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The Effect of Penicillin on the Vitality of Bull Spermatozoa

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Abstract

Antibiotic supplementation into semen extenders is an important way to control several microorganisms that can affect semen quality by their presence. The objective of the present work is to estimate the effect of two different concentrations (300 µg/mL and 600 µg/mL) of penicillin on the selected quality parameters of spermatozoa collected from bulls (motility, mitochondrial activity, acrosome integrity and membrane integrity) after 0, 2 and 24 h of *in vitro* culture. Sperm motion was examined using HTM IVOS computer-aided sperm analysis (CASA), cell viability was assessed with the metabolic activity (MTT) assay. The acrosomal integrity was evaluated following the fast green – rose bengal staining protocol and the eosin – nigrosin staining method was used to assess the functional integrity of the sperm membrane. Our results indicate that penicillin at lower amount significantly ($p>0.05$) decreased the sperm motility, mitochondrial activity and membrane integrity after 24 h of *in vitro* culture. Supplementation of higher doses of this substance led to a significant decrease of the sperm motion during 0, 2 ($p>0.05$) as well as after 24 h ($p>0.01$), of the viability after 2 h ($p>0.05$) and 24 h ($p>0.01$), of the acrosomal integrity after 24 h ($p>0.05$) and of the membrane integrity at 24 h ($p>0.001$) too. We can consider, that the effect of penicillin addition to bovine spermatozoa during *in vitro* incubation is time and dose dependent.

1. Introduction

Semen quality may be highly challenged by the presence of bacteriospermia. Microorganisms cause lower reproductive functions of males, by agglutination of motile sperm, by alternations in cell morphology and by reducing the occurrence of acrosome reaction (**Azawi and Ismaeel, 2011**). Natural mating or artificial insemination (AI) are a way for transmission of bacteria that can negatively affect the reproductive function of females and decrease the fertility rates (**Akhter *et al.*, 2007**). Semen samples are usually collected from the farm animals using an open-ended artificial vagina. This process can be easily compromised with bacterial contamination, which eventually causes lower semen quality during storage and contaminates the female reproductive tract (**Yániz *et al.*, 2010**). The presence and the multiplication of the bacteria in semen may deteriorate the semen samples during *in vitro* culture (**Moretti *et al.*, 2009**).
Over the last 60 years bovine semen samples have been extended in the presence antibiotics to control bacterial growth. Bacterial populations that could be present in semen samples are controlled by antibiotics present in semen extenders (**Boonthai *et al.*, 2015; Avilés *et al.*, 2019**). The most common combination of antibiotics added to bovine semen are penicillin and streptomycin, followed by gentamycin, tylosin, lincomycin and spectinomycin (**Visser *et al.*, 1999**), but some microorganisms may have a resistance to them (**Morrell and Wallgren, 2014**).

There is currently limited data regarding the effectiveness of antibiotics on the semen sample quality. The aim of this study was to investigate the effect of two different concentrations of penicillin during *in vitro* culture on the bovine reproductive cells.

2. Material and methods

2.1 Semen sample collection and processing

Semen samples (n=20) were collected from four adult Holstein Friesian breeding bulls (Slovak Biological Services, Nitra, Slovak Republic). Ejaculates were collected regularly (once a week for five consecutive weeks) from each bull using an artificial vagina. After the collection, semen concentration and motility were measured by phase-contrast microscopy (200 x). For further experiments, we chose ejaculates with required quality (min. 70 % motility and concentration of 1 x 10^9 sperm/mL). Institutional and national guidelines for the care and use of animals were followed, and all experimental procedures were approved by the State Veterinary and Food Institute of Slovak Republic (no. 3398/11-221/3) and Ethics Committee.
Samples were diluted in PBS (Dulbecco's Phosphate Buffer Saline without calcium chloride and magnesium chloride; Sigma Aldrich) containing various concentrations of the penicillin (300; 600 µg/mL) using a dilution ratio of 1:40. The samples were cultured at laboratory temperature (22-25°C). After

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culture periods of 0, 2, 24h, sperm motility, membrane integrity, acrosome integrity and mitochondrial activity were assessed in each group

2.2 Spermatozoa motility analysis

Spermatozoa motion parameters were evaluated using the computer-aided sperm analysis CASA; Version 14.0 TOX IVOS II.; Hamilton-Thorne Biosciences, Beverly, MA, USA). The system was set up as follows: frame rate – 60 Hz; minimum contrast – 20; static head size – 0.25 – 5.00; static head intensity – 0.40 – 2.00; static elongation – 20 – 100; default cell size - 4 pixels; default cell intensity – 40. Ten µL of each sample were placed into the Makler counting chamber (depth 10 µm, 37 °C; Sefi Medical Instruments, Haifa, Israel) and spermatozoa motility (MOT; percentage of motile spermatozoa; motility > 5 m/s; %) was evaluated immediately. Ten microscope fields were subjected to each analysis to include at least 300 cells **(Tvrdá *et al.*, 2018).**

2.3 Eosin nigrosin staining

The functional integrity of the sperm membrane was assessed using the eosin - nigrosine staining method. This technique is based on the ability of eosin to penetrate into non – viable cells **(Moskovtsev and Librach, 2013)**. Five microliters of each sample were placed on tempered glass slide, mixed with 10 µL 5 % eosin (Sigma – Aldrich), followed by 10 µL 10 % nigrosine (Sigma – Aldrich). The mixture was smeared on glass slide and left to air – dry at 37 °C. The slides were observed using bright field microscopy at x 1000 using oil immersion. At least 200 sperm per slides were identified as either dead (red heads) or live (white heads), and expressed as a percentage rate. All slides were evaluated blindly by one observer.

2.4 Acrosomal integrity

The fast green – rose bengal staining protocol designed by **Pope *et al.* (1991)** was used to evaluate the acrosomal status of spermatogenic cells. This single – step staining method applies a mixture consisting of 1 % fast green (Sigma - Aldrich), 1 % rose Bengal (Sigma – Aldrich) and 40 % ethyl alcohol (Centralchem, Bratislava, Slovak Republic) in 0.1 M citric acid – 0.2 M disodium phosphate buffer (Sigma – Aldrich). Twenty microliters of the thawed sample were mixed with 20 µL of the staining solution and incubated for 70 s at room temperature. Ten microliters of the mixture were smeared on the tempered glass slide and air – dried at 37 °C. Acrosomal integrity was assessed using bright field microscopy at x 1000 using oil immersion. At least 200 cells per slide were evaluated for the presence or absence of acrosome, and expressed as a percentage rate. All slides were followed blindly by one observer.

2.5 Mitochondrial activity (MTT test)

Mitochondrial activity of the bovine reproductive cells was assessed using the colorimetric metabolic activity (MTT) test, which is based on the conversion of a yellow tetrazolium salt (3 – (4,5 – dimetylthiazol – 2 – yl) – 2,5 – diphenyltetrazolium bromide; MTT) to blue formazan particles by mitochondrial succinate dehydrogenase of intact mitochondria within living cells. The tetrazolium salt (Sigma – Aldrich) was dissolved in PBS (Dulbecco's Phopshate Buffer Saline without calcium chloride and magnesium chloride; Sigma – Aldrich) at 5 mg/mL. Ten µL of the tetrazolium solution was mixed with each sperm suspension. After a 2 h incubation (shaker, 37 °C, 95 % air atmosphere, 5 % CO_2), the formazan crystals were dissolved in 80 µL of acidified (0.08 mol/L HCl; Centralchem) isopropanol (Centralchem). Optical density was determined at a wavelength of 570 nm against 620 nm as reference using a Multiskan FC microplate photometer (Thermo Fisher Scientific Inc., Waltham, MA, USA). Data are expressed as percentage of the control set to 100 % (**Knazická *et al.*, 2012).**

2.6 Statistical analysis

Statistical analysis was carried out using the GraphPad Prism program (version 3.02 for Windows; GraphPad Software, La Jolla, CA, USA, http://www.graphpad.com). Initially, descriptive statistical characteristics were evaluated. One-Way ANOVA with the Dunnett's test were used for specific statistical evaluations, based on the assumption that values in each row represent paired observations. The level of significance was set at $***p < 0.001$, $**p < 0.01$, $*p < 0.05$.

3. Results

Our experiment focused on the *in vitro* effects of penicillin on four quality parameters of bovine sperm. All assessments we performed at 0, 2 and 24 h using working solutions with 600, 300 µg/mL of penicillin administration.

The bovine sperm motility was performed using the CASA system. According to our results we can say, that higher concentration of penicillin (600 µg/mL) had a negative effect by decreasing of activity of the sperm cells at 0 and 2 h ($p>0.05$) as well as after 24 h ($p>0.01$) of *in vitro* culture. The lower dose addition had a similar effect, after 24 hours ($p>0.05$) when compared to the control group (Figure 1).

The second measured parameter was the mitochondrial activity, as mitochondria are the source of energy for the movement of the reproductive cells. At the beginning we did not register any significant changes. At 2 h, the higher applied concentration, significantly decreased ($p < 0.05$) mitochondrial viability. After 24 h of incubation, we observed a significantly decreasing mitochondrial activity ($p < 0.05$; $p < 0.01$) at both of the given doses (Figure 2).

The eosin – nigrosin staining was used as a method to assess membrane integrity of the bovine spermatozoa. Penicillin addition showed a decreasing membrane integrity at 2 h, but after 24 h of *in vitro* culture, administration of this substance led to a significantly decreased membrane integrity at the lower dose ($p < 0.05$) and at the higher dose ($p < 0.001$) as well (Figure 3).

The evaluation of the acrosomal status was prepared by the fast green - rose bengal staining method. Our results indicate, that exposure to penicillin led to a significantly decreased acrosome integrity at a higher dose application at 2 h ($p < 0.05$) and after 24 h ($p < 0.05$) (Figure 4).

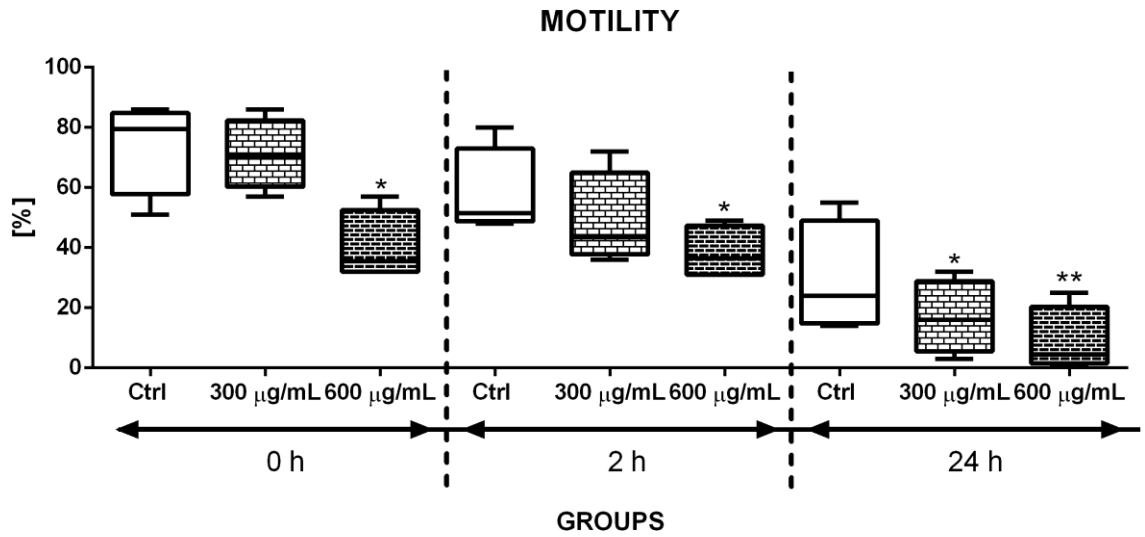

Figure 1. The effect of various concentrations of the penicillin on the motility of bovine spermatozoa at 0 h, 2 h and 24 h. The level of significance was set at * $p < 0.05$; ** $p < 0.01$.

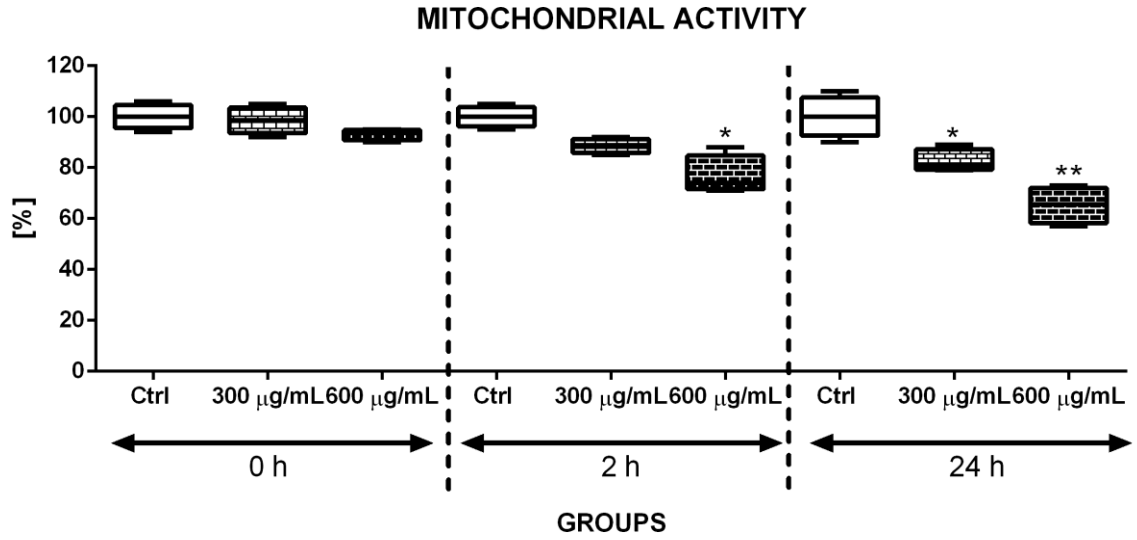

Figure 2. The effect of various concentrations of the penicillin on the mitochondrial activity of bovine spermatozoa at 0h, 2 and 24 h. The level of significance was set at * $p < 0.05$; ** $p < 0.01$.

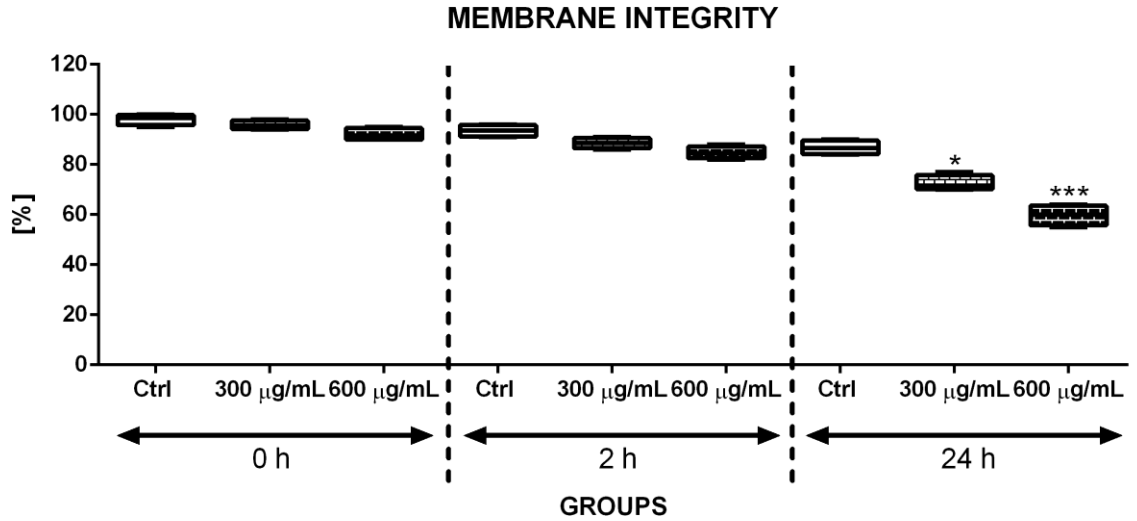

Figure 3. The effect of various concentrations of the penicillin on the motility of bovine spermatozoa at 0 h, 2 h and 24 h. The level of significance was set at * $p < 0.05$; *** $p < 0.001$.

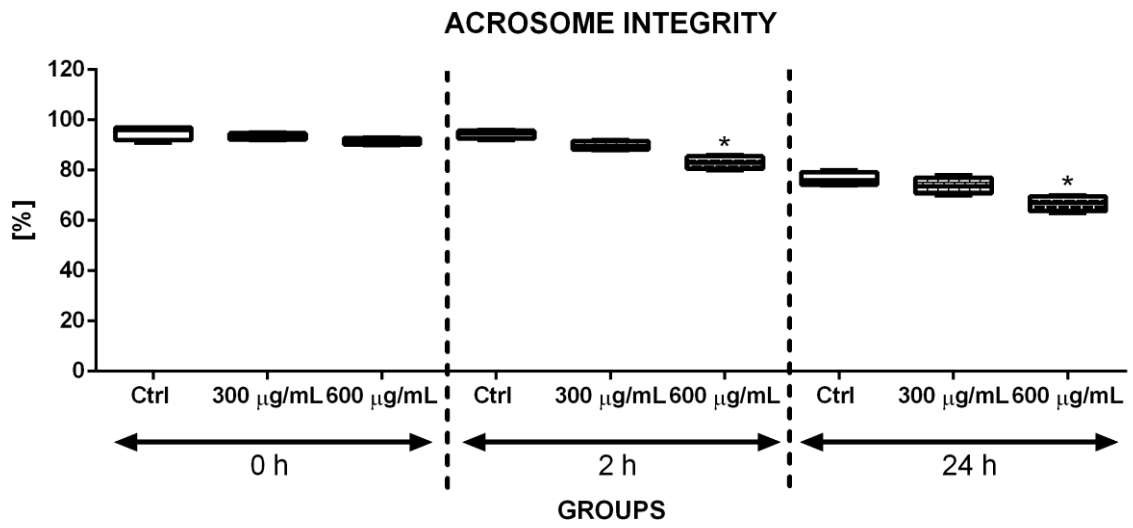

Figure 4. The effect of various concentrations of the penicillin on the motility of bovine spermatozoa at 0 h, 2 h and 24 h. The level of significance was set at * $p < 0.05$.

4. Discussion

In our study we found negative effects of penicillin. The same results were observed by **Stoss *et al.* (1978)**. They applied penicillin and streptomycin to rainbow trout semen, leading to bacterial growth inhibition, but with a detrimental effect on the spermatozoa too. Furthermore, **Schlegel *et* al. (1991)** monitored the effect of therapeutic doses of penicillin G and cephalothin on mammals. The authors recorded a gonadotoxic effect of both agents expressed as spermatogenesis arrest by this 8-day antibiotic treatment in rats.

Inversely, **Miraglia *et al.* (2003)** compared four antibiotics used to inactivate leptospires in Holstein Friesian bull semen. During their experiment, egg yolk citrate (EYC) extender was used with different concentrations of antibiotics. After 24 h there were no significant effects on the progressive motility or individual progressive motility of spermatozoids, but penicillin-streptomycin addition showed the best way to inactivate leptospires.

The first study focused on the effect of this antibiotic on male gametes was published by **Almquist *et al.* (1946).** Their experiment showed that the addition of penicillin into diluted semen of bulls with relatively high breeding efficiency did not cause any significant changes in the quality of bull semen. Subsequently, **Almquist (1949)** tested the impact of penicillin on the fertility of spermatozoa from relatively infertile bulls. Surprisingly the report showed that the administration of this agent in different amounts (500 and 1000 units per ml of extender) highly significantly increased the fertility of the inseminated cows. This conclusion was explained by the fact, that bulls with quite lower fertility might contain types of bacteria which can cause breeding difficulties, and penicillin may raise efficiency of the bovine semen only by eliminating the microorganisms which cause a disbalanced internal milieu for semen viability.

Alavi-Shoushtari *et al.* (2007) investigated the effects of various antibiotics on controlling bacterial contaminations in tris-egg yolk diluted buffalo bull semen. Each semen sample was exposed to the recommended and twice the recommended dose of antibiotic. The next step was to find out the impact of these additives on the buffalo spermatozoa diluted in the same extender (tris-egg yolk +glycerol) after freezing and thawing. They summarized, that penicillin G revealed to be an effective substance at both doses against bacterial growth and proved to be suitable as an additive to the semen extenders. The discrepancies between their report and our data may be explained by the fact that our results were measured at laboratory temperature (22-25 °C) during 24h, revealing no positive effects of the antibiotic on the bull semen quality parameters.

On the other hand, **Oplinger and Wagner (2015)** studied the ability of penicillin and streptomycin to reduce bacterial coldwater disease in Rainbow Trout semen. After 15 min exposure of these antibiotics in extender mixed with spermatozoa, the bacterial amount was reduced and the sperm motility was not changed. But, during production-scale trials, the authors observed a reduction of fertilized egg production by stored sperm in the antibiotic containing extender. This decreasing ability of fertilization can be caused by the penicillin content what finally points to the negative effect of this antibiotic on the basic semen parameters such as sperm motility, acrosome integrity, mitochondrial activity and membrane integrity as was showed in our results. On the other hand, the effects of penicillin and streptomycin sulphate were examined by **Rahimi *et al.* (2016)**. They focused on the application of these two antibiotics to semen samples gained from caspian brown trout during 12 days short-term storage at 4°C. After the experiment, the results showed, that the percentage and duration of semen viability were significantly higher in the samples enriched with antibiotic than in the antibiotic free group. They concluded that application of these antibiotics may improve the viability of caspian brown trout during short-term storage.

The effects of antibiotics on the sperm survival have become a fruitful topic in animal andrology. **Aurich and Spergser (2007)** investigated the impact of gentamicin addition to the semen extender on the motility, velocity and membrane activity. Addition of this antibiotic during cooled storage decreased the motility and velocity and may negatively affect the sperm functions in extended semen samples.

While our study showed a negative effect of penicillin, **Akhter *et al.* (2007)** tried to find out the effect of two antibiotic combination (streptomycin and penicillin; SP) (gentamycin, tylosin, lincomycin and spectinomycin; GTLS) in extender on the bacterial control and sperm quality of liquid bovine semen stored at 5 °C. After three days of storage at 5 °C, there were no differences in the sperm motility, longevity and plasma membrane integrity, but after five days, the measured semen parameters were significantly better in extender containing SP compared to GTLS and control group without antibiotic addition. The same conclusion was determined by **Dissanayake *et al.* (2014)**. The objective of their study was to detect the effect of penicillin and streptomycin in the elimination of bacteria and to

the human sperm motility, survivability and pregnancy rates. They found that this antibiotic combination did not seem to have any harmful effects on human reproductive cells.

Tylosin is an antibiotic belonging to macrolides. **Slanina *et al.* (2016)** examined the effects of tylosin on selected motion parameters of turkey spermatozoa during short-term *in vitro* incubation at 41 °C. Their results indicated, that tylosin did not have any negative effects on the motility parameters of the sperm samples. Finally, **Sone *et al.* (1982)** reported that after seven days of storage at 15 °C, about 80 % of the semen samples were without any bacterial growth and the mean amounts of the motility and normal acrosomes were 75.4 % and 82.7 %, in the presence of dibekacin.

5. Conclusion

In our study we focused on the determination of the *in vitro* effect of the penicillin on male reproductive markers: sperm motility, mitochondrial activity, membrane integrity and acrosomal integrity. The higher added concentration of the antibiotic (600 µg/mL) significantly decreased the motility as well as mitochondrial activity after 2 h. After 24 h both of the applied doses showed a detrimental effect on the viability and sperm metabolism of the bovine spermatozoa. The protective dose (300 µg/mL) significantly reduced the membrane integrity, same as the higher dose did, after 24 h of incubation. The acrosome integrity was significantly lower after 2 h as well as after 24 h in the presence of the higher applied dose. We may conclude that penicillin addition, as an antibiotic control against bacteriospermia, can significantly inhibit the selected parameters, revealing its potential negative effect on the bovine reproductive cells.

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Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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Comparative Studies of Phytochemical and Antimicrobial Activity oc *Carica papaya* L. Extracts against *Escherichia coli*, *Staphylococcus aureus* and *Candida albicans*

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Abstract

Carica papaya extracts are known for their traditional medicinal uses. The ability of its parts to control the growth of common pathogens in the laboratory has been tested in different parts of the world using different varieties of *C. papaya*. This study was initiated to compare the phytochemical and antimicrobial activity of different plant parts extracts of *C. papaya* var. papayi GAV4 on *Escherichia coli*, *Staphylococcus aureus* and *Candida albicans*. *C. papaya* plant parts were collected from a farm in Kiboswa (Kisumu): coordinates 0.0245°S and 34.7474°E, and then were transported to Maseno University Botany Laboratory. Seeds, green leaves and bark were washed thoroughly with tap water, rinsed in sterile water and dried after which they were ground using a grinder. From each of the three plant parts, three types of extracts were prepared using water, acetone and ethanol in the concentrations 25%, 50%, 75% and 100%. The antimicrobial activity of the extracts was tested on microbes growing on agar plates by inoculation with the different concentrations using diffusion method and replicated 3 times. Extracts were isolated using Soxhlet apparatus and MIC determined by serial dilution, zone of inhibition was measured in millimeters. Means from the measurements were separated and compared at significance level P = 0.05. Phytochemicals present included alkaloids, flavonoids, tannins, phenols, saponins, glycosides, anthocyanins and terpenoids while anthraquinones were absent. Ethanol bark extract on *C. albicans* showed higher inhibition and there were significant differences in inhibition among the plant parts and extracts used. In concentrations used, 25% was significantly different from 50%, 75% and 100%. The results obtained in this study confirm that C. papaya has antimicrobial activity on *E. coli, S. aureus* and *C. albicans*; and has also shown high potentials for use as a potential source of antibiotics to treat diseases caused by these microorganisms.

1. Introduction

In traditional system of medicine, plant preparations in the forms of decoctions, concoctions, macerations, or infusions are used to treat a wide range of diseases (**Tsobou *et al.*, 2016**). Current estimates indicate that about 80 million people worldwide still depend on plants for their health needs (**Dwivedi *et al.*, 2020**). Limited access to primary health care in developing countries has resulted into widespread use of herbal medicines due to the availability, accessibility, affordability and cultural acceptance across different ethnic backgrounds (**Muhwana *et al.*, 2020**). There is widespread use of broad-spectrum antibiotics which has led to the emergence of nosocomial infections caused by drug resistant microbes (**Abubakar, 2009**). Multi drug resistance and the presence of several virulence factors in the strains of many pathogens responsible for different diseases pose an increasing threat to disease treatment. There are several varieties of this plant spread throughout the world. Papaya also known as pawpaw (*Carica papaya* Linn) is commonly known for its nutritional and medicinal values throughout the world (**Alabi *et al.*, 2012**). It is a giant herbaceous non woody plant resembling a tree from the family *Caricaceae* (**Akujobi *et al.*, 2010).**

Each part of papaya tree possesses economic value when it is grown on a commercial scale (**Krishna et al., 2008**; **Orchue and Momoh, 2013**). Even though the active compounds are normally extracted from all parts of the plant, the concentration of these compounds varies from structure to structure (**Aruljothi *et al.*, 2014**). However, parts known to contain the highest concentration of the principles are preferred for therapeutic purposes and it can either be the leaves, stem, barks, roots, bulks, corms, rhizomes, woods, flowers, fruits, and the seeds (**Kafaru, 1994; Emitaro *et al.*, 2020**). Various parts of the papaya plant, which include the leaves, fruit, seed, latex, and root, are known to contain bioactive compounds that contribute to reported medicinal properties (**Anibijuwon and Udeze, 2009**; **Aravind *et al.*, 2013**).

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In Kenya there exists more than 65 varieties of *C. papaya* [**Asundu *et al.*, 2010**], yet most *C. papaya* farmers around Kiboswa (Kisumu) grow the *C. papaya* L. var. papayi GAV4 variety. It was therefore easy to obtain *C. papaya* L. var. *papayi* GAV4 plant materials for these studies. Extracts from different varieties of the same plant may respond differently to bioassay tests conducted on microorganisms due to varying physiological and chemical characteristics where they may occur (**Nirosha and Mangalanayaki, 2013**; **Jyotsna *et al.*, 2014**; **Brij *et al.*, 2015**). Therefore, evaluating *C. papaya* var. papayi GAV4 extracts for possible microbial control of *E. coli, S. aureus* and *C. albicans* was appropriate because research work carried out in other parts of the world was only conducted on other varieties of *C. papaya* (**Nirosha and Mangalanayaki, 2013**; **Jyotsna *et al.*, 2014**; **Brij *et al.*, 2015**). It is imperative therefore that before this study, little was known about the antimicrobial effect of bark, leaf and seed extracts of *C. papaya* var.papayi GAV4.The choice of *C papaya* var. papayi GAV4 was guided by earlier findings that showed that it is the most abundant variety in Kiboswa (Kisumu) (**Asundu *et a*l., 2010**).

2. Material and methods

2.1 Study area

After identification of the plant (Figure 1) was conducted using a taxonomic key (**Cowan, 1999**), the materials used in this study were collected from a farm located at Kiboswa (Kisumu)within the geographical coordinates 0.0245°S and 34.7474°E. All plant parts including; fruits, leaves and bark were then transported to the botany laboratory at Maseno University (Maseno Kenya) located within the geographical coordinates 0.0°0S and 34.36°E where analysis was conducted.

Figure 1. *C papaya* var. papayi GAV4tree with ripe (yellow) and unripe (green) fruits.

2.2 Preparation of seed extract

Seeds were obtained when fruits earlier acquired from Kiboswa (Kisumu County), and taken to Maseno University botany laboratory were washed with clean tap water, rinsed in sterile distilled water and cut open using a kitchen knife, then left to dry for 30 days at 25^0 C at the Botany Laboratory, Maseno University (**Jyotsna *et al.*, 2014**). The seeds were then powdered using a grinder to produce a fine powder (Figure 2).

Figure 2. *C. papaya* var. papayi GAV4 seed powder.

2.3 Preparation of leaf extract

Disease free green leaves of *C. papaya* var. papayi GAV4 earlier collected from Kiboswa were washed in tap water, rinsed in sterile distilled water, dried at 25°C for 20 days before being grounded into a green powder using a grinder to produce a fine powder (**Jyotsna *et al.*, 2014**) (Figure 3).

Figure 3. *C. papaya* var. papayi GAV4 leaves powder.

2.4 Preparation of bark extract

Diseases free pawpaw bark were cut from the tree using a sharp kitchen knife, washed in tap water, rinsed in sterile distilled water then dried at 25°C for 30 days before being ground to produce a brown powder using a grinder (**Jyotsna *et al.*, 2014**) (Figure 4).

Figure 4. *C. papaya* var. papayi GAV4 barkpowder.

2.5 Ethanol extraction of seed, leaf and bark extracts

One hundred grams of powdered dried seeds, leaf and bark were weighed using a weighing machine and powder transferred into 500 ml glass conical flasks as earlier described by **Okunola *et al.* (2012**). Ninety five percent of 500 ml ethanol was measured and poured onto the conical flask containing the seed, leaf and bark powder and stirred to produce mixtures that were allowed to stand for 24 hours before decantation and filtration through a Whatman filter paper No 1. (**Okunola *et al.*, 2012**). The resulting filtrates were concentrated in a rotary evaporator at 79^0C resulting in concentrates that were stored in the refrigerator at 4^0C until required for use.

2.6 Water extraction of seed, leaf and bark extracts

One hundred grams of powdered dried seeds, leaf and bark extracts were transferred into 500 ml glass conical flask into which two hundred millimeters distilled water was poured then stirred to produce mixtures that were allowed to stand for 24 hours before decantation to produce filtrates using a Whatman filter paper No 1.

2.7 Acetone extraction of seed, leaf and bark extracts

One hundred grams of powdered dried leaves, seed and bark extracts were transferred into 500mls glass conical flask into which five millimeters of 95% acetone was poured then stirred to obtain mixtures that were allowed to stand for 24 hours before decantation and filtration through Whatman filter paper No 1. resulting in filtrates that were concentrated with a rotary evaporator at 45^0C to produce concentrates that were later stored in the refrigerator at 4^0C until required for use.

2.8 Test microorganisms

The test organisms that were used in this study were pure strains of human pathogenic organisms of clinical origin obtained from Centre for Disease Control (KEMRI/CDC) located in Kisian (Kisumu) and maintained on Mueller Hinton Agar (Oxoid, UK) medium as stock cultures in the laboratory refrigerator set at 4 ^{0}C.The isolates included pure strains of gram negative bacteria *E. coli (ATCC 25922)* gram positive bacteria *S. aureus (ATCC 25923)* and an imperfect yeast *C. albicans (ATCC 1405)*. The *C. albicans* strain was grown in Nutrient agar (NA) media, *E. coli* grown in Nutrient agar (NA) media while the S. *aureus* was grown in Potato Dextrose agar (PDA).

2.9 Phytochemical compounds extraction and screening

Twenty-five grams of dried leaves, seed and bark powder were extracted in Soxhlet apparatus by using 25 ml of solvent having polarity of ethanol for 48hrs and then concentrated by evaporation. These prepared extracts were used for phytochemical screening for alkaloids, flavonoids, tannin, phenols, saponin, terpenoids, anthraquinones, Cardiac glycosides and anthocyanins as earlier described by **Mibei *et al.* (2012)**; **Musyimi *et al.* (2007)**; **Musyimi *et al.* (2017)**; **Opande *et al.* (2017)**; **Akinyemi *et al.* (2005)** and **Opande et al. (2017)**.

2.9.1 Determination of alkaloids
Two grams of the extract were extracted by warming it for 2 minutes with 20ml of 1% H_2SO_4 acid in a 50ml conical flask on a water bath, with intermittent shaking. It was then centrifuged and the supernatant was pipetted off into a small conical flask. One drop of Meyer's reagent was added to 0.1ml supernatant in a semi micro tube. A cream precipitate indicated the presence of alkaloids.

2.9.2 Determination of flavonoids
5ml of dilute aqueous ammonia solution were added to a portion of the plant extract followed by addition of concentrated sulphuric acid. Positive test was indicated by yellow colouration which disappeared on standing.

2.9.3 Determination of tannins
About 0.5 g of the dried powdered samples was boiled in 20ml of water in a test tube and then filtered through Whatman No. 42 filter paper. A few drops of 0.1% ferric chloride was added. A brownish green or a blue-black coloration indicated the presence of tannins.

2.9.4 Determination of phenols
Ferric chloride test was carried out where the extract was diluted to 5ml with distilled water. To this, a few drops of neutral 5% Ferric chloride solution was added. A dark green or a blue-black color indicated the presence of phenolic compounds.

2.9.5 Determination of saponins
About 2 g of the powdered sample was boiled in 20ml of distilled water in a water bath and filtered. Ten milliliters of the filtrate were mixed with 5ml of distilled water and shaken vigorously to form a stable persistent froth. The froth was mixed with 3 drops of olive oil and shaken vigorously, and then was observed for the formation of emulsion.

2.9.6 Test for anthraquinones
Powdered plant material was boiled with 10% HCl for a few minutes, then filtered and allowed to cool. This was then partitioned against equal volume of chloroform. Formation of rose-pink color upon addition of 10% aqueous ammonium solution, indicated the presence of anthraquinones.

2.9.7 Test for Cardiac glycosides
Five ml of extract was treated with 2ml of glacial acetic acid containing a drop of $FeCl_3$ solution. This was then underplayed with 1ml conc. H_2SO_4. A brown ring of the interface indicated a deoxy-sugar characteristic of cardenolides.

2.9.8 Test for Anthocyanins
2ml of 2M sodium hydroxide (NaOH) solution was added to few extracts in a test tube. The formation of blue-green colour compound confirmed the presence of anthocyanins.

2.9.9 Test for terpenoids
5ml of the extract was mixed with 2ml of chloroform followed by addition of 3ml of concentrated sulphuric acid to form a layer. Positive test was indicated by formation of a red colouration at the interface.

2.10 Antimicrobial susceptibility test for bacteria

The disc diffusion method on Mueller Hinton agar (**Yahaya *et al.*, 2017**) was used to determine the antibacterial activity of the plant extracts. An overnight culture of the bacterium was diluted to 10^5 cells/ml using a spectrophotometer at a wavelength of 625 nm. One milliliter of the bacterial suspension was introduced into sterile petri dishes and 20 ml of Mueller - Hinton agar at 40^0C poured into the inoculated dishes before the plates were allowed to cool and solidify. A sterile filter circular discs, 8mm in diameter each were cut from Whatman No.1 filter paper

using a paper punch and each dipped in a known concentration of 25, 50, 75 and 100% of the extracts for about 2 minutes, then gently transferred to the centre of the inoculated agar media. Petri dishes inoculated with bacteria and fungi were kept for incubation for 24 hrs at 37^{0}C and 25^{0}C respectively. The diameter of inhibition zone was measured using 12.5 cm Vernier calipers. This was carried out in triplicates in a completely randomized design.

2.11 Determination of Minimum Inhibitory Concentration (MIC)

The MIC of the extracts was determined by using Muller-Hinton broth dilution (**Anibijuwon and Udeze, 2009**) made and sterilized using an autoclave. Serial dilutions of the extract in liquid medium were prepared and 1.0 ml of the prepared broth dispensed into the test tubes labeled from 1 to 4 using sterile syringe and needle. A stock solution containing 100mg/ml of the extract was prepared. 1.0 ml of the solution was dispensed into the tube 1. Subsequently, from tube 1 solution was serially transferred until 4.0-1.0 ml of the solution was discarded from it. An overnight culture of each of the test isolates was prepared in sterile nutrient broth. 1 ml inoculum was transferred into each tube from tube 1 to tube 4. The final concentrations of the extract in each of the test tubes after dilution i.e. 100, 50, 25 and 12.5 mg/ml was incubated at 37°C for 24 hrs and examined for emergent growth.
To measure the MIC values, various concentrations of the stock, 25, 50, 75 and 100 mg/ml were assayed against the test bacteria where the minimum inhibitory concentration was defined as the lowest concentration able to completely inhibit any visible microorganism growth after overnight incubation with media (**Prescort, 1999; Yahaya *et al.*, 2017**).

3. Results

3.1 Phytochemical screening

Information on the phytochemical constituents of plant materials are generally required for the discovery of novel drugs. The phytochemical screening of *C. papaya* plant materials carried out revealed the presence of alkaloids, flavonoids, tannins, terpenoids, anthraquinones, phenolic compounds and saponins in the extracts of leaf, seed and bark (Table 1), while anthraquinones were not detected in the seed and bark extracts.

Table 1. Screening for secondary metabolites in plant ethanol extracts of *C. papaya*

Phytochemical grouping	Leaves	Seeds	Bark
Alkaloids	+	+	+
Flavonoids	+	+	+
Tannins	+	+	+
Phenols	+	+	+
Saponins	+	+	+
Anthraquinones	+	-	-
Cardiac glycosides	+	+	+
Anthocyanins	+	+	+
Terpenoids	+	+	+

Key: + Present; - Absent

3.2 Determination of the effect of *C. papaya* seed, leaf and bark extracts on growth of *C. albicans, E. coli* and *S. aureus*

The results obtained when the effect of *C. papaya* bark on growth of *E. coli, C. albicans* and *S. aureus* was conducted, indicated that the highest zone of inhibition was demonstrated against *S. aureus* was 9.82 mm by ethanol extracts of dried bark, while the lowest zone of inhibition was demonstrated against *C. albicans* measured 0.89mm as shown by water extracts of dried bark (Table 2). There was no visible inhibition exhibited by *C. albicans* on the acetone extracts, yet when comparison was made for the 3 extracts, there were significant differences among the extracts (Table 2).

Table 2. Diameter of zone of inhibition (mm) exhibited by *C. papaya* var. papaya GAV 4 bark extracts against *C. albicans, E. coli and S. aureus*

Test Microorganism	Ethanol extract	Water extract	Acetone extract
E. coli (ATCC 25922)	7.16a	5.96b	6.29b
C. albicans (ATCC 1405)	8.84 a	0.89b	0 c
S. aureus (ATCC 25923)	9.82 a	3.80 b	5.13 c
LSD at **P=0.05**	**0.35**		

Data presented are the means of three replicates. Means with the same letter down the same column are not significantly different at P=0.05.

Figure 5. Zone of inhibition for water extracts of bark on E. coli, and C. albicans

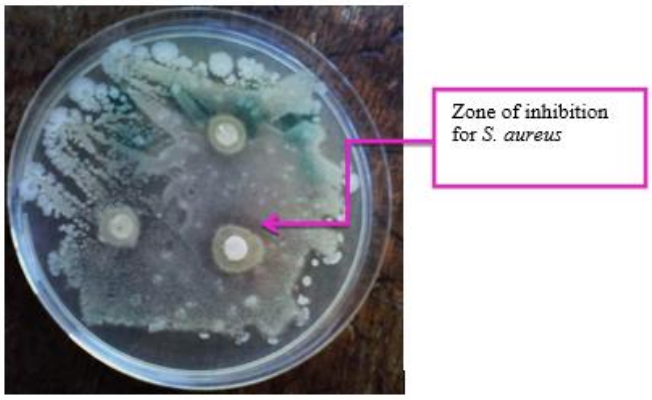

Figure 6. Zone of inhibition for water extracts of bark on *S. aureus.*

The effect of *C. papaya* water bark extract on growth of the 3 microorganisms (Figure 5 & 6) indicate that even the water extract has visible inhibitory effects against the growth of *E. coli, C. albicans* and *S. aureus* in the Petri dishes. Yet the effect of *C. papaya* seed extract on growth of *E. coli, C. albicans* and *S. aureus* when measured indicated that the highest zone of inhibition was demonstrated against *E. coli* at 8.13 mm by ethanol extract of dried seeds (Table 3). The lowest zone of inhibition was demonstrated against *E. coli* at a measurement of 5.09 mm by the acetone extracts of dried seed (Table 3). There was no inhibition at all for the acetone extract of *C. albicans* and *S. aureus.* There appeared to be no significant differences among the extracts.

Table 3. Diameter of zone of inhibition (mm) exhibited by *C. papaya* var. papayi GAV4 seed extracts *against E. coli, C. albicans* and *S. aureus*

Microorganism	Ethanol	Water	Acetone
E. coli (ATCC 25922)	5.13 a	4.51 b	2.97 c
C. albicans (ATCC 1405)	7.27 a	5.22 b	0 c
S. aureus (ATCC 25923)	8.87 a	5.78 b	5.93c
LSD at **P=0.05**	**0.27**		

Data presented are the means of three replicates. Means with the same letter down the same column are not significantly different at P=0.05.

Table 4. Diameter of zone of inhibition (mm) exhibited by *C. papaya* leaf extracts against *E. coli, C. albicans* and *S. aureus*

Microorganism	Ethanol	Water	Acetone
E. coli (ATCC 25922)	8.13 a	5.09 b	5.22 b
C. albicans (ATCC 1405)	7.8a	6.29b	0 c
S. aureus (ATCC 25923)	8.11 a	5.49b	0 c
LSD P=0.05	**0.27**		

Data presented are the means of three replicates. Means with the same letter down the same column are not significantly different at P=0.05.

When the effect of C. *papaya* leaves extract on growth of *E. coli, C. albicans* and *S. aureus,* was compared, the highest zone of inhibition was demonstrated against *S. aureus* with a measurement of 8.87 mm by ethanol extract of dried leaves (Table 3). The lowest zone of inhibition was demonstrated against *E. coli* with a measurement of 2.97 mm by the water extract of dried seeds. There was no inhibition for the acetone extracts on *C. albicans* and *S. aureus* (Table 4). The results obtained indicated that there were significant differences among the extracts (Table 4).

The disc diffusion method on Mueller Hinton agar was used to determine the antimicrobial activity of *C. papaya*var. *papaya* GAV4 leaf, seed and bark extracts with different concentrations (Table 5). As shown in table 5, increase in the concentration has different effects on the microorganism, plant part and extract used. It is shown that ethanol, acetone and water leaf extracts did not inhibit the growth of *C. albicans* and acetone leaf extracts did not inhibit the growth of *S. aureus*. At 25% concentration, there was no inhibition of growth of *E. coli* by ethanol, water and acetone leaf extracts and water seed extracts.

3.3 Determination of Minimum Inhibitory Concentration (MIC)

Table 6 shows Minimum Inhibitory Concentration (MIC) of various extracts of leaf, seed and bark extracts of *C. papaya* var. papayi GAV4 on the microorganisms, the minimum inhibitory concentration was defined as the lowest concentration able to inhibit any visible bacterial or fungal growth, the MIC varies with the microorganism, plant part and extract used.

4. Discussions

Plants are important source of potentially useful structures for the development of new chemotherapeutic agents. They synthesize bioactive compounds which are of great potential in agriculture, antimicrobial and anti-insect activity (**Emitaro *et al.,* 2020**). Phytochemical screening of the leaf, seed and bark extracts of the plant revealed the presence of flavonoids, alkaloids, saponins, tannins, phenolic compounds, glycosides and anthocyanins in agreement with other workers (**Ekaiko *et al*., 2015a; Ekaiko *et a*l., 2015b; Sikandar *et al*., 2013; Ayoola and Adeyeye, 2010**), anthraquinones were only present in leaves but absent in seeds and bark. The medicinal value of these plants lies in some chemical substances that produce a definite physiological action on the human body (**Musyimi *et al*., 2007**). For example, alkaloids isolated from plants have been found to have antimicrobial properties (**Sikandar *et al*., 2013**), and are one of the most efficient therapeutants that were isolated from the plant extracts during these studies. Flavonoids represent the common and widely distributed group of plant phenolics; their biological functions include protection against allergies, inflammations, platelets aggregation microbes, ulcer, viruses and tumors (**Okwu and Okwu. 2004**). The presence of tannins in the *C. papaya* can support its strong use for healing of wounds, ulcers, hemorrhoids, frost-bites and burns in herbal medicine (**Igboko, 1983**). Tannins have astringent properties which hasten the healing of wounds and inflamed mucous membrane (**Igboko, 1983; Maduinyi, 1983**).

The presence of phenolic compounds in the extracts of *C. papaya* shows that the extracts may have antimicrobial potential, because phenols and phenolic compounds have been extensively used in disinfections and remains the standard with which other bactericides are compared (**Oakenful, 1981**).The presence of saponins supports the fact that *C. papaya* extracts may have cytotoxic effects (**Okwu and Okwu, 2004**; **Okigbo *et al*., 2009**). Saponins exhibit broad range of pharmacological actions, such as ability to heal wounds and inflamed mucous membranes. Therefore, in view of the occurrence of phytochemicals in the extracts it is more appropriate to state that the antimicrobial activity of the *C. papaya* extracts may be attributed to the presence of the bioactive compounds.

Table 5. Effect of different concentrations of leaf, seed and bark extracts of water, ethanol and acetone on the growth (mm) **of** *E. coli, C. albicans* and *S. aureus.*

Plant part	Extract	Microorganism	Concentration mg/ml			
			25	**50**	**75**	**100**
Seed	Ethanol	*E. coli (ATCC 25922)*	11.33 a	10.56 b	5.78 c	8.11 d
		C. albicans (ATCC 1405)	10.78 a	10.67 a	10.56 a	12.22 b
		S. aureus (ATCC 25923)	14.22 a	12b	11.78 b	11.11c
	Water	*E. coli (ATCC 25922)*	0 a	8.11 b	8.89 c	8.67 d
		C. albicans (ATCC 1405)	8.67 a	9.44 a	9.67 a	8.56 b
		S. aureus (ATCC 25923)	10.78 a	12.44 b	11.89 b	9.22 c
	Acetone	*E. coli (ATCC 25922)*	9.60a	11.80 b	9.90 c	8.83 d
		C. albicans (ATCC 1405)	9 a	10.44 b	9.78c	9.78 c
		S. aureus (ATCC 25923)	9.78 a	10.00 a	10.89 b	9.89 c
Bark	Ethanol	*E. coli (ATCC 25922)*	8.78 a	6.44 b	7.56 c	7 d
		C. albicans (ATCC 1405)	0 a	0 a	4.44 b	0 b
		S. aureus (ATCC 25923)	0 a	0 a	9.33 b	9.67 c
	Water	*E. coli (ATCC 25922)*	5.56 a	5.8 a	5.33 b	5.22 b
		C. albicans (ATCC 1405)	9.44 a	0 b	7.78 c	8.70 d
		S. aureus (ATCC 25923)	0 a	10.11 b	10 b	8.78 c
	Acetone	*E. coli (ATCC 25922)*	6.78 a	6.60 a	5.75 b	6.22 c
		C. albicans (ATCC 1405)	8.44 a	7.78 b	7.44 c	7.88 d
		S. aureus (ATCC 25923)	2.78 a	9.78 b	10.89 c	4d
Leaf	Ethanol	*E. coli (ATCC 25922)*	10.33 a	9.89 b	5.78 c	5.44 c
		C. albicans (ATCC 1405)	0 a	0 a	0 a	0 a
		S. aureus (ATCC 25923)	6.67 a	3.56 b	2.71b	5.51c
	Water	*E. coli (ATCC 25922)*	0 a	6.44 b	0 c	8.44 d
		C. albicans (ATCC 1405)	0 a	0 a	0 a	0 a
		S. aureus (ATCC 25923)	10.56 a	10.67 a	0b	8.44 c
	Acetone	*E. coli (ATCC 25922)*	0 a	8.56 b	8.33 b	9.22 c
		C. albicans (ATCC 1405)	0 a	0 a	0 a	0 a
		S. aureus (ATCC 25923)	0 a	0 a	0 a	0 a

Data presented are the means of three replicates. Means with the same letter across the same column are not significantly different at P=0.05.

Table 6. Minimum Inhibitory Concentration (MIC) of various extracts of leaf, seed and bark extracts of *C papaya* on *E. coli, C. albicans* and *S. aureus*

Microorganism	Plant part	Minimum Inhibitory Concentration MIC (mg/ml)		
		Water	**Ethanol**	**Acetone**
E. coli (ATCC 25922) P=0.05	Bark	0.1	0.05	0.1
	Seed	0.1	0.05	0.025
	Leaf	0.05	0.025	0.1
C. albicans (ATCC 1405) P=0.05	Bark	0.05	0.1	0.05
	Seed	0.05	0.1	0.05
	Leaf	-	-	-
S. aureus (ATCC 25923) P=0.05	Bark	0.025	0.025	0.025
	Seed	0.025	0.025	0.025
	Leaf	0.025	0.05	-

Key: - No inhibition

4.1 Inhibitory effects *C.papaya*var.papayi GAV4seed, leaf and bark extracts on *E.coli, C.albicans* and *S.aureus*

The confirmed presence of bioactive substances in these extracts is very important, such substances has been reported to confer resistance to plants against bacteria, fungi and other microorganisms, this therefore may explain the reasons for the demonstrated antibacterial activity by the plant extracts used in this study. Antimicrobial properties infer that any of these properties: i.e. anti-bacterial (antibiotics), anti-fungal (anti-mycotic), anti-cancerous (anti-oncogenic) or anti-viral is inherent (**Baskaran *et al*., 2012**).

The present study has clearly shown that the different parts of *C. papaya* possess antimicrobial potential against *S. aureus*, *E. coli* and *C. albicans.* In line with the present findings, several other studies have reported other varieties of *C. papaya* leaves (**Kafaru, 1994**; **Rahman *et al*., 2011**) have antimicrobial potentials. Additionally, the reports of other workers (**Yahaya *et al*., 2017**) have also shown that other varieties of *C. papaya* leaves and stem barks have significant antibacterial activity in extracts from different tree parts. Other workers concur with our findings that *C. papaya* had significant antibacterial activity (**Nirosha and Mangalanayaki, 2013**; **Douhari *et al*., 2007**). The gram-negative bacteria display some particularities that inhibit antibiotics penetration, as the lipopolysaccharide layer that determines the permeability and susceptibility to antibiotics.

In the antimicrobial test for bacteria, it was observed that the potency of the activity of *C. papaya* depends on the extraction solvent used; organic extracts such as ethanol were more effective than *C. papaya* in aqueous extracts may be as a result of the better solubility of the active components in organic solvents. The ethanol extracts clearly demonstrated a higher activity than the acetone and water extracts, the better efficacy of the ethanol extract against the acetone and extract may be because different solvents have different polarities, hence different degrees of solubility of the various phytoconstituents (**Rahman *et al*., 2011**).Based on the limited spectrum of activity of the other extracts compared with the ethanol extracts, it suggests that the active component is more soluble in ethanol than in the other solvents. This is in agreement with **Aruljothi *et al*. (2014)** and **Ekaiko *et al*. (2015a)** whose findings can be attested to other works by **Okunola et al. (2012)** that reported the effect of *C. papaya* on similar microorganisms including *Klebsiella pneumonia, Enterococcus* and *Proteus* spp. However, these results are in disparity with others (**Sumathi and Gowthami, 2014**) who reported that the zone of inhibition was observed only in leaf extracts.

4.2 Minimum inhibitory concentration of extracts on *E. coli, C. albicans* and *S. aureus*

The results obtained during this study have clearly shown that the MIC values varied from 0.025-0.1mg/ml for the three extracts. Lowest MIC value 0.025mg/ml was recorded against *E. coli* and *S. aureus* where against *C. albicans* the lowest MIC observed was 0.05mg/ml. these results indicates significant antimicrobial potential of extracts. High minimum inhibitory concentration observed for *Candida albicans,* high MIC may be an indication of low efficacy or that the organism has higher potential for developing resistance to the bioactive compounds in the plant, which is said to be related to a thick layer in their outer membrane which prevents the entry of inhibition substances (**Chima *et al*., 2016**). High MIC may also mean that a higher concentration of the extract is required to inhibit the organism's growth.

The low MIC value observed for *E. coli* and *S. aureus* is a good indication of high efficacy against these microorganisms. This also means that lower concentration of the extract is required to inhibit the organism's growth. On the other hand, disparity in Minimum Inhibitory Concentration may be due to variable sensitivity to the chemical substances related to different resistant levels among strains.

5. Conclusions

Carica papaya var. papayi GAV4 leaves, seeds and bark contain alkaloids, saponins, tannins, glycosides, phenols, terpenoids, anthocyanins and flavonoids. Anthraquinones were absent in seeds and bark. This plant extracts showed antibacterial and antifungal activities against *S. aureus, E. coli* and *C. albicans,* thus an indication that the plant can be a potential source for production of drugs with a broad spectrum of activity. Additionally, the Minimum inhibitory concentration for *S. aureus* and *E. coli* was 0.025mg/ml while that of *C. albicans* was 0.05mg/ml.

Further pharmacological evaluations, toxicological studies and possible isolation of the therapeutic antibacterial substances from plants are some of the future challenges that will be faced by workers studying new substances with antimicrobial properties. *C. papaya* var. papaya GAV4 may be recommended as a useful source to prepare natural bioactive products from which we can develop new antimicrobial drugs which will be cost-effective. We suggest that in the search for new pharmaceuticals substances, screening of various natural organic compounds and the identification of active agents must be considered as a fruitful approach.

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Competing interests

The authors declare that they have no competing interest.

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Aflatoxins: A Brief Review of their Chemical Properties, Toxicological Effects and Control Measures

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Abstract

Aflatoxins are toxic secondary metabolites produced by the fungi of the genus *Aspergillus*. These substances cause food poisoning with clinical manifestations that vary according to the time of exposure and concentration of the dose ingested, representing a serious public health problem for compromising the food security, also causing considerable economic losses both in the production of stocked vegetable foods, as well as in the livestock contaminated with these substances through the feed. Therefore, this literature review aims to introduce some aspects related to the contamination of food by the fungi of the genus *Aspergillus*, the chemical and toxicological properties of the aflatoxins, as well as the strategies of control to avoid them in food.

1. Introduction

The provision of food containing nutrients and devoid of food hazards is an essential factor for the establishment and maintenance of the health and quality of life of the population. However, often in the food production, there are critical points during the processes of raw matter processing, packaging, transport, and storage that can favor the food contamination by physical, chemical, or biological hazards, highlighting that the microbial contamination can be mitigated or enhanced by factors intrinsic to the food, or pertinent to the conditions of the environment of the processes mentioned above. **(Vågsholm *et al*. 2020)**.

In this context, many foods of plant origin are susceptible to microbial contamination by fungi that produce toxic substances called mycotoxins. And this situation represents an economic problem due to the deterioration of foods by the fungal growth and release of metabolites that turn their use in the human and animal nutrition unfeasible, generating economic losses reinforced by the sanitary barriers imposed on the international market; and also a public health concern due to the harmful effects that these substances can cause in the long term (**Pereira; Santos, 2011; Baquião, 2012; Sacramento,2016; Sousa, 2018**).

Therefore, this work aims to expose, through a literature review, the biological aspects of the genus *Aspergillus*, the chemical and toxicological properties of the main group of mycotoxins produced by this genus - the aflatoxins-, and also expose the main strategies of control and prevention of aflatoxins in foods.

2. Material and methods

For the development of this work, we conducted an online literary survey with the terms aflatoxin and *Aspergillus* that resulted in the obtaining of 70 works, being 28 selected, including 12 articles, 5 thesis, 2 monographs, and 1 book. As selection criteria, we adopted the pertinence to the theme and the economic viability regarding the strategies of control of aflatoxin in foods.

The results obtained are presented in an explanatory way, according to the methodology described by **Cooper (1988),** presenting a focus on the analysis of the literature about the chemical properties of the aflatoxins, their toxicological effects, and control measures, aiming to raise data that provide subsidies for future works of applied nature. Therefore, this review is directed to the public of the agrarian, biological, and health sciences.

3. Results and discussion

The genus *Aspergillus*

The genus *Aspergillus* belongs to the group of the Hyphomycetes, which is characterized by the formation of specialized hyphae and conidia with variable shapes and architecture. It is a genus strongly associated with the deterioration of dry foods that are hardly attacked by other microorganisms that require higher water activity, also deteriorating foods stored in improper conditions of drying and storage (**Santos, 2008**). This fungic genus can develop and produce toxic secondary metabolites called mycotoxins, which can be contained inside the spores and

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mycelia, and then be released into the food contaminated by the fungi of this genus (**KwiatkomoskI; Alves, 2007**).

Among the mycotoxins produced by the genus *Aspergillus,* it is possible to mention the aflatoxins, penicillic acid, citrinine, sterigmatocystin, ochratoxin, and patulin, which are substances that, depending on the dose, can cause acute, subacute, or chronic intoxications, mainly affecting the liver, and also possessing the potential to cause or induce cancer. Such toxins are produced mainly by the species *Aspergillus flavus*, *Aspergillus melleus*, *Aspergillus niveus*, *Aspergillus rugulosus*, *Aspergillus ochraceus*, and *Aspergillus clavatus*, respectively; emphasizing that the same toxin can be also produced by more than one species of *Aspergillus* (**Diniz, 2002**).

The identification of this genus is easily accomplished by analyzing their main morphological features, which are the formation of colonies with white mycelium, and spores that may present a coloration that varies from green to yellow, gray, brown, or black; possessing at the microscopical level hyaline hyphae with septa (**Morais, 2018**), and the presence of conidiophores that generally present an enlarged structure called vesicle in which a layer of sterile cells called metula are present giving support to the cells that produce the spores, which can be arranged in rows (phialides) over the *Aspergillus* "head" covering it totally or just at the top (**Francisco, 2017**).

The classic microbiological identification for this genus and its species is carried out through morphological analysis and the growth profile of the fungus in the media recommended by the literature, such as CYA (Agar Czapek Yeast Extract - K_2HPO_4 1.0g, Czapek Concentrate 10 mL, 1 mL metallic solution, Yeast Extract 5.0g, Agar 15.0g, Sucrose 30.0 g, and distilled water 1000 mL), MEA (Agar Malt extract - Malt extract 20.0g, Peptone 1.0 g, Glucose 20.0g, Agar 20 and distilled water 1000 mL), and the BDA medium (Potato Dextrose Agar - Potato infusion 4.0g, Dextrose 20.0g, Agar 15g, and 1000 mL of water) with an incubation period of approximately 7 days at the appropriate temperature (**Monteiro, 2012; Sia, 2012; Gava, 2002**).

Since the great majority of the fungi are mesophiles, with an optimal growth temperature between 20 ° C and 30 ° C, the genus *Aspergillus* can be easily distinguished from other molds because it is thermotolerant, managing to germinate their spores at temperatures above 37 ° C, presenting an optimal temperature ranging from 30°C to 40 ° C (**Poester *et al*., 2015; Shabo, 2014**).

Although its morphological characteristics have great value in the classification and taxonomy, the genus can also be identified by analyzing the profile of the secondary metabolites produced, and molecular biology techniques that allow the obtaining of results in a short time with great precision (**Monteiro, 2012**).

Aflatoxins

Mycotoxins are fungal products that are dependent on the occurrence of a consecutive series of reactions catalyzed by enzymes that lead to their production, being accepted that their biosynthesis is the result of accumulations of metabolic intermediaries in the fungi primary metabolism, which in order to maintain the primary pathways operating, performs deviations of the intermediates in excess for the production of mycotoxins (**Dias, 2018**).

Being the aflatoxins mycotoxins produced by the genus *Aspergillus* usually detected in foods containing glucose, sucrose, and fatty acids at temperatures between 25 and 30 ° C, mostly in foods from tropical countries due to the high temperature, such as peanuts, rice, beans, corn, barley, and almonds such as the Brazilian nuts (**Dias, 2018; Lopes, 2012 Gonçalves *et.al*, 2017**).

The aflatoxins are classified in groups designated by the letters B, G, and M, in which the letter B is derived from the Blue fluorescence emitted by the substances of the group B when exposed to UV radiation, the letter G is derived from the Green fluorescence that the aflatoxins of the group G emit when exposed to UV radiation; and the letter M designates the aflatoxins mainly found in the Milk (**Baquião, 2012**).

The most common aflatoxins in foods are the aflatoxins B1, B2, G1, and G2, where those of the group of B are produced mainly by the fungi of the species *A. flavus* and *A. parasiticus*, while those of the group G are produced by the species *A. parasiticus*, being the aflatoxins M1 and M2 derived from the animal metabolism over the toxins of the group B, and they affect humans through the consumption of milk or meat contaminated by the urine of animals exposed to the fungal toxins of the group B, or from animals whose food was made with raw matter contaminated by B aflatoxins of the group (**Moreira, 2018**).

Generally, when isolated, the aflatoxins are crystalline, colorless or yellowish, insoluble in non-polar solvents (except chloroform), and moderately soluble in polar solvents such as methanol, and dimethyl sulfoxide, presenting a solubility of approximately 10-20 μg. mL^{-1} in water; they also are substances extremely resistant to the heat (thermostable) that start their degradation process at temperatures in the order of 220 ºC (**Pierezan, 2013; Cruz, 2010**).

The aflatoxins can be differentiated by 1) their low molecular weight, in which the aflatoxin B1 has a molecular mass of 312 g. mol^{-1}, the aflatoxin B2 has a molecular weight of 314 g. mol^{-1}, the aflatoxins G1 and M1 328 g. mol^{-1}, and the aflatoxins G2 and M2 have a molecular weight of 330 g.mol^{-1}; and 2) by their melting points, in which the aflatoxin B1 has a melting point of 269 ºC, the aflatoxin B2 has a melting point in values ranging between 286-289 ºC, the aflatoxin G1 changes from the solid to the gaseous state at temperatures between 244-246 ºC, aflatoxin G2 at temperatures between 237-240 ºC, aflatoxin M1 at values of 299 ºC), and the aflatoxin M2 melts at a temperature of 293 ºC (**Pierezan, 2013**).

In structural terms, it can be noted that the difference between the aflatoxins B1 and B2 is due to a double bond present in the position C15, differentiating both from the aflatoxins belonging to group G by the presence of a second cyclic ester between C3 and C4, with a double bond at the position C15 in the aflatoxin G1, and its absence in the aflatoxin G2 (**Bordini *et al*., 2013**); the same difference is also found in the toxins of the group M concerning the C15 position, differentiating them from the others by the hydroxylation after the hepatic metabolism in the C14 position. Figure 1 shows the structures of aflatoxins B1, B2, G1, G2, M1, and M2.

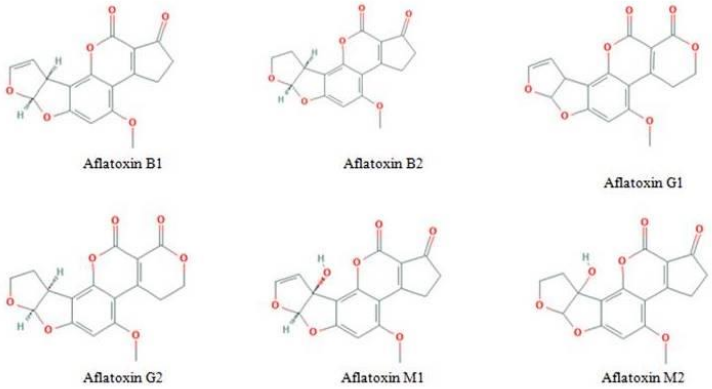

Figure 1. Molecular structure of the aflatoxins. **PubChem (2020)**

Aflatoxins can be detected by various analytical methods, such as gas chromatography (GC), high-performance liquid

chromatography (HPLC), thin-layer chromatography (CCD), and ELISA (**Baquião, 2012**).

Mycotoxicosis

The poisoning caused by mycotoxins is called mycotoxicosis, and their main effects on the human and animal organism are the production of injuries to the liver, kidney, system nervous system, and behavioral changes, as well as a high potential, to induce cancer. Highlighting that the mycotoxins present as the main forms of access to the organism the oral, inhalation, and topical routes, where the oral and inhalation routes are the commonest in cases of intoxication, respectively through the consumption of contaminated food, or by inhaling spores. (**Gonçalves *et al*., 2017; Pereira; Santos, 2011**).

The mycotoxicoses caused by aflatoxins receive the name of aflatoxicosis, and they tend to present in humans a degree of severity that depends on variables such as age, nutritional status, pre-existing disease, dose, and the manifestation of the symptoms is strongly dependent on the dosage and time of exposure, where high doses cause acute intoxication with the occurrence of damage in the liver, changes in the digestion, absorption of nutrients, edema, and hemorrhages that can happen along with a high chance of evolution to death in a short time; being the development of hepatic cancer common in cases of chronic intoxications due to low exposure for a long time. (**Pereira; Santos, 2011; Vitorino, 2011; Rosmaninho, *et al*., 2001**).

In animals, the main symptoms are reduced growth, motor, and behavioral changes, as well as reduced or loss of the reproductive capacity with progression to death, and it is often difficult to identify this type of intoxications because the adverse effects tend to be sub-clinical (**Gonçalves *et al.*, 2017; Vitorino, 2011; Rosmaninho, *et al*., 2001**).

Considering the effects of aflatoxins over the animal production, **Pierezan *et al.* (2012)** report that the ingestion of aflatoxin B1 in the feeding and water of calves in concentrations of 1.250 ppb, 2.500 ppb, and 5.000 ppb resulted in diarrhea and profound weight loss a few days after the beginning of the administration. And **Michellin (2014)** reports that the administration of aflatoxin B1 to fishes of the genus Astyanax in the feed at concentrations of 10 µg.kg-1, 20 µg.kg-1, and 50 µg.kg-1 can directly affect humans through the food route because this toxin accumulates in the muscle tissue and liver of the fishes.

In other animals, **Oliveira *et al.* (1997)** report the development of liver cancer after prolonged administration of doses of aflatoxin B1 ranging between 15-1000 µg.kg-1 to rats, while **Guterres *et al.* (2017)** report that prolonged exposure of dogs to the same aflatoxin through the feeding in concentrations between 100-300 ppb resulted in vomiting, diarrhea, anorexia, melena, coagulopathies, jaundice, and sudden death.

Such studies demonstrate the severity of the effects of the aflatoxins on food as a public health problem, and its potential to generate significant economic losses in animal production. Therefore, it reinforces the importance of the theme and development of research seeking strategies to fight the fungal contamination in food, as well as the contamination by aflatoxins.

Control methods

The foods most susceptible to contamination by mycotoxigenic fungi are those that present favorable conditions for this to occur, where the intrinsic and extrinsic factors inherent to the food influence the development of these microorganisms, emphasizing that all they act in a combined manner (**Pereira et al., 2002**). Highlighting that the fungal contamination can occur during the harvesting, transportation, and storage, mainly due to the lack of proper hygienic-sanitary conditions that consequently favors the contamination by mycotoxins after fungal development (**Schabo, 2014**). What makes relevant the application of strategies of control to avoid this problem through preventive or corrective (**Sassi, 2015; Lopes, 2012**).

The preventive forms of control can be applied in the pre-harvest and the post-harvest period, wherein the pre-harvest, actions such as the correct irrigation, protection against insects, the supply of nutrients to the plants, biological control of diseases, crop rotation, elimination of residues can reduce the susceptibility of the plants against the fungal infection, and improve the quality and safety of the harvest. While in the post-harvest, measures such as harvesting in the dry weather, the removal of the damaged grains, adequate drying conditions (below 10%), proper temperature and humidity control during the storage, pest control, and cleaning of the processing and storage facilities can avoid the contamination of the food by fungi in general, and consequently prevent the contamination by mycotoxins (**Lopes, 2012**).

While the corrective actions employ methods whose purpose is to degrade the mycotoxins in the food and reduce their concentration to an acceptable level through approaches that use physical, chemical, or biological agents.

In the physical approaches, the foods are exposed to physical agents such as the sunlight, high temperatures in the process of roasting, wet grinding, and radiation as the ultraviolent, and the gamma radiation, highlighting that this last physical agent is still under study. While in the chemical methods, the foods are exposed to substances such as ammonia, hydrogen peroxide, sodium bicarbonate, ozone; and the biological approaches can be exemplified by the use of lactic acid bacteria fermentation that reduces the levels of aflatoxin in some foods, and the use of adsorbents during the livestock production that prevents the absorption of mycotoxins by the animals, therefore reducing their toxicity (**Lopes, 2012; Moreira *et al.*, 2018**).

4. Conclusion

The aflatoxins constitute a public health problem, mainly because they can affect many people in the population, and their signs and symptoms are often confused with many diseases, also compromising the production of livestock and food, causing losses at different scales.

In this review, different approaches for the detection, prevention, and correction of mycotoxin contamination in foods were exposed, being their cost of application variable depending on the size of the production and the financial resources of the producer. Where in general, the detection of mycotoxigenic fungi is the most viable measure because it directly indicates the presence of fungi in the production and also favors the detection of critical points where the hygienic-sanitary measures must be reinforced.

However, in cases where the fungal contamination has already happened, to avoid large losses of the production, the adoption of corrective measures is desired, but their costs may represent a limitation for producers who don't have the financial resources available to invest in the mitigation of the chemical hazard in the food.

Therefore, this work strongly suggests the adoption of preventive methods to avoid the deterioration of foods by fungi, as well as the contamination by mycotoxins. And in this context, it is also important that the academic community direct efforts to seek means to lower the costs of the corrective methods, making them more affordable for different scales of production to assure the safety of the food reaching the final consumer.

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Chemical Components and Antimicrobial Activity of Essential Oils of *Petiveria alliacea* Leaves Extracts

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Abstract

A steam distillation technique was employed to obtain oil from the leaf of *P. alliacea*. The oil obtained was subjected to GC/MS analysis to determine the chemical components, which showed the presence of sulphur heterocyclic compounds, 1,2,3-trithiolane (**3**), 1,2,5 trithiepane (**4**) and 1,2,5,6-tetrathiocane (**7**) as well as benzenecarbothioic acid (**8**) that have not been reported previously as components in the crude extracts of *Petiveria alliacea*. The crude extracts showed antimicrobial activity on the following microorganisms *Salmonella typhi, Staphylococcus aureus, Bacillus subtilis, Escherichia coli, Rhizopus sp., Aspergillus niger*. The tests showed that the extract was most effective at limiting the growth of *Salmonella typhi* and the *Rhizopus sp.* (MIC 3.125 µg/mL and MBC value of 6.25 µg/mL). The ethanol extract using the Soxhlet technique was the most effective on *Staphylococcus aureus, Escherichia coli, Rhizopus sp.,* and *Aspergillus niger (*MIC 3.125 µg/mL).

1. Introduction

Medicinal plants are of great importance to the health of individuals and communities. *Petiveria alliacea* is one such medicinal plant that has been used in different parts of the world, with numerous bio-active compounds **(Cseke et al. 2006)**. The leaf, stem and root decoctions of *Petiveria alliacea* have been employed as a diuretic, antispasmodic agents in traditional medicine and the treatment of cancer, diabetes, nervous disorders, respiratory and pulmonary infections, and as antirheumatic, antifungal, anti-HIV agents, and to enhance memory **(Silva et al. 2015)**. As a result of these uses, this plant has the potential to be applied in the treatment of several ailments **(de A Neves et al. 2011; Gomes et al. 2005; Kerdudo et al. 2015; Lopes-Martins et al. 2002; Lowe et al. 2015; Luz et al. 2016)**.
Some important and interesting compounds have been identified in the extracts from different parts of the plant, including phytol, dibenzyl trisulphide, coumarins, benzaldehyde, benzoic acid, isoarborinol, fredelinol, pinitol and allantonin have been detected in the extracts of various parts of *P. alliacea* **(Ayedoun et al. 1998; Castellar et al. 2014; de A Neves et al. 2011; de Andrade et al. 2012; Gomes et al. 2005; Kerdudo et al. 2015; Kim et al. 2006; Lopes-Martins et al. 2002; Oluwa et al. 2017; Randle et al. 2018; Sathiyabalan et al. 2014; Silva et al. 2018; Zavala-Ocampo et al. 2017)**.

2. Material and methods

Fresh leaves of *P. alliacea* were collected from Iju town in Ado-Odo/Ota local government area of Ogun State. The plant was identified by Dr. Jacob Popoola, Department of Biological Sciences, Covenant University, Ota, and authenticated at the Forestry Research Institute of Nigeria (FRIN). A voucher specimen with the taxonomic identification FHI number (FHI 112438) was deposited in the herbarium of University of Ibadan, Ibadan. The leaves were separated and chopped into small pieces, which were weighed, then first washed with tap water and then with distilled water. The leaves were then allowed to dry at room temperature over three (3) days, after which 480 g of the dried leaves were stuffed into a 1000-mL two-necked flask and 500 mL distilled water was added. The flask was placed on a 1-L heating mantle and steam distillation was carried out .over 4 h. The distillate was poured into the separating funnel and 40 mL of diethyl ether was added, the mixture was shaken. Two layers formed, the upper ether layer was then drained off into a 50-mL beaker and then concentrated on a rotary evaporator to obtain the essential oil.
The crude extracts were obtained from the leaves of *P. alliacea* by two different extraction methods namely, cold maceration and Soxhlet extraction (exhaustive and successive) using hexane and ethanol as solvents. For example a total of 1287.87 g powdered leaves samples was weighed and about 400. g portions of the leaves samples were placed in the thimble; 1.2 L hexane was measured and poured into the round bottom flask. The Soxhlet apparatus was set up then placed on the heating mantle. The procedure was carried out exhaustively until the

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extracting solvent was colourless. Further exhaustive extraction was carried out using ethanol solvent on the same leaves samples after the hexane solvent was allowed to dry. The extraction process was handled the same way as the hexane extraction.

For the cold extraction, 1 kg of the leaves sample of *P. alliacea* was weighed and placed in two separate tanks (one of hexane and one of ethanol). The tanks were then covered and left to soak for about 14 days. At the end of the two weeks the samples were decanted and rotary evaporator was used to get the crude extract out.

The antimicrobial analysis was conducted using the Micro-titre technique for the determination of antimicrobial activity using an indicator. Standard micro-tube dilution bio-assay of 96-well micro-titre plates was used to determine the end point, which was taken as the minimum inhibitory concentration (MIC) of the extract samples against the microorganisms. To each of well, 100 µL of sterile 1% glucose peptone water is placed. To well number 1, 100 µL of extract sample was placed and a serial doubling dilution was used to prepare the samples from well number 2 to well number 10. 100 µL broth culture of 0.5 McFarland turbid identified bacteria was added to all the dilution ranged from well number 1 to well number 10. Overnight broth culture of 100 µL was placed in well number 11 and 100 µL of 1% sterile glucose peptone water was added to serve as control while 100 µl of sterilised 1% glucose peptone was added to 100 µL sterile water in well 12 (blank). The plate was incubated at 37°C for 24 hours. After incubation, 10 µL of phenol red solution (0.025%) was added to each well and colour changes were detected to determine the MIC.

Gas chromatography-mass spectrometry analysis was carried out on the essential oil on GCMS-QP2010SE SHIMADZU instrument. The conditions for the GC analysis are as detailed here.

Column oven temperature was set at 60.0°C; injection temperature was 250.0°C; injection mode was Split with a split ratio of 1:1. The temperature programme was 60.0°C for 2 min then increased at the rate of 13.0°C/min to 260.0°C and held for 2 min. the flow control velocity was linear at 46.3 cm/s and the column flow rate was 3.22 mL/min. The injection quantity was 1 µL. For the mass spectrometry, the ion source temperature was 230.0°C. The solvent cut off time was 4.50 min, with a relative detector mode set at 1.38 kV and threshold value of 2000. The acquisition scan speed was set at 1428.

3. Results and discussion

The percentage yield of the crude extract from cold maceration in hexane and ethanol was 11.02% and 8.75% respectively while the Soxhlet extraction it was 54.75% and 40.42% in hexane and ethanol, respectively. The cold extraction yielded less extract compared to Soxhlet extraction. It was also noted that the use of hexane solvent gave better yield compared to that of using ethanol as solvent.

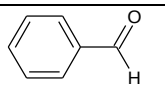
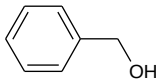
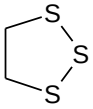
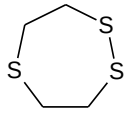
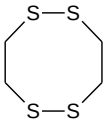
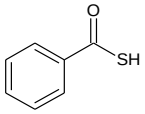
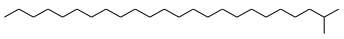
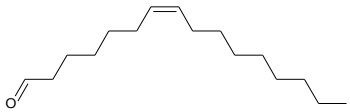
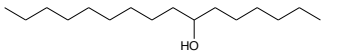
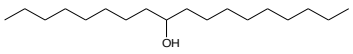
In Table 1, the results of the antimicrobial study are presented. The extracts had effects at different concentrations on the test organisms. The study showed that the hexane and ethanol extracts form cold extraction were most effective at limiting the growth of *Salmonella typhi* and the *Rhizopus sp.* respectively with an MIC value of 3.125 µg/mL and MBC value of 6.25 µg/mL. The ethanol extract using Soxhlet extraction technique was most effective at MIC value of 3.125 µg/mL on *Staphylococcus aureus, Escherichia coli, Rhizopus sp., and Aspergillus niger*. It was also found that the hexane extract with the Soxhlet extraction was effective in inhibiting the growth of *E. coli* and *Rhizopus sp* and the hexane extract inhibited the growth of *S. typhi* micro-organisms all at a MIC value of 3.125 µg/mL. Similar results on the effectiveness of the leaf extracts of *P. alliacea* on some of these organisms were reported by **Silva et al (2018)**. They also reported that the hexane extract was more effective at inhibiting the growth of some these organisms than the polar ethanol extract **(Silva et al., 2018)**.

Table 2 shows the retention times and names of compounds identified from the chromatogram, Figure 1. The compounds are numbered in the chromatogram based on the similarity index of greater than 85%. The components identified here made up to 71% of the total distillate. Crude extract from the leaf of *P. alliacea* showed antioxidant activity **(Olomieja, 2020)**, confirming the antioxidant activity reported by other researchers as well as corroborating the antioxidant activity of some sulphur containing compounds.

Table 1. MIC assay against test microorganisms *P. alliacea* leaves extracts

	Organism	*S. typhi*	*S. aureus*	*B. subtilis*	*E. coli*	*Rhizopus sp.*	*A. niger*
Cold maceration	Hexane	3.12	12.5	12.5	12.5	12.5	6.25
	Ethanol	12.5	6.25	12.5	6.25	3.12	6.25
Soxhlet	Hexane	6.25	6.25	6.25	3.12	3.12	6.25
	Ethanol	12.5	3.12	6.25	3.12	3.12	3.12

Table 2. Identification of chemical components of *P. alliacea* essential oils using steam distillation

S/N	Retention time, t_R (min)	Similarity Index (%)	Compound Name	Compound Structure	Percent composition
1	6.450	96	Benzaldehyde, C_7H_6O		3.06
2	7.450	95	Benzyl alcohol, C_7H_8O		3.97
3	8.675	89	1,2,3-trithiolane, $C_2H_4S_3$		0.58
4	11.617	88	1,2,5-trithiepane, $C_4H_8S_3$		1.13
7	14.410	86	1,2,5,6-tetrathiocane, $C_4H_8S_4$		1.27
8	15.667	87	benzenecarbothioic acid, C_7H_6SO		2.03
9	16.842	87	2-methyltetracosane, $C_{25}H_{52}$		1.26
10	17.517	87	(*Z*)-7-hexadecenal, $C_{16}H_{30}O$		1.65
11	17.567	88	2-hexyldecan-1-ol, $C_{16}H_{34}O$		1.20
12	18.310	89	2-octyldecan-1-ol, $C_{18}H_{38}O$		0.91

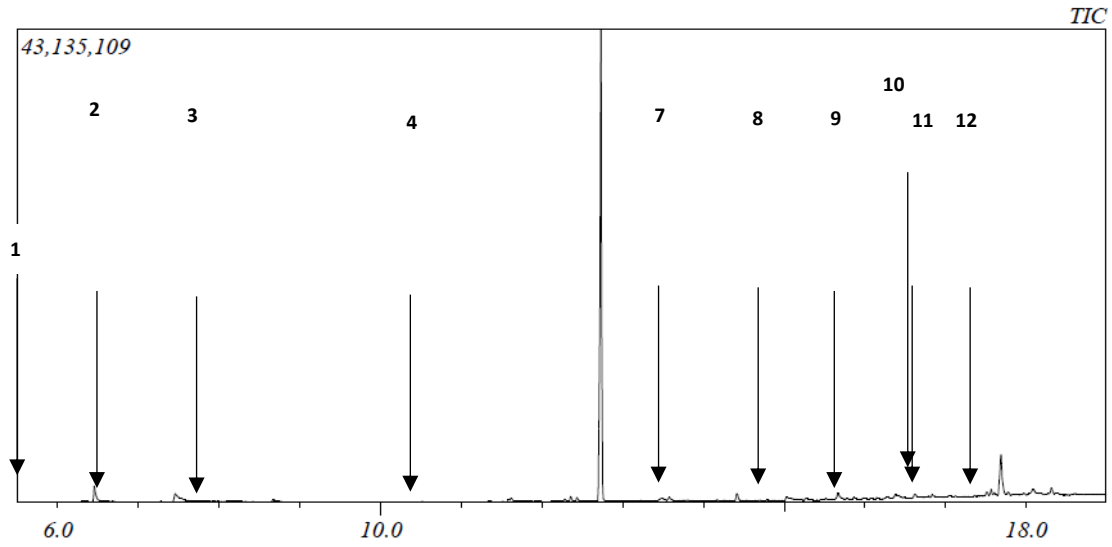


Figure 1. Chromatogram of the distillate from the steam distillation of the leaf of *Petiveria alliacea* (the numbers are as shown in Table 1).

Many sulphur compounds have been reported in various plant extracts and several have been identified in the extracts of *P. alliacea* in particular that are biologically active (Kim et al., 2006; Lowe, et al., 2015). We report here for the first time the presence of some ringed-sulphur compounds (heterocycles) (**3, 4 and 7**) as well as benzenecarbothioic acid (**8**) as components of *Petiveria alliacea*. These compounds make up more than 65% of the mixture. The mass spectral data for these four (4) compounds are shown here. The values for each compound are shown in decreasing order of abundance. 1,2,3-trithiolane, (**3**)

m/z: 124 (M^+ $C_2H_4S_3^+$), 60 ($C_2H_4S^+$), 59 ($C_2H_3S^+$), 96 (S_3^+), 45 (CHS^+), 64 (S_2^+); 1,2,5-trithiepane, (**4**) m/z: 152 (M^+ $C_4H_8S_3^+$), 59 ($C_2H_3S^+$), 60 ($C_2H_4S^+$), 45 (CHS^+), 87, 124 ($C_2H_4S_3^+$), 78; 1,2,5,6-tetrathiocane, (**7**) m/z: 184 (M^+ $C_4H_8S_4^+$), 124 ($C_2H_4S_3^+$), 92 ($C_2H_4S_2^+$), 64 (S_2^+), 59 ($C_2H_3S^+$), 128 and benzenecarbothioic acid (**8**) m/z: 105 ($C_7H_5O^+$), 77 ($C_6H_5^+$), 51. These values match those in the literature where available. As with other sulphur-containing compounds reported here, this is the first time as far as we are aware, that benzenecarbothioic acid has been reported as a compound of *Petiveria alliacea*. The presence of these sulphur heterocycles in the distillate points us to evidence of the antimicrobial and antioxidant properties of the extracts from the *Petiveria alliacea*.

Conclusion

With the results reported here, it should be noted that the chemical constituents of *P. alliacea* essential oil obtained by steam distillation differ from the chemical constituents obtained by hydrodistillation reported in previous studies. The presence of three new sulphur heterocyclic compounds and benzenecarbothioic acid as constituents in *P. alliacea* essential oil is reported. These compounds were identified by examination of the mass spectral data. The extracts showed antimicrobial activities against some microorganisms studied here.

Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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Decolorization of Textile Dye by *Brevibacillus laterosporus* (TS5) and Influencing Factors Optimization through Response Surface Methodology

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Abstract

The dye removal bacteria *Brevibacillus laterosporus* (TS5) was isolated from dye contaminated soil, and it's identified by 16S rDNA sequencing method. The prospective bacterial strain exhibited a highest decolorization (97.8%) in Luria-Bertani broth medium. Among the operational factors, Plackett-Burman design, experimental results indicated that pH, incubation period, and yeast extract significantly contributed for the dye decolorization. Also, dye concentration, starch, temperature, and inoculum size noted as insignificant factors on dye decolorization. Central composite design applied for optimization of important factors to enhance the dye decolorization by Brevibacillus laterosporus (TS5). The optimal values of significant factors were determined by the Response surface methodology (RSM) as follows: 0.60% (w/v) yeast extract, 7.23 pH and 61.45 hrs incubation period, which assisted for Brevibacillus laterosporus (TS5) to attain 90.66% dye removal. *Brevibacillus laterosporus* (TS5) showed 90.08% decolorization in validation experiments by the support of optimal factors, and implies that explored strain could be a suitable candidate for bioremediation of dye containing effluents.

1. Introduction

The disposal of waste from textile industries is considered as major environmental problem in the worldwide. Particularly, the discharging of textile industry effluent into the ecosystem is hazardous one, as it contains bio-recalcitrant dye stuffs **(Sarayu and Sandhya 2012)**. Textile effluent includes a mixture of dyes, organic and inorganic chemicals **(Doble and Kumar 2005)**. Dyes are recognized as a first pollutant in textile waste water, since it possesses higher visibility in colored effluent **(Kilic *et al.* 2007)**.

Azo dyes are normally recognized as the most imperative group of synthetic dyes. They are carcinogenic aromatic compound that disturbs the transparency, gas solubility nature of water bodies, and inhibits the growth of aquatic plants **(Rodriguez Couto *et al.* 2009)**. Some of the azo dyes induce bladder cancer, chromosomal aberrations in humans **(Medvedev *et al.* 1988)**. The effluents containing complex structure of azo dyes makes it difficult to treat in conventional methods **(Jadhav *et al.* 2016).**

Various physical and chemical technologies are employed in textile industries for the removal of dye molecules from colored effluents **(Yaseen *et al.* 2018)**. They are utilizing the large amount of chemicals, and electricity for their mode of action, which produce the secondary pollutants **(Karthik *et al.* 2015; Sweety *et al.* 2018)**. These secondary pollutants described as sludge, inorganic salts, metals, and carcinogenic substance possess risk to human health and aquatic fauna **(Vikas and Sandip 2013)**. From these points, the investigation aimed on environmental friendly methods for removal of dyes using microorganisms. Several textile dye decolorization studies have been reported with bacteria, fungi, and yeast.

Among microbial decolorization, the use of bacteria is comparatively faster, economical, and can be applied to wide range of dye degradation and mineralization **(Saratale *et al.* 2011)**. Bacterial dye degradation mainly occurs in aerobic, anaerobic, anoxic, microaerophilic, and sequential process **(Ajaz *et al.* 2019)**. Generally, azo dye reduction take place in bacteria through synthesizing of azoreductase enzyme and cleavage of azo bonds under oxygen limited conditions **(Coughlin *et al.* 2003)**.

The most important cultural factors are carbon source, nitrogen source, dye concentration, pH, inoculum size, temperature, and the incubation period is dynamically affected the efficiency of microbial activity and decolorization processes **(Garg *et al.* 2017)**. Consequently, their relevant factor optimization is prerequisite one, prior to real effluent studies, to avoid any inhibitory effect on dye degradation process. Therefore, the necessity found in the screening of robust bacteria which are able to adopt, and decolorize the textile effluent in an adverse condition. Also, key factors optimization by response surface methodology (RSM) approach can enhance the performances of bacterial dye decolorization and its effectiveness **(Mohana *et al.* 2008)**.

Mostly statistical tools employed to simplify the operations, chemical consumption, and the time involved in a process. Moreover, RSM is an efficient chemometric empirical approach that can be used to eliminate insignificant variables, and to study the relationship between a set of significant factors in optimized

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process **(Chen, 1994)**. In this view, the present study focused on the exploration of textile dye decolorizing bacteria *Brevibacillus laterosporus* (TS5) isolated from the dye contaminated soil, and its dye decolorization efficacy was enhanced via optimization by screening of influencing factors in dye removal process with Plackett-Burman design, followed by application of response surface methodology with central composite design.

2. Material and methods

2.1 Screening of dye decolorizing bacteria

The dye contaminated soil sample was collected from a textile dyeing industry located in Tirupur district of Tamil Nadu, India. The bacterial colonies were isolated and enumerated by nutrient agar medium (pH 7.0±0.2) containing, 5 (g/l) Peptone, 5 (g/l) Sodium chloride, 3 (g/l) Yeast extract, 20 (g/l) Agar. The pour plate method employed plates were incubated at 35±2 °C, and 45±2 °C for 24 - 48 hrs incubation. The decolorizing ability of bacterial colonies was determined in Luria Bertani agar plates containing, 10 (g/l) Casein enzymic hydrolysate, 5 (g/l) Yeast extract, 10 (g/l) NaCl, 15 (g/l) Agar, amended with various concentrations (50, 100, 150, 200 and 250 mg/l) of remazol golden yellow (RNL) dye, and incubated at 37 °C for 4 days. The bacterial colonies which showing clear zones on the agar plates were selected for decolorization studies.

2.2 Identification of dye decolorizing bacteria

The dye decolorizing bacterial strain TS5 was identified by 16S rDNA sequencing method. The genomic DNA isolation, extraction, PCR amplification, and 16S rDNA sequencing was carried out in Xcelris Labs Ltd, Ahmedabad, India. The generated 16S rDNA sequences were compared to sequences of the NCBI server using BLASTN tool. The closely related sequences were aligned with multiple alignment program Clustal W. Phylogenetic tree was constructed by the neighbour-joining (NJ) method in MEGA version 5. The sequences have been deposited in NCBI GenBank (http://www.ncbi.nlm.nih.gov) under accession number JQ885974.

2.3 Effect of nutritional substrates in dye decolorization

The effect of co-substrates on dye decolorization using *Brevibacillus laterosporus* (TS5) was carried out in the various broth compositions (100 ml each) Luria Bertani broth (g l^{-1}): Casein enzymic hydrolysate 10.0, yeast extract 5.0, NaCl 10.0; Yeast extract broth (g l^{-1}): yeast extract 5.0, NaCl 5.0; Bushnell and Hass broth (g l^{-1}): $MgSO_4$ 0.2, K_2HPO_4 1.0, $CaCl_2$ 0.02, $FeCl_3$ 0.05, NH_4NO_3 1.0 (remazol golden yellow dye concentration, 100 mg/l). About 1 ml of inoculum with an optical density of 1.0 was inoculated. Experimental flasks were incubated statically at 37 °C for 3 days with abiotic control. The samples were withdrawn at 24 hrs intervals, and centrifuged at 5000 rpm for 20 min. The absorbance value of the cell free supernatant was analyzed in UV spectrophotometer (Cyberlab UV-100 USA) at 412 nm. Triplicate experiments were performed in simultaneously, and decolorization percentage was calculated using the Equation (1).

$$\text{Decoloriza tion } (\%) = \frac{\text{Initial absorbance value - Final absorbance value}}{\text{Initial absorbance value}} \times 100$$

Equation (1)

2.4 Plackett- Burman design - Screening of significant factors for dye decolorization

The Plackett–Burman design, a fractional factorial design, was used to select the most important factors that influencing in remazol golden yellow dye decolorization by strain *Brevibacillus laterosporus* (TS5). **Table 1** provides the information about testing factors range and their actual values. The levels of the factors were selected based on previous literatures, selective carbon and nitrogen sources were obtained from preliminary experiments. The effect and relative importance of each factor on dye decolorization was determined in 12 combinations of Plackett- Burman design matrix shown in **Table 2**, which are based on the following first-order model Equation (2)

Table 1. Actual values of the factors Plackett-Burman design screening

Test variables	Starch % (w/v)	Yeast extract % (w/v)	pH	Temperature (°C)	Inoculum size % (v/v)	Dye concentration (mg/l)	Incubation period (hrs)
Low level (+)	0.1	0.1	5	30	5	100	24
High level (-)	1.0	1.0	9	45	10	300	72

Table 2. Plackett-Burman design for screening of major factors on dye decolorization

Run order	Starch % (w/v)	Yeast extract % (w/v)	pH	Temperatures (°C)	Inoculum size % (v/v)	Dye concentrations (mg/l)	Incubation periods (hrs)	DV-1	DV-2
1	1.0	0.1	9	30	5	100	72	1	1
2	1.0	1.0	5	45	5	100	24	1	1
3	0.1	1.0	9	30	10	100	24	-1	1
4	1.0	0.1	9	45	5	300	24	-1	-1
5	1.0	1.0	5	45	10	100	72	-1	-1
6	1.0	1.0	9	30	10	300	24	1	-1
7	0.1	1.0	9	45	5	300	72	-1	1
8	0.1	0.1	9	45	10	100	72	1	-1
9	0.1	0.1	5	45	10	300	24	1	1
10	1.0	0.1	5	30	10	300	72	-1	1
11	0.1	1.0	5	30	5	300	72	1	-1
12	0.1	0.1	5	30	5	100	24	-1	-1

$Y= \beta_0 + \Sigma \beta_i X_i + \Sigma \beta_{ij} X_i^2 + \Sigma \beta_{ij} X_i X_i$

Equation (2)

Where Y is the response (Percentage of dye decolorization), X_i is factor levels, i is factor number, β_0 is the model intercepts term, βi is the linear effect, βii *is* the squared effect, βij is the interaction effect between X_i and X_j on dye decolorization process. As per the design, experiments were carried out in flask culture under static incubation. All the runs were performed in triplicate, and the averages of decolorization percentage were considered as the response. Statistical analysis of the Plackett-Burman design results were performed by using statistical software Minitab Version 15.0.

2.5 Response surface methodology - Optimization of dye decolorization

The concentrations of yeast extract, incubation period, and pH were the significant factors for effective dye decolorization was identified from results of Plackett–Burman design. Response surface methodology was used to study the optimal region of significant factors, and their interactions on remazol golden yellow dye decolorization using strain *Brevibacillus laterosporus* (TS5), a full factorial central composite design was employed. Each significant factor was studied at five levels (-α, -1, 0, +1 and +α) and their actual values are shown in **Table 3**. Where $\alpha = 2^{n/3}$; here "n" corresponds to the number of factors and "0" corresponds to the central point. The Equation (3) was adopted to calculate the actual values of significant factors.

$$\text{Coded value} = \frac{\text{Actual value} - (\text{high level} + \text{low level}) / 2}{(\text{High level} - \text{low level}) / 2}$$

Equation (3)

Table 3. Actual values of significant factors in central composite design

Variables	Unit	Five levels of variables				
		-α (-1.68179)	-1	0	1	+α (+1.68179)
Yeast extract	% (w/v)	-0.20681	0.1	0.55	1	1.306807
pH	-	3.636414	5	7	9	10.36359
Incubation Period	Hrs	7.636972	24	48	72	88.36303

The experimental model of central composite design was given in **Table 4**. The other cultural factors were maintained in constant range, such as, sodium chloride 0.5% (w/v), starch 0.55% (w/v), dye concentration 200 mg/l, inoculum size 7.5% (v/v), and temperature 37 °C. The experimental results obtained from the central composite design were established in second order polynomial model Equation (4) for the prediction of decolorization efficiency, and the significant factor interactions within testing range.

$Y= \beta_0 + \beta_1X_1 + \beta_2X_2 + \beta_3X_3 + \beta_{11}X_{12} + \beta_{22}X_{22} + \beta_{33}X_{32} + \beta_{12}X_1X_2 + \beta_{13}X_1X_3 + \beta_{23}X_2X_3$

Equation (4)

Where Y is the response, β_0 is the intercept term, β_1, β_2 and β_3 are linear coefficient of tested factors, β_{11}, β_{22}, β_{33} are quadratic coefficient, β_{12}, β_{13}, β_{23} are interaction coefficient, and X_1, X_2 , X_3, X_4 are coded factors. All the experiments were carried out in triplicate, and the statistical software package Minitab Version 15.0 was used to mathematical interpretation of experimental results. The significant factors optimal value was established from Central composite design results with response surface analyzer. Under optimal values of these key factors, the validation study was performed, and enhancement of dye decolorization efficiency was evaluated by comparing the experimental results with predicted values

Table 4. Central composite design for optimization of dye decolorization

Run Order	Pt type	Blocks	Yeast extract % (w/v)	pH	Incubation Period (hrs)
1	1	1	0.1	5	24
2	1	1	1	5	24
3	1	1	0.1	9	24
4	1	1	1	9	24
5	1	1	0.1	5	72
6	1	1	1	5	72
7	1	1	0.1	9	72
8	1	1	1	9	72
9	-1	1	-0.20	7	48
10	-1	1	1.31	7	48
11	-1	1	0.55	3.63	48
12	-1	1	0.55	10.36	48
13	-1	1	0.55	7	7.63
14	-1	1	0.55	7	88.36
15	0	1	0.55	7	48
16	0	1	0.55	7	48
17	0	1	0.55	7	48
18	0	1	0.55	7	48
19	0	1	0.55	7	48
20	0	1	0.55	7	48

3. Results and Discussion

3.1 Isolation and Screening of dye decolorizing bacteria

A total of 42 mesophilic and thermotolerant bacterial strains were isolated and enumerated from the dye contaminated soil. The development of a significant number of bacteria in the dye contaminated soil denotes its ability to degrade the toxic dye substances **(Rajee *et al.* 2011)**. Among these, eighteen isolates made the clear zone around the colony in Luria-Bertani agar plates amended with 100mg/l concentration of remazol golden yellow dye. The colony which showed >1.0 cm of clear zone was found to be effective dye decolorizer **(Tiwari *et al.* 2012)**. Whereas, the isolated bacteria TS5 proficiently showed the clear zone with diverse concentrations of dye amended plates. **Wang *et al.* (2012)** stated that indigenous microorganisms have the capability to decolorize various classes of dyes with high efficiency.

3.2 Identification of dye decolorizing bacteria

The BLAST analysis of the 16S rDNA amplicon, indicated that the strain TS5 was belonging to the member of *Brevibacillus* genus. The phylogenetic relationship of strain TS5 with other *Brevibacillus* species was shown in **Figure 1** exhibited 100% similarity with *Brevibacillus laterosporus* IAM 12465 (D16271) from the GenBank database. The bacterial strain was identified as *Brevibacillus laterosporus* (TS5). Earlier on this, few studies reported the enzymatic role of *B. laterosporus* in the textile dye decolorization **(Gomare *et al.* 2009a and Kurade *et al.* 2011)**.

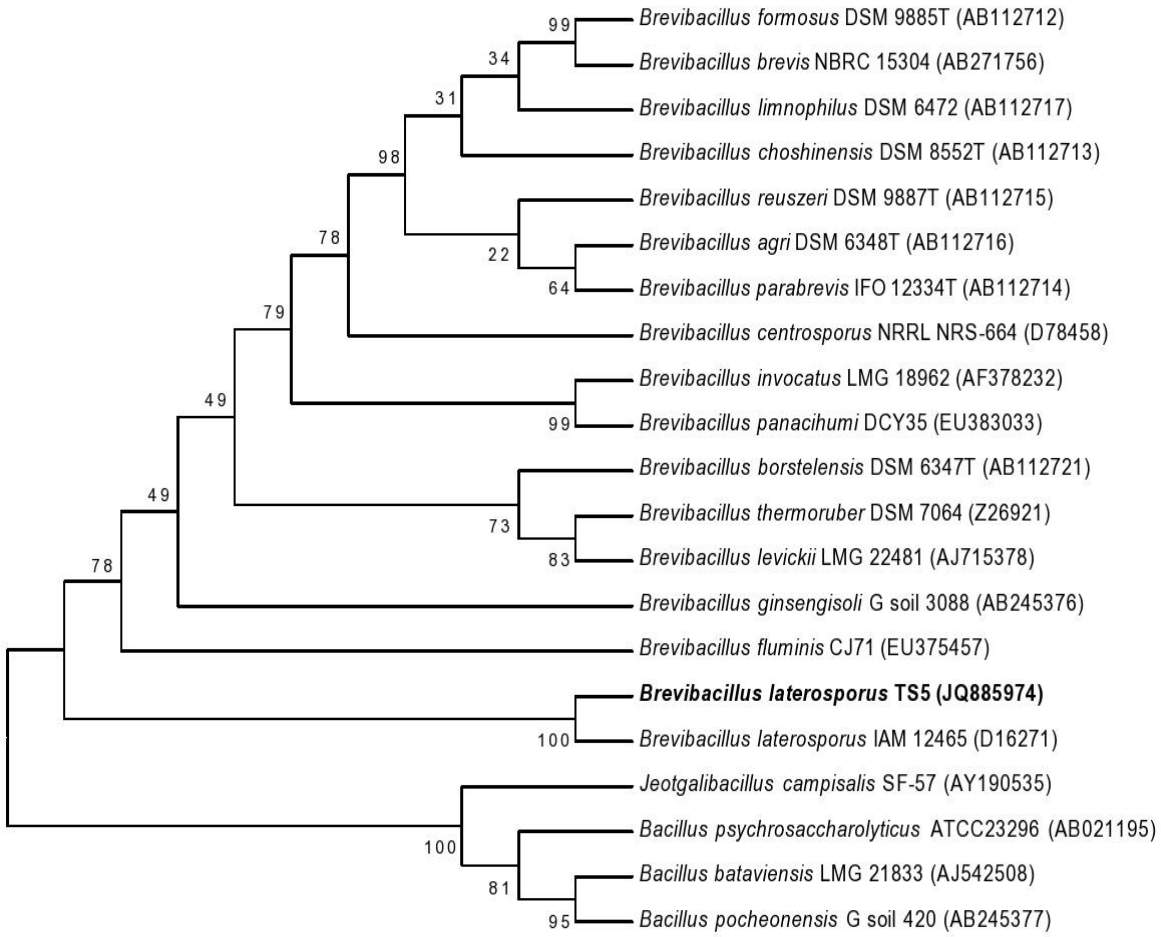


Figure 1. Phylogenetic tree of *Brevibacillus laterosporus* (TS5) showing relationship between selected bacterial strains. The percent numbers at the nodes indicate the levels of bootstrap support based on neighbour-joining analyses of 1000 replicates. Brackets represents sequence accession numbers.

3.3 Effect of nutritional substrates

Suitable substrates of broth composition on decolorization by *Brevibacillus laterosporus* (TS5) were investigated in batch studies. The dyes are recalcitrant to biodegradation in nature, and it's not acting as a nutritional source for microorganisms **(Lellis *et al.* 2019; Jamee *et al.* 2019)**. Thus require additional co-substrates for its degradation, and mineralization **(Dos Santos *et al.* 2007)**. In the present study, *Brevibacillus laterosporus* (TS5) showed 2.55% and 29.31% of dye reduction in yeast extract broth composition at 24 hrs intervals. On this, incubation time extended up to 72 hrs, and the 73.6% decolorization was found. **Moosvi *et al.* (2007)** observed that 25% removal of Reactive Violet 5R (100ppm) by bacterial consortium JW-2 with yeast extract broth composition. The absence of carbon substances in the yeast extract medium confirms the reason for lower decolorization of dyes. Whereas, 25.49%, and 97.8% dye removal observed in Luria-Bertani broth, and its components found as effective nutritional substrates. Casein enzymic hydrolyzate in LB broth enhanced the bacterial growth characteristics and decolorization ability. The Luria-Bertani broth combined nutritional composition indicates the induction for an efficient dye decolorization. Similarly, *Marinobacter* sp. strain HBRA effectively decolorized the 100 mg/l concentration of Direct Blue-1 dye in LB broth under static conditions **(Arun Prasad *et al.* 2013)**. Diversified results revealed that broth composition playing a vital role in dye decolorization experiments. There was no sign of decolorization was observed in bushnell hass broth medium without nutritional substrates. It indicates the inability of bacteria for utilizing the dye as a sole of energy, and agreed with many confirmed reports that the obligate requirement of co-substrate for proficient dye decolorization **(Karim *et al.*2018)**.

3.4 Screening of significant factors on dye decolorization

The influence of operational factors in remazol golden yellow decolorization by *Brevibacillus laterosporus* (TS5) was scrutinized in Plackett-Burman design and the results are ranging from 0.68 to 53.69% **(Table 5)**, it reflected the significant effect of factors in dye removal process. The diverse culture conditions were responsible for the decolorization performance of *Brevibacillus laterosporus* was denoted by **Gomare *et al.* (2009c)**. Standardized effects in a pareto chart **(Figure 2)** illustrates the significant factors of dye decolorization, which was yeast extract, pH and incubation period respectively. Analysis of estimated effects and regression coefficient represents the factors significance level **(Table 6)**. The coefficient value of yeast extract, pH, and incubation period indicates the positive effect accumulated via increased their concentration. The availability of carbon/nitrogen sources determines the rate of dye decolorization, since it acts as an

electron donor for the reduction of the dye **(Kurade *et al.* 2011)**. **Waghmode *et al.* (2012)** found that addition of yeast extract regenerated the NADH, which acts as an electron donor for decolorization of Rubin GFL. Similarly in presence of yeast extract, *A. hydrophila* effectively decolorized, and reduced RED RBN dye into azo bonds **(Chen *et al.* 2003)**. Microbial decolorization of dyes is generally reported in neutral to alkaline pH range. **Kurade *et al.* (2012)** also reported that neutral pH and 40 °C temperature was an effective factor for significant decolorization of Rubine GFL by *Brevibacillus laterosporus*. Thus the influence of finest pH on dye decolorization could be a supportive factor in industrial scale application. On the other hand, concentration of dye, starch, temperature, and inoculum size indicates the insignificant condition for dye decolorization. Beyond the optimum temperature, loss of its cell viability, and inhibition of oxido-reductive enzyme activities lead the reduced decolorization **(Kurade *et al.* 2012)**. In general, lower decolorization was observed with higher dye concentration demonstrates the toxicity of dye molecules effect on microorganisms. **Kurade *et al.* (2013a)** reports the decreased Disperse Brown 118 decolorization by *B. laterosporus* with an increasing of initial dye concentration. In the present study, minimum decolorization with decreasing inoculum volume was observed, because to lesser bacterial cells rapidly entered the death phase from the exponential growth phase, and cause the declined decolorization. **(Kolekar *et al.* 2008)**. **Gomare *et al.* (2009b)** reported the better decolorization of Navy Blue-3G by exponential phase of *B. laterosporus*, as at this stage produced the highest amount of biotransformation enzymes for their dye degradation activity. Starch is a carbon source often utilized in the various processes of textile industries **(Ramesh babu *et al.* 2007)**. Thus the use of starch as a co-substrate by bacteria would be favorable for the removal of dye from textile effluents. However, previous studies stated that carbon sources like glucose and starch have an inhibitory effect on the enzyme system responsible for dye decolorization **(Kurade *et al.* 2011)**. Hence, the interactive effect of factors could be a reason for distinguished observation noted in this study, compared to previous reports.

Table 5. Plackett-Burman design decolorization (%) results

Run order	Starch % (w/v)	Yeast extract % (w/v)	pH	Temperatures (°C)	Inoculum size % (v/v)	Dye concentrations (mg/l)	Incubation periods (hrs)	Percentage decolorization	
								Experimental	Predicted
1	1.0	0.1	9	30	5	100	72	19.43	17.59
2	1.0	1.0	5	45	5	100	24	18.90	23.03
3	0.1	1.0	9	30	10	100	24	38.48	34.83
4	1.0	0.1	9	45	5	300	24	6.19	6.01
5	1.0	1.0	5	45	10	100	72	35.30	31.03
6	1.0	1.0	9	30	10	300	24	18.46	22.54
7	0.1	1.0	9	45	5	300	72	53.69	48.05
8	0.1	0.1	9	45	10	100	72	18.67	26.31
9	0.1	0.1	5	45	10	300	24	1.63	0.20
10	1.0	0.1	5	30	10	300	72	0.68	0.91
11	0.1	1.0	5	30	5	300	72	29.48	33.12
12	0.1	0.1	5	30	5	100	24	5.56	3.37

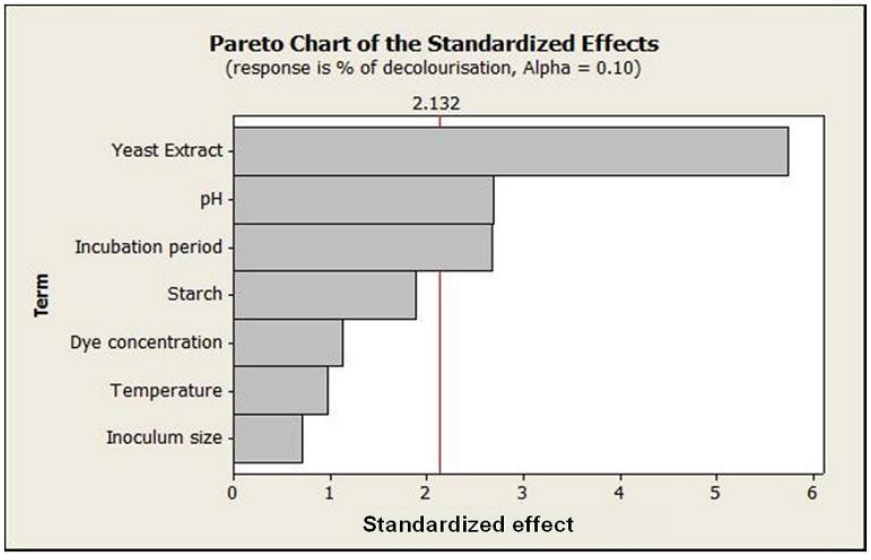


Figure 2. Effects of operational factors on dye decolorization in Pareto chart

Table 6. Statistical analysis of Plackett-Burman design

S. No	Variables	Effects	Coef	SE Coef	T	P
1	Constant	-	20.400	2.036	10.02	0.001*
2	Starch	-7.697	-3.848	2.036	-1.89	0.132
3	Yeast extract	23.407	11.703	2.036	5.75	0.005*
4	Ph	10.983	5.492	2.036	2.70	0.054*
5	Temperature	3.947	1.973	2.036	0.97	0.387
6	Inoculum size	-2.930	-1.465	2.036	-0.72	0.512
7	Dye concentration	-4.597	-2.298	2.036	-1.13	0.322
8	Incubation period	10.933	5.467	2.036	2.68	0.055*
	R-Sq = 93.08% R-Sq (adj) = 80.98%					

*Significant

Usually large t-value associated with low P-value specifies the high significance of the model term. The significant factors effect on the decolorization process is explicated in regression equation number 5. Determination of correlation coefficient (R^2 = 0.9308) is nearer to 1, demonstrates the model was fittest one. It showed a good statistical simulation between the experimental and predicted response, which implies that the model can be explain up to 93.08% variation in the experiment.

Y= 20.400 - 3.848 × starch + 11.703 × yeast extract + 5.492 × pH + 1.973 × temperature -1.465 × inoculum size - 2.298 × dye concentration + 5.467 × incubation period
Equation (5)

ANOVA of linear model explains the factors affecting on remazol golden yellow decolorization **(Table 7)**. The fisher's F – test value of the response is 7.69 and its delivered that the model was significant one, the value of Probability (P) > F is less than 0.034. The operational factors P-value were less than 0.10 indicates, that the model and those factors are highly significant.

3.5 Optimization of dye decolorization

From the results of Plackett-Burman design, yeast extract, pH and incubation period found as the most important factors and their levels were optimized in response surface methodology (RSM) for maximum decolorization of remazol golden yellow by *Brevibacillus laterosporus* (TS5). The experimental results of % dye decolorization involving central composite design were varied from 5.62 to 87.15% **(Table 8)**.

Table 7. Analysis of variance for Plackett-Burman design

S.No	Source	DF	Seq SS	Adj SS	Adj MS	F	P
1	Main effects	7	2677.72	2677.72	2677.72	7.69	0.034*
2	Starch	1	177.72	177.72	177.72	3.57	0.132
3	Yeast extract	1	1643.62	1643.62	1643.62	33.04	0.005*
4	pH	1	361.90	361.90	361.90	7.27	0.054*
5	Temperature	1	46.73	46.73	46.73	0.94	0.387
6	Inoculum size	1	25.75	25.75	25.75	0.52	0.512
7	Dye concentration	1	63.39	63.39	63.39	1.27	0.322
8	Incubation period	1	358.61	358.61	358.61	7.21	0.055*
9	Residual error	4	198.99	198.99	49.75	-	-
	Total	11	2876.71				

*Significant

Table 8. Central composite design decolorization (%) results

Trails	Yeast extract (X_1)	pH (X_2)	Incubation period (X_3)	Percentage decolorization		Residual
				Experimental	Predicted	
1	0.1	5	24	10	12.52	-2.52
2	1	5	24	8.5	6.08	2.41
3	0.1	9	24	6.45	1.67	4.77
4	1	9	24	5.62	5.60	0.01
5	0.1	5	72	17.5	19.55	-2.05
6	1	5	72	24	30.81	-6.81
7	0.1	9	72	25.5	29.95	-4.45
8	1	9	72	52.07	51.58	0.48
9	-0.20	7	48	8.45	6.89	1.55
10	1.31	7	48	21	19.66	1.33
11	0.55	3.64	48	7.5	3.14	4.35
12	0.55	10.36	48	10	11.47	-1.47
13	0.55	7	7.63	24	27.76	-3.76
14	0.55	7	88.36	79	72.34	6.65
15	0.55	7	48	85.65	86.63	-0.98
16	0.55	7	48	87	86.63	0.36
17	0.55	7	48	86	86.63	-0.63
18	0.55	7	48	86.5	86.63	-0.13
19	0.55	7	48	87.15	86.63	0.51
20	0.55	7	48	87	86.63	0.36

The decolorization result (%) was fitted with second order polynomial model equation (6) to explain the confidence of dye removal percentage. The estimated regression coefficient for the model was given in **Table 9**. The small p-value corresponding to larger t-value explains that the factors and model term are significant. Also, correlation coefficient (R^2) was found to be 99.18% that reveals the presence of a good correlation between studied and predicted decolorization (%).

$$Y= 86.632 + 3.796 \times X_1 + 2.478 \times X_2 + 13.253 \times X_3 - 25.933 \times X_1^2 - 28.045 \times X_2^2 - 12.931 \times X_3^2 + 2.592 \times X_1 \times X_2 + 4.425 \times X_1 \times X_3 + 5.313 \times X_2 \times X_3 \quad \textbf{Equation (6)}$$

Where Y is response (percentage of dye decolorization), X_1, X_2 and X_3 were the coded values of yeast extract, pH and incubation period respectively. In analysis of variance determined data **(Table 10)**, the calculated F-value = 134.59 and probability value P=0 shows the model as a significant one. The enhanced effects, linear (p=0.000), quadratic (p=0.000) and interaction (p=0.006) are found between the factors in dye removal. The significant factors interaction and their effects on dye decolorization were graphically shown in response contour plots **(Figure 3a, b, c)**.

Table 9. Estimated regression coefficients of CCD results

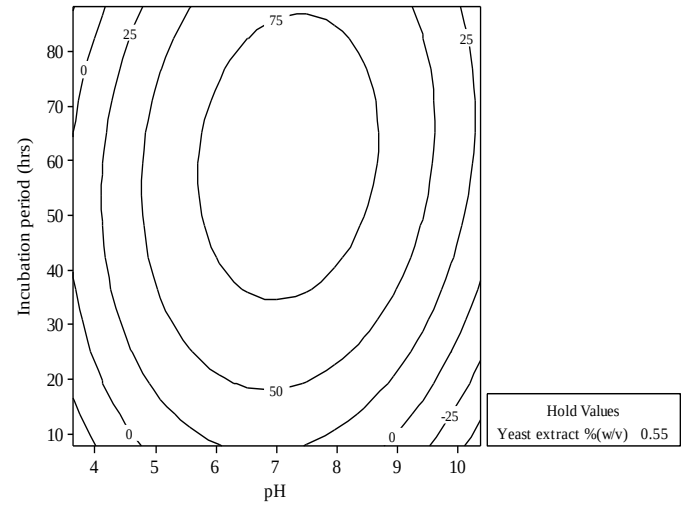
S. No	Variables	Coef	SE Coef	T	P
1	Constant	86.632	1.785	48.534	0.000*
2	Yeast extract	3.796	1.184	3.206	0.009*
3	pH	2.478	1.184	2.093	0.063*
4	Incubation period	13.253	1.184	11.191	0.000*
5	Yeast extract*Yeast extract	-25.933	1.153	-22.494	0.000*
6	pH*pH	-28.045	1.153	-24.326	0.000*
7	Incubation period*Incubation period	-12.931	1.153	-11.216	0.000*
8	Yeast extract*pH	2.592	1.547	1.675	0.125
9	Yeast extract*Incubation period	4.425	1.547	2.860	0.017*
10	pH*Incubation period	5.313	1.547	3.433	0.006*
R-Sq = 99.18% R-Sq (adj) = 98.44%					

*Significant

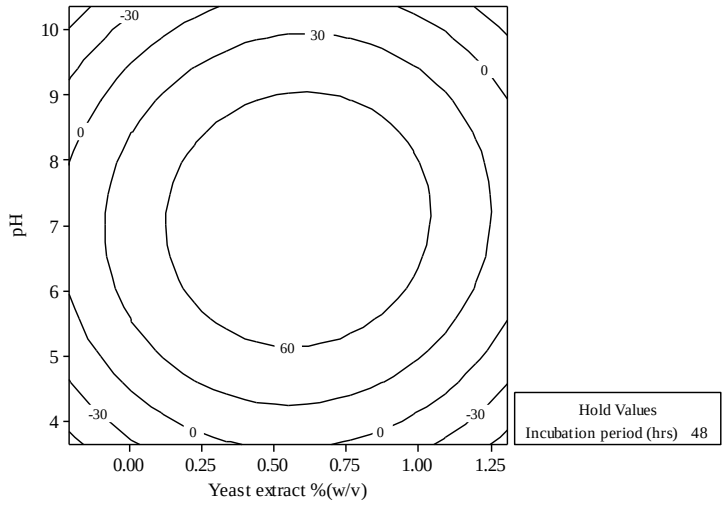
Table 10. Analysis of variance for central composite design

S. No	Source	DF	Seq SS	Adj SS	Adj MS	F	P
1	Regression	9	23201.6	23201.6	2577.95	134.59	0.000*
2	Linear	3	2679.5	2679.5	893.18	46.63	0.000*
3	Square	3	20085.9	20085.9	6695.29	349.55	0.000*
4	Interaction	3	436.2	436.2	145.40	7.59	0.006*
5	Residual error	10	191.5	191.5	19.15	-	-
6	Lack-of-fit	5	189.7	189.7	37.93	100.88	0.000*
7	Pure error	5	1.9	1.9	0.38	-	-
	Total	19	23393.1	-	-	-	-

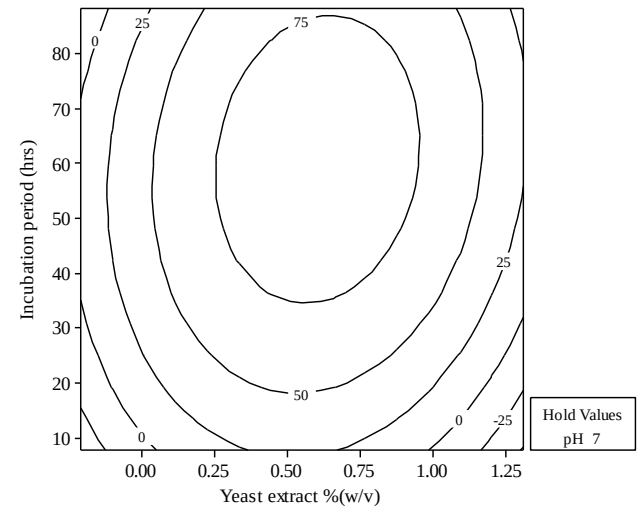
*Significant



(a)



(c)



(b)

Figure 3. Response contour plots on decolorization efficiency (%); (a) Effect of incubation period and pH on dye decolorization (%), (b) Effect of incubation period and yeast extract on dye decolorization (%), (c) Effect of pH and yeast extract on dye decolorization (%).

The response surface methodology predicated optimal values of significant factors were 0.60% (w/v) yeast extract, 7.23 pH and 61.45 hrs incubation period, which capable to yield 90.66% removal of remazol golden yellow dye by *Brevibacillus laterosporus* (TS5). In the optimal values validation, the *Brevibacillus laterosporus* (TS5) using predicted concentrations of significant factors attained the color removal by 90.08%, and it's agreed with expected result (90.66%). Whereas, *Brevibacillus laterosporus* MTCC 2288, was reached 74% color removal for Golden Yellow HER dye by pH 7 and 30 °C temperature **(Gomare *et al.* 2009c)**. Under optimized condition, Acid Red GR color removal by *Dyella ginsengisoli* LA-4 achieved at 98.36%, its resemblance to predicted value 98.47% from RSM **(Zhao *et al.* 2010)**. These studies support that the RSM was effective and reliable for optimization of dye decolorization. It was found that a range of pH mainly affects the bacterial decolorization rate. Similar to present results, the 93.06% of Direct Orange 39 (Orange TGLL) color removal occurred at pH 7 by *Pseudomonas aeruginosa* **(Jadhav *et al.* 2010)**. Microorganisms show differences in the uptake of nutritional sources, as it depends upon their dye degradation mechanisms. The optimization study confirmed the yeast extract contribution, and their significant role in the dye decolorization by *Brevibacillus laterosporus* (TS5), and that carbon source was found as insignificant one. It indicates the carbon sources have an inhibitory effect on the dye decolorizing enzymes. **Senthilkumar *et al.* (2012)** reported that *Pseudomonas* sp. effectively decolorizes the reactive dyes with RSM accounted concentrations of dye, carbon source, and nitrogen source. Likewise, decolorization was found between 3 to 4% in the presence of glucose and starch, as cells preferred the utilization of additional carbon substrates, and ensured the dye derivatives was not functioning as carbon source **(Kurade *et al.* 2011)**. While in extend incubation hours, cultured cells could augment the various biotransformation enzymes responsible for dye decolorization. Moreover, the maximum decolorization of Navitan Fast Blue S5R was found in the late exponential growth phase of *Pseudomonas aeruginosa* **(Valli Nachiyar *et al.* 2003)**. Inspite of, **Abd el-rahim *et al.* (2003)** reported that low decolorization efficiency of bacteria at the end of 21 days incubation, which was 20% for direct yellow and 25% for red dyes (10 mg/l). Since, the toxic nature of dye intermediates, and their concentration varied among dyes inhibits the growth of bacteria, the subsequently reduced decolorization occurred. These are the findings denotes the operational factors influence, and significant factor optimized levels enhances the decolorization rate of *Brevibacillus laterosporus* (TS5).

Conclusion

The present study demonstrated that the decolorization potentiality, and statistical optimization of bacterium *Brevibacillus laterosporus* (TS5) for an effective dye decolorization process. The precise ranges of various operational factors affect the dye decolorization rate of bacteria. Plackett-Burman design approach eliminated the insignificant factors from multiple factors of the optimization process. In these, the contribution of starch seems to be less effective in promoting decolorization. However, decolorization augmented with the addition of yeast extract as a nitrogen source. It indicates the bacterial cells, either utilized dye as carbon source instead of added starch. The bacteria showed a maximum dye removal capacity with neutral pH highlights their positive interaction of dye molecules. The robust dye decolorization obtained with assistance of significant factors, optimal level, and confirms the important factors interactions on decolorization. Hence, this result suggested that the isolated bacteria and their optimized condition could be an effective role in the removal of dyes from textile waste water treatment system.

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Declaration of interest

The authors reports no conflicts of interest.

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