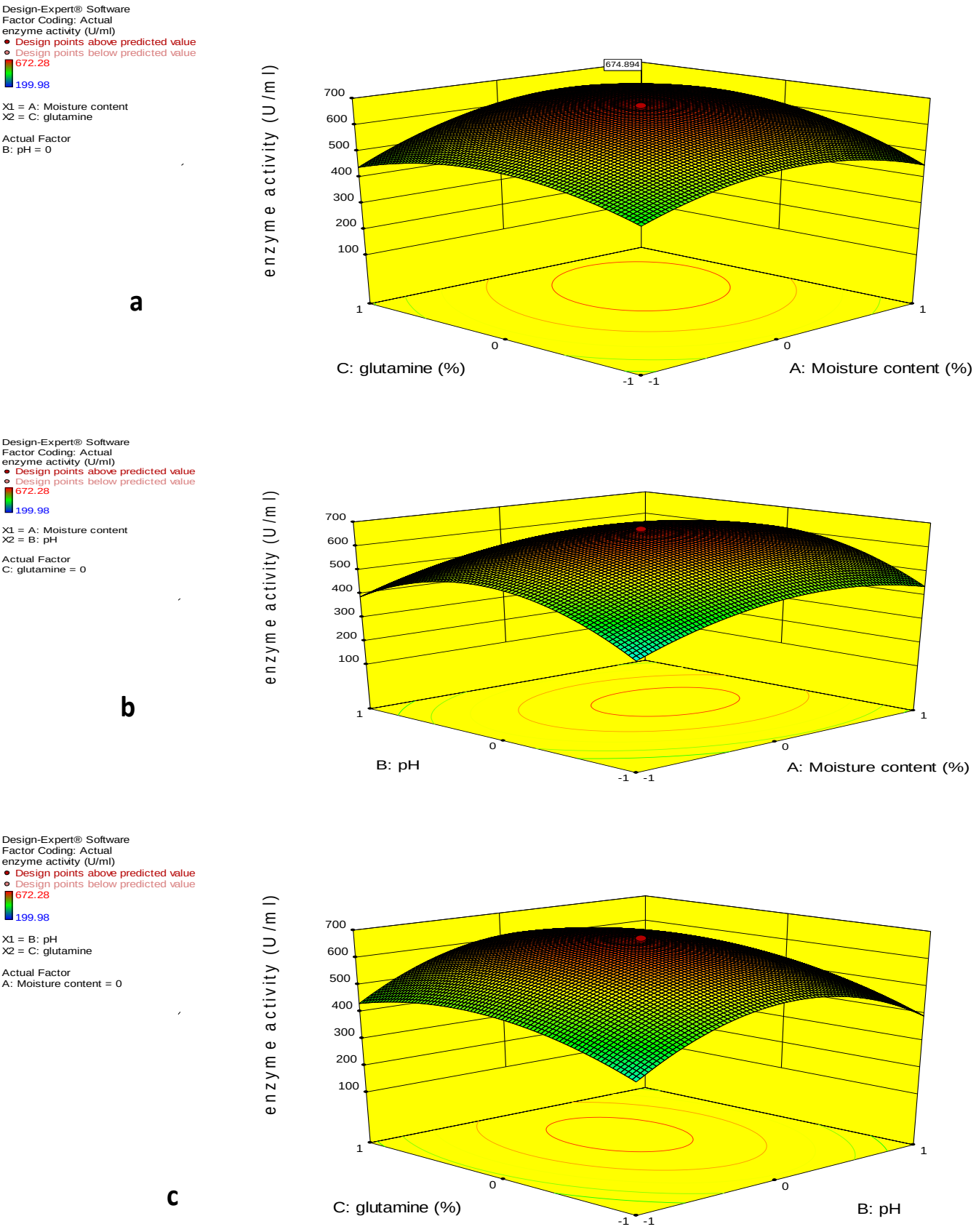

Figure 15. 3D response surface plots showing the interaction between two variables considered in the optimization with other two variables at their central point. a) Response behavior of moisture content and glutamine concentration under constant level of pH; b) Response behavior of moisture content and pH concentration under constant level of glutamine; c) Response behavior of glutamine and pH concentration under constant level of moisture content.

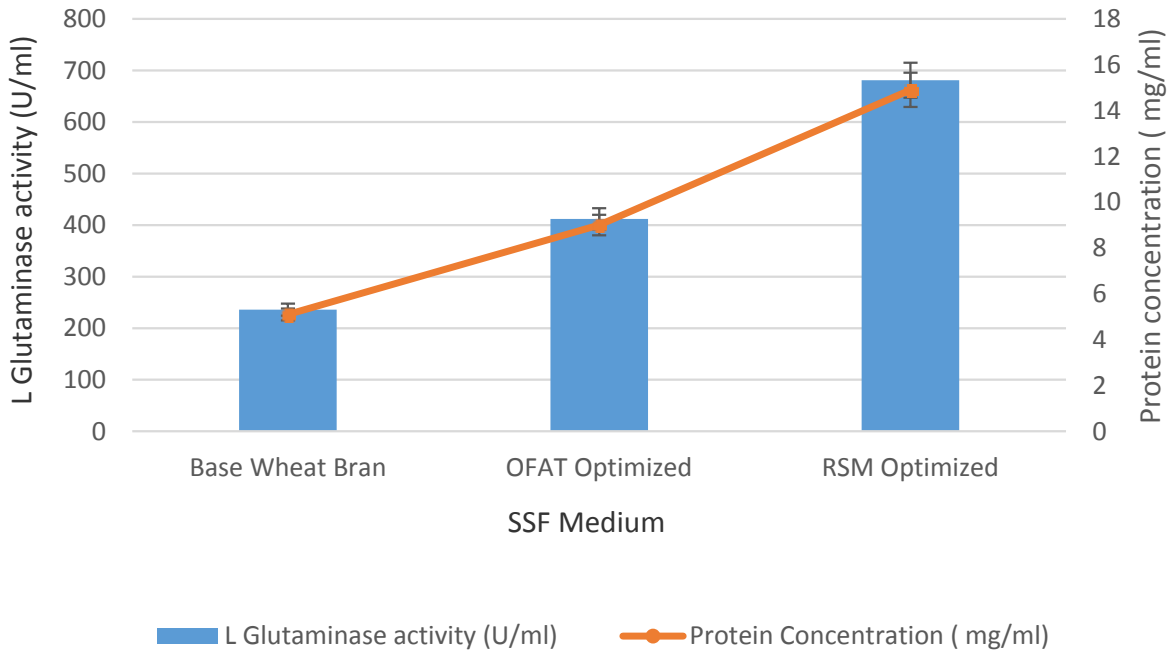

Figure 16. Comparision of L-glutaminase production by marine *Bacillus subtilis* JK-79 in basal and optimized SSF medium.

fold increase in L-glutaminase production under SSF by *T. koningii* and *Aspergillus wentii* MTCC 1901, respectively.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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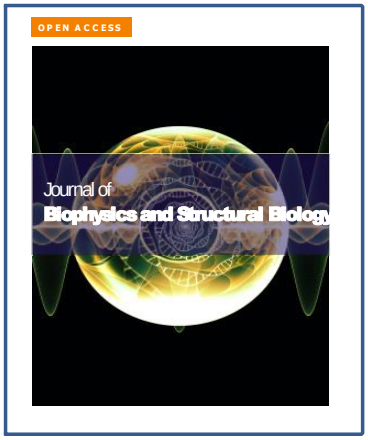

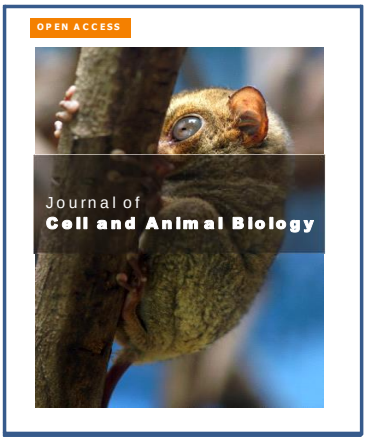

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