OPEN ACCESS

BACTERIAL EMPIRE

2019, VOL. 2, NO. 2, 33-36

SCICELL

REGULAR ARTICLE

ANTIBACTERIAL ACTIVITY OF ETHANOL CRUDE EXTRACTS OF WHOLE PLANT OF THE UGANDAN PHYLLANTHUS AMARUS SCHUMACH. & THONN AGAINST SHIGELLA DYSENTERIAE

Magimbi Arthur[1], *Adamu Almustapha Aliero*[2,3*] *and* John Odda[1]

Address (es): Mr. Adamu Almustapha Aliero,
[1] School of Pharmacy, Kampala International University Western Campus, Uganda.
[2] Departments of Microbiology and Immunology, Kampala International University Western Campus, Uganda .
[3] Department of Microbiology, Faculty of Life Sciences, Kebbi State University of Science and Technology Aliero, Kebbi State, Nigeria.

*Corresponding author: aimustapha.adamu@kiu.ac.ug, adamualieroa@gmail.com

ABSTRACT

Apart from being used for the treatment of malaria fevers, mono-herbal preparation of Ugandan *Phyllanthus amarus* is widely by local communities in central Uganda, for treatment of bacterial dysentery. However, efficacy studies regarding the mono-herbal extracts of the Ugandan *Phyllanthus amarus* against *Shigella dysenteriae* is lacking. The present study was aimed at determined the antibacterial activity of aqueous ethanol crude extracts of whole plant of *Phyllanthus amarus* against *Shigella dysenteriae*. Whole plant of *Phyllanthus amarus* was collected from Wakiso district central of Uganda. Different concentrations (128µg. mL^{-1}, 256µg.mL^{-1} and 512µg.mL^{-1}) of the ethanol crude extract of whole plant of *Phyllanthus amarus* were tested against *Shigella dysenteriae* using agar well diffusion method. Minimum inhibitory concentration (MIC) was determined using broth tube dilution method while Minimum bactericidal concentration (MBC) was determined by culture method. The results of this study showed that, *Shigella dysenteriae* was sensitive to different concentrations (128µg.mL^{-1}, 256µg.$mL^{-1}$and 512µg.mL^{-1}) of ethanol crude extract of whole plant of *Phyllanthus* amarus possessed antibacterial against *Shigella dysenteriae* with mean and standard error mean zone of inhibition ranging from 11.5±0.5 to 13.5±0.5mm, while the positive control had mean and standard error mean zone of inhibition 24.5±0.5mm. The MIC and MBC of the ethanol crude extract of whole plant of *Phyllanthus amarus* against *Shigella dysenteriae* was found to be 32µg.mL^{-1} and 64µg. mL^{-1} respectively. This study found that the whole plant of *phyllanthus amarus* of ethanol crude extract was active against *Shigella dysenterie*. Further study should be carried out test whole plant *Phyllanthus amarus* crude extract on different microorganisms that cause dysentery to find out its activity against them and spectrum, determine lead compound in the whole plant *Phyllanthus amarus* crude extract responsible for the anti-microbial activity against *Shigella dysenteriae* and also determine the mechanism of action by which this plant crude extract inhibits *Shigella dysenteriae.*

Keywords: Antibacterial activity, whole plant of *Phyllanthus amarus* and *Shigella dysenteriae*

INTRODUCTION

The use of medicinal plants as alternative medicine is currently being given more attention worldwide (**Flora and Folasade, 2008; WHO, 2017)**. In Africa, traditional herbal medicines are widely used informed of concoction in hot or cold water, foods and drinks for the treatment of many infectious diseases including dysentery caused by bacteria and non-bacterial agents **(Senjobi *et al*., 2017)**. Antibiotic resistant organisms have created a global threat as far as management of many diseases especially among immunocompromised patient **(UNAS, 2015; WHO, 2017; CDC, 2017)**. Worldwide, the burden of infections caused by *Shigella* spp was estimated to be 80– 165 million cases of disease and 600,000 deaths annually **(CDC, 2017)**. In Uganda, the burden of infections caused by all bacteria was reported to be 20% including dysentery caused by shigellae **(UNAS, 2015)**. The burden of antibiotics resistance caused by S*higella* spp in Uganda was established by some researchers **(UNAS, 2015).** In studies conducted between 1997-2007 from different part of the country **(Legros *et al*.,1998; Mpairwe, 2000; Atwiine , 2007; Kajumbula, 2014)** showed that, *Shigella* isolates were resistance to most commonly prescribed antibiotics between 36 and 100% to chloramphenicol, ampicillin and co-trimoxazole, but remained lower – between 0 and < 3% (in three of the four studies) – to quinolones. These showed the needs for new, safe and chief antimicrobial agent(s) against *Shigella* spp. Literatures have shown that *Phyllanthus amarus* is used in many countries to treat different diseases. In Africa like Nigeria, **Senjobi *et al*., 2017**, reported that, *Phyllanthus amarus* is used in treatment of gonorrhea, genito-urinary diseases, asthma, diabetes, typhoid fever, jaundice, stomach ache, dysentery, hypertension ringworm and ophthalmic condition. Furthermore, **Ogunjobi and Abiala, 2013** reported that the leave of this plant is used in the treatment of stomach pains and haemorrhoids in Kenya and Southwestern Nigeria respectively. Odda and colleagues reported this herb as being used locally to treat malaria fevers in Tororo, Eastern **Uganda (Odda *et al*., 2018)**. In central Uganda, *Phyllanthus amarus* (In Luganda local language called Nakitembe/Kabalira mugongo) is used for treatment of bacterial dysentery and this is done by boiling fresh leaves of *Phyllanthus amarus* in water for about 30 minutes and when cool the patient is given the resultant soup orally amounting to mug cup of about 500ml twice a day for about a week. Despite the wide herbal use of *Phyllanthus amarus* in the treatment of diarrheal diseases to include dysentery there is a lack of scientific publication as to its efficacy against dysentery caused by *Shigella dysenteriae*. The present work was aimed to determine the antibacterial activity of whole plant of *Phyllanthus amarus* ethanol crude extract against *Shigella dysenteriae*.

MATERIALS AND METHODS

Study design/area and Plant collection

This was experimental laboratory study that determined *Phyllanthus amarus* ethanol crude extract activity on *Shigella dysenteriae*. The study was conducted in January - April 2018 at Kampala International Teaching Hospital Western Campus (KIUTH-WC) microbiology laboratory and pharmacology laboratories. The study was conducted according Clinical Laboratory Standard Institute **(CLSI, 2009; 2018)**
The fresh whole plant of *Phyllanthus amarus* was collected in January, 2018 from Wakiso district central Uganda (00°24′N 32°29′E) where it grows naturally in a banana garden according to the method described by **(Sekar *et al*., 2012).**

Drying and pulverization whole plants of *Phyllanthus amarus*

The collected herb sample was washed, chopped in small pieces and dried in a shade to avoid direct sunshine that could degrade phytochemical due to ultra violet light because the active is not known. The herbs were air dried at room temperature by displaying it on a dry cemented table in pharmacology lab and it was turned daily to prevent fungal attack until complete dryness that was confirmed by weighing every week for three weeks and the constant weight was achieved then the plant was confirmed dry. The dried sample was ground into powder by grinding it using a blender **(Md. Murad *et al*., 2006).**

Extraction of whole plant of *Phyllanthus amarus*

The plant extracts was prepared by using the method of **(Alade and Irobi 1993; Bhasha *et al*., 2014)**. Extraction was performed by macerating air-dried, powdered of *Phyllanthus amarus* in 70% ethanol. The ground whole Plant material was weighed, the initial weight of powder was 375g and it was soaked

in 3.7 liters of 70% ethanol and put on a mechanical shaker for 72h. After 72h of agitation on mechanical shaker, the mixture was sieved using a clean sieve and after the filtration was made using DRs Watts filtering paper of (0.5µm) pore size in a funnel into a measuring cylinder. The filtrate was put in the drying oven at 50°C for concentration, after concentration the weight of the concentrate (extract) was 17.6g then it was stored in the refrigerator at 4°C for further study.

Isolation and Identification of *Shigella dysenteriae*

Stool sample was collected and inoculated in Deoxycholate citrate agar (DCA) and Salmonella Shigella agar (SSA) and incubated at 37°C for 24 hour. Shigella isolates was identified base on colony size, color, texture, on Salmonella Shigella agar (SSA medium) colorless on the media. Gram stain, and further confirmation was done using biochemical test such triple sugar iron (TSI) MR, VP, Indole, Citrate, Urease and Motility test **(Gaurav *et al*.,2013).**

Preparation of test crude extracts stock solution

The stock solution of the whole plant crude extract was achieved by diluting 512mg of the extract in 1000ml of sterile distilled water in order to achieve 512mg.L^{-1} concentration of extract.

Assay of anti-bacterial activity of whole plant ethanol crude extract of *Phyllanthus amarus*

The test organism was aseptically inoculated (approx. 1-2 x 108cfu.mL^{-1}) on sterile Mueller Hinton agar using sterile cotton swab. Using sterile glass cork borers (6 mm in diameter), wells were carefully made on the agar plate without distorting the media. Two hundred micro liters (100µL) of ethanol crude extracts of different concentration (128µg.mL^{-1}, 256µg. mL^{-1} and 512µg.mL^{-1}) was dispensed into the well. Cipro 5µg.mL^{-1} sterile distilled water was used as positive and negative controls respectively. Plates were left on the bench for 10 minutes for crude extract to diffuse in medium. The culture plates were then incubated at 37°C for 24 hours. The diameter of the zones of inhibition was taken in millimeters (mm) using a metric ruler **(Obiagwu *et al*., 2011)**.

Determination of Minimum Inhibitory Concentration (MIC) using Broth dilution method

Minimum inhibitory concentrations (MIC) of whole plant of *Phyllanthus amarus* ethanol crude extract were determined on *Shigella dysenteriae* using tube dilution method **(Obiagwu *et al*., 2011)**. Different concentration was prepared from initial concentration512 µg.mL^{-1} (2µg. mL^{-1} to 256µg.mL^{-1}). Bacterial concentration was adjusted to 0.5 McFarland standard (1-2.0x10^6cfu.mL^{-1}) and 1ml was inoculated in each test tube and incubated for 24h at 37ºC. Two controls were used: one test tube with broth and test organism while the other one contained the broth only. After incubation, the tube with no turbidity next to the one showing turbidity (Microbial growth) was considered as MIC of the extract in question. All extracts that exhibited MIC of 100µg. mL^{-1} and below were considered worth further investigation and Vice versa **(Obiagwu *et al*., 2011)**.

Determination of Minimum Bactericidal Concentration (MBC)

Minimum Bactericidal Concentration was determined according to the method described by **(Obiagwu *et al*., 2011).** Using a sterile wire loop (0.01mL) samples were picked from the broth dilution that didn't show growth (physical turbidity) and inoculated on freshly prepared Mueller Hinton agar and incubated at 37°C for 24 hours. The MBC was determined as the lowest concentration of the extract that allowed less than 0.1% of the original inoculums of 5x10^5cfu.mL^{-1} to grow. The plate that shows no growth of the microorganism was considered as the Minimum Bactericidal Concentration (MBC).

Data analysis

The data collected was entered into Excel. All experiment was done in triplicate and the results were presented as Mean ± SEM. Data was analyzed using STATA v14. One way ANOVA test was used to compare between different concentration of whole plant crude ethanol crude extract and positive control and p value ≤ 0.05 was considered statistically significant.

RESULTS AND DISCUSSION

The use of medicinal plants as alternative medicine is given more attention worldwide **(Flora and Folasade, 2008; WHO, 2017)**. The percentage yield obtained was 4.7% from 375g whole plant powder of *Phyllanthus amarus* used for extraction using 70% ethanol. The antibacterial activity of whole plant of *Phyllanthus amarus* ethanol crude extract was determined by agar well diffusion method and diameter zones of inhibition were measured in mm. The mean and standard error mean zone of inhibition of different concentration (128µg.mL^{-1}, 256µg.mL^{-1}, and 512µg. mL^{-1}) the whole plant of *Phyllanthus amarus* ethanol crude extract against *Shigella dysenteriae* ranged from 11.5±0.5 to 13.5±0.5mm, while the positive control had 24.5±0.5mm **(Table 1)**. There was no activity observed from negative control (sterile distilled water). The comparison between different concentration of whole plant crude extract and positive control using one way ANOVA showed significant different ($p < 0.05$). The ability of the extract to showed antibacterial activity against *Shigella dysenteriae* could be due to the presence of phytochemical property of the plant (tannins, sapoins, cardiac glycosides and alkaloids) as reported by **(Flora and Folasade, 2008)**. Furthermore, **Ogunjobi and Abiala, 2013** also reported the presence of steroids, flavonoid, alkaloids, tannins, phylobatannins and phenol from aqueous and ethanol crude extract leaf of *Phyllanthus amarus*. These compounds have been reported to have effect on bacterial cell wall including Gram negative bacteria which have complex cell wall due to the high content lipid compound. The mean and standard error mean zone of inhibition obtained from this study was high than those reported by **Flora and Folasade, 2008; Ogunjobi and Abiala,2013** from the leaf crude extracts (cold, hot water and ethanol) of *Phyllanthus amarus* against Salmonella typhi which is also Gram negative bacteria. **Ogunjobi and Abiala, 2013** reported higher men and standard error mean zone of inhibition of water and ethanol crude extracts leaf of *Phyllanthus amarus* against *Salmonella typhi* and Escherichia coli which are also enteric bacteria. It was observed that, the sensitivity increased with increasing concentration of the crude ethanol extract. According to **Oluduro and Omoboye (2010)** the antibacterial activities of most plant extracts are concentration dependent as zone of inhibition increased with increasing concentration of the extracts. **Ekwenye and Elegalam (2005) and Azu and Onyeagha (2007)** reported that the efficacy of most plant extracts is concentration dependent. The ability of the whole plant *Phyllanthus amarus* crude extract to inhibit the growth of *Shigella dysenteriae* could due to the presence of phytochemicals constituents of the plants which includes alkaloids, flavonoids, saponins and tannins **(Umbare *et al*., 2009)**. However, **Obianime and Uche (2009)** reported the presence of Flavonoids, tannins, alkaloids, steroids, terpenoids, saponins and glycosides from *Phyllanthus amarus* in their study. **Oluduro and Omoboye (2010)** indicated that the presence of phytochemicals in plant extracts is a function of their antimicrobial activities against the test pathogen as they play important roles in bioactivity of medicinal plants. They further explained that phytochemicals exert antimicrobial activity through different mechanisms. **Chonoko and Rufai (2011)** also indicated that there was a link between the antibacterial activity exhibited by the plant extracts to the presence of steroids flavonoids, tannins, alkaloids and saponins. Tannins, for example, act by iron deprivation, hydrogen binding or specific interactions with vital proteins such as enzymes in microbial cells **(Scalbert, 1991; Akinpelu *et al*., 2008)**. Herbs that have tannins as their component are astringent in nature and are used for the treatment of gastrointestinal disorders such as diarrhoea and dysentery **(Dharmananda, 2003; Asquith and Butler, 1986)**. The test pathogen, *Shigella dysenteriae* was more susceptible to the ciprofloxacin (5µg.mL^{-1}) as the positive control with zone of inhibition at 24.5±0.5mm higher than the ethanol crude extract at all concentrations. The negative control which is the solvent used for dissolving the extract (sterile distilled water) showed no activity. The difference between the activity of the crude extract and the standard antimicrobial drug may be due to the mixtures of bioactive compounds in the crude extract which probably have antagonistic effects against the major bioactive(s) present in the crude extracts compared to the pure compound contained in the standard antibiotic ciprofloxacin.

Table 1 Mean and standard error of the mean activity of different concentration of whole plant of *Phyllanthus amarus* ethanol crude extracts on *Shigella dysenteriae*

Test extract/drug	Zone of Inhibition (mm) Mean ± SEM	Activity Index	p value
128µg.mL^{-1}	11.5±0.5	0.47	
256µg. mL^{-1}	12.5±0.5	0.51	
512µg. mL^{-1}	13.5±0.5	0.55	0.0000
Ciprofloxacin (5µg. mL^{-1})	24.5±0.5	1.00	
Distilled water	0±0	0.00	

The minimum inhibitory concentration of the whole plant of Phyllanthus amarus ethanol crude extracts against *Shigella dysenteriae* was determined using broth tube dilution method. The MIC of ethanol crude extract was found to be 32µg. mL^{-1} (Table 2). Furthermore, Minimum bactericidal concentration (MBC) of whole plant of Phyllanthus amarus ethanol crude extracts against Shigella dysenteriae was determined. The MBC of the crude extract was found to be 64µg. mL^{-1} (Table 2). For test extract whose MIC is <100µg. mL^{-1} is considered as good candidates to be developed into new drugs. However, the results of this study found that the MBC of the ethanol crude extract of whole plant of Phyllanthus amarus was 64µg/mL^{-1}. MBC is the lowest concentration of the extract that allowed less than 0.1% of the original inoculum. The crude extract is said to be bacteriostatic if the ratio of MBC: MIC is equal to 4, whereas it is said to be bactericidal when the ratio of MBC: MIC is equal to 2 **(Kipre *et al.*, 2017).**

Table 2 Minimum inhibitory concentration (MIC) and Minimum Bacteriocidal concentration of *Phyllanthus amarus* ethanol crude extracts against *Shigella dysenteriea*

Type of extract	Concentration of extracts in different test tubes (µg.mL^{-1})									
	128	**64**	**32**	**16**	**8**	**4**	**2**	**1**	**MIC**	MBC
Ethanol	-	-	-	+	+	+	+	+	32	64
Control 1	+	+	+	+	+	+	+	+	+	+
Control 2	-	-	-	-	-	-	-	-	-	-

Key: + means there was growth, - Means there was no grow.

CONCLUSION

The crude extracts of *P. amarus* whole plant exhibited significant in-vitro antibacterial activity against *Shigella dysenteriae* thus indicating their positive role, and justifying the ethno medicinal use of the plant parts in traditional medicine. Further study should be carried out to test whole plant *Phyllanthus amarus* crude extract on different microorganisms that cause dysentery to find out its activity against them and spectrum, determine lead compound in the whole plant *Phyllanthus amarus* crude extract responsible for the anti-microbial activity against *Shigella dysenteriae*, determine the mechanism of action by which this plant crude extract act against *Shigella dysenteriae.*

Acknowledgments: We would like to thanks all staff of Microbiology and Pharmacognosy Laboratories of Kampala International University-Western Campus for their kind support during this research.

REFERENCES

AKINPELU, D.A., AIYEGORO, D.A. AND OKOH, A.I. 2008. In Vitro antimicrobial and Phytochemical Properties of Crude extract of Stem bark of *Afzelia africana* (Smith). *AJB.* 7(20); 3662-3667.

ALADE, P.I., IROBI, O.N. 1993. Antimicrobial activities of crude leaf extracts of Acalyphawilkensiana. *J Ethnopharmacol*. 39; 171–174. https://doi.org/10.1016/0378-8741(93)90033-2

ASQUITH, T.N. AND BUTLER, L.G. 1986. Interaction of condensed tannins with selected proteins. *Phytochemistry*. 25 (7); 1591-1593. https://doi.org/10.1016/S0031-9422(00)81214-5

ATWIINE, A. K. 2007. Determination of Circulating *Shigella* Serotypes and their antimicrobial Susceptibility Patterns in selected Districts of Uganda. (Bachelor of Biomedical Laboratory Technology), Makerere University, Kampala.

AZU, N.C. AND ONYEAGBA, R.A. 2007. Antimicrobial properties of extracts of *Allium cepa* (onions) and *Zingiber officinale* (ginger) on *Escherichia coli*, and *Bacillus subtilis*. *The Internet Journal of Tropical Medicine*.3 (2); 277-286. https://doi.org/10.5580/1f5e

BHASHA, S., KONDETI R. S., SAHUKARI R., VENKATA S.G., KORIVI M., KESIREDDY, S.R. 2014. Antibacterial Activity and Phytochemical Screening of *Phyllanthus niruri* in Ethanolic, Methanolic and Aqueous Extracts. *Int. J. Pharm. Sci. Rev. Res*. 27(2); 85-89.

CENTRE FOR DISEASE CONTROL AND PREVENTION (CDC). 2017. Infectious Diseases Related to Travel. Available on https://wwwnc.cdc.gov/travel/yellowbook/2018/ infectious-diseases-related-to-travel/shigellosis. Accessed5/8/2018.

CLINICAL AND LABORATORY STANDARDS INSTITUTE (CLSI) . 2009. Document M39-A3. Analysis and Presentation of Cumulative Antimicrobial Susceptibility Test Data; Approved Guideline, Third Edition. CLSI, 940 West Valley Road, Suite 1400, Wayne, Pennsylvania 19087-1898, USA.

CLINICAL AND LABORATORY STANDARDS INSTITUTE (CLSI). 2018. Document M100. Performance Standards for Antimicrobial Susceptibility Testing, 28th Edition A CLSI supplement for global application.

DHARMANANDA, S. (2003). Golinuts and the uses of tannins in Chinese Medicine. In proceedings of Institute for Traditional Medicine Portland, Oregon,USA, 25 September, 2003.

EKWENYE, U.N. AND ELEGALAM, N.N. 2005. Antibacterial activity of ginger (Zingiber officinales Roscoe) and garlic (*Allium sativum* L) extracts on *Escherichia coli* and Salmonella typhi. *Journal of Molecular Medicine and Advanced Science* 1(4): 41-416.

FLORA, O. AND FOLASADE, D. 2008. Antimicrobial Effect of *Phyllanthus amarus* and *Parquetina nigrescens* on *Salmonella typhi. African Journal of Biomedical Research*, Vol. 11; 215 – 219. http://dx.doi.org/10.4314/ajbr.v11i2.50712.

GAURAV, A., SINGH, S.P., GILL, J.P.S., KUMAR, R. AND KUMAR, D. 2013. Isolation and identification of *Shigella* spp. from human fecal samples collected from Pantnagar, India, *Vet World* 6(7):376-379. https://doi.org/10.1016/b978-070203481-7.50010-4

CHONOKO, U. G. AND RUFAI, A. B. 2011. Phytochemical screening and antibacterial activity of *Cucurbita pepo* (pumpkin) against *Staphylococcus aureus* and *Salmonella typhi*. *BAJOPAS.* 4(1); 145 –147. http://dx.doi.org/10.4314/bajopas.v4i1.30

KAJUMBULA, H. 2014. Antimicrobial Resistance (T. D. o. M. Microbiology, Trans.): Makerere University College of Health Science.

KIPRE, B. G., GUESSENND, N.K., KONÉ, M.W., GBONON V., COULIBALY, J. K., DOSSO, M. 2017. Antibacterial activity of the stem bark of Tieghemella Heckelii Pierre ex. A Chev against methicillin-resistant *Staphylococcus aureus*. *BMC Complementary and Alternative Medicine*. 17:170. https://doi.org/10.1186/s12906-017-1681-8.

LEGROS, D., OCHOLA, D., LWANGA, N., & GUMA, G. 1998. Antibiotic sensitivity of endemic *Shigella* in Mbarara, Uganda. *East Afr Med* . 75(3); 160-161.

MD. MURAD, H., MOHAMMAD, S. A., ACHINTO, S. AND MD. A. 2006. Antinociceptive Activity of Whole Plant Extracts of *Paederia foetida*. Dhaka Univ. *J. Pharm. Sci*. 5(1-2); 67-69.

MPAIRWE, Y. 2000. Resistance Pattern of *Shigella* Isolates at Naguru Medical Laboratory, Kampala (1995 and 2000) Naguru Medical Laboratory, Kampala.

OBIAGWU, I. N., OKECHALU, O.B. AND NJOKU, M.O. 2011. Studies on Antibacterial Effect of The Leaves of *Phyllanthus niruri* on Some Enteric Pathogens. *Nig J. Biotech*. 23; 22- 27 I.

OBIANIME A.W. AND UCHE F.I. 2009. The comparative effects of methanol extract of Phyllanthus amarus leaves and Vitamin E on the Sperm parameters of Male guinea pigs. *Journal of Applied Sciences and Environmental Management* 13(1): 37-41. https://doi.org/10.4314/jasem.v12i4.55222

ODDA, J., WAAKO, P., OBUA, C., KABASA, J. D. 2018. In vivo antiplasmodial activity and Safety of the aqueous ethanolic extract of *Phyllanthus amarus* Schum. and Thonn . *International Journal of Pharmacy and Pharmaceutical Research*. 12(3):265-280.

OGUNJOBI, A.A. AND ABIALA, M.A. 2013. Antimicrobial Activity of Senna alata and *Phyllanthus amarus. Global Journal of Pharmacology* 7 (2): 198-202

OLUDURO, A. AND OMOBOYE, O. 2010. In Vitro Antibacterial Potentials and Synergistic Effect of South-Western Nigerian Plant Parts Used in Folklore Remedy for *Salmonella typhi* infection. *Nature and Science*, 8(9): 52-59.

SCALBERT, A. (1991). Antimicrobial Properties of tannins.Phytochemistry. 30: 3875-3883. https://doi.org/10.1016/0031-9422(91)83426-l

SEKAR D., KOLANJINATHAN K., SARANRAJ P., GAJENDIRAN K. 2012. Screening of *Phyllanthus amarus*, *Acalypha indica* and *Datura metel* for its antimicrobial activity against selected pathogens. *International Journal of Pharmaceutical & Biological Archives*; 3(5): 1231-1235.

SENJOBI, C.T., ETTU, A. O. AND OTUJO, C.O. 2017. Antibacterial and antifungal activities of leaf extracts of *Phyllanthus amaru* Schum and Thonn. *Journal of Pharmacognosy and Phytotherapy*. Vol. 9(1), pp. 6-10. https://doi.org/10.5897/JPP2013.0261.

UGANDA NATIONAL ACADEMY OF SCIENCES (UNAS) 2015. "Antibiotic Resistance in Uganda: Situation Analysis and Recommendations, ISBN: 978-9970-424-10-8. A4 Lincoln House Makerere University P.O. Box 23911, Kampala, Uganda, 2015.https://cddep.org/wp-content/uploads/2017/08/garp-uganda_sa.pdf. accessed 20/9/2016. https://doi.org/10.20319/pijss.2018.41.477493

UMBARE, R. P., MATE1, G. S., JAWALKAR, D.V., PATIL, S. M. AND DONGARE, S. S. 2009. Quality evaluation of *Phyllanthus amarus* (Schumach) leaves extract for its hypolipidemic activity. *Biology and Medicine*1 (4): 28-33.

WHO 2017. WHO guidelines on good herbal processing practices (GHPP) for herbal medicines. Available at http://www.who.int/medicines/areas/quality_safety/quality_assurance/V-2ndGlobalReview-RevisedDraft-WHO-GHPP-March2017.pdf. Accessed 9/8/2018

OPEN ACCESS

BACTERIAL EMPIRE

2019, VOL. 2, NO. 2, 37-40

SCICELL

SHORT COMMUNICATION

THE PREVALENCE OF FOODBORNE PATHOGENS RECOVERED FROM READY-TO-EAT FOOD FROM RESTAURANTS IN OKADA, EDO STATE, NIGERIA

Akpoka Augustine Obhioze [1], Imade Odaro Stanley[1], Maureen U. Okwu[1] and Solanke Ezekiel Olatoye*

Address (es):

[1] Department of Biological Sciences, College of Natural and Applied Sciences, Igbinedion University Okada, Edo State, Nigeria

*Corresponding author: ausbones@gmail.com , ausbones@iuokada.edu

ABSTRACT

This study assessed the microbiological quality of various ready-to-eat cooked food and their contact surfaces in selected restaurants in the university town of Okada, Edo state, Nigeria. Microbial analysis was carried out on the samples obtained from six restaurants. The restaurants sampled were the ones with the highest patronage within the study area. The results obtained indicated that most of the ready-to-eat food did not meet the required quality standards, and therefore, posed potential risks of foodborne infections to consumers. Exposure assessment was used to evaluate the level of hygiene practices in the selected restaurants with results indicating poor hygiene practice. The organisms isolated from the ready- to-eat cooked foods in the restaurants and their prevalence were, *Bacillus* species (12.50%), *Enterobacter* species (50.00%), *Streptococcus* species (12.50%), *Micrococcus* species (12.50%) and *Staphylococcus aureus* (12.50%), from the food contact surfaces (Hands of ready-to-eat food servers) were, *Enterobacter* species (36.36%), *Streptococcus* (9.09%), *Micrococcus* (9.09%) *Staphylococcus* (45.46%) and ready-to-use serving plates (*Streptococcus* (33.33%) and *Enterobacter* sp. (50%). Haemolysis test was employed as a phenotypic marker of pathogenicity for *Staphylococcus aureus* and *Streptococcus* sp. The prevalence of the pathogenic strains isolated from the ready-to-eat food were *Streptococcus* sp. (9.09%) and *Staphylococcus* sp. (45.46%) and that of the one isolated from the serving plate was *Streptococcus* sp. (33.33%).

Keywords: Microbial quality, Prevalence, Foodborne pathogens, Food poisoning, Food intoxication, Food contamination, Public Health, ready-to-eat food, Nigeria.

INTRODUCTION

Foodborne diseases include an array of illnesses triggered by ingestion of foods contaminated with microorganisms or chemicals **(WHO, 2014).** It has become a major cause of morbidity and mortality, posing a major public health challenge globally and contributing to a marked economic loss, reduction in quality of life and productivity **(Scharff, 2012**). Although there are no reliable estimates for the burden of diarrheal diseases alone, it however makes up a sizeable proportion of foodborne diseases. It is estimated that 1.9 million children die globally every year due to foodborne infections. **(WHO, 2014).** Cooked Foods are ideal culture medium for growth of many organism and these Ready-to- eat cooked food can be contaminated at any stage in the process of preparation or serving by foodborne pathogens capable of causing foodborne illnesses.

Foodborne pathogens are a leading cause of illness and death in developing countries amounting to billions of dollars in medical care and social costs. There is now an increased public health concern as a result of the frequent reports of food poisoning in relation to the potential presence of pathogenic organisms in food. This problem is aggravated by the massive changes in eating habits, mass food preparation, unsafe food storage conditions and poor hygiene practices amongst food handlers.

It is worthy of note that the microbial assessment of restaurants is not carried out as part of the inspection process of restaurants. This is probably due to the time limitations of traditional microbial analyses which takes up to 48 hours for culturing, isolation and subsequent evaluation. This is a major challenge since bacterial and viral contaminations are not detectable by visual assessment. The use of hygiene swabs and agar contact plates have shown that visual inspection is a poor indicator of cleaning **(Griffith *et al.*, 2000; Moore & Griffith, 2002).** Most restaurants even do not consistently clean certain surfaces outside the kitchen particularly true for furniture, equipment and other frequently used items. Some of the factors that contribute to the outbreaks of the foodborne disease are (i) inadequate food manipulation (ii) improper holding temperatures (failing to properly refrigerate food) (iii) inadequate cooking (iv) contaminated equipment (failure to clean and disinfect kitchen or processing plant equipment) and (v) poor personal hygiene (vii) preparing food a day or more before serving with improper holding and reheating (vii) cross contamination (from raw to cooked products) and (viii) adding contaminated ingredients to the previously cooked food. After foods are contaminated, the main factor is the appropriate temperature that supports the growth of the potentially hazardous microorganisms or its toxin production in the food.

Foodborne infections are caused by the ingestion of food containing pathogenic microorganisms which multiply within the gastrointestinal tract, producing widespread inflammation and have become a significant public health problem with major economic and social effects **(Altekruse and Swerdlow, 1996).** They have an incubation periods usually from 6 to 24-hour longer after ingestion and the causative organism may be identified by laboratory examination of the vomits, faeces, or blood of the infected individual and the suspected food **(Sudershan *et al.*, 2014).** It is usually characterized by diarrhea (which may be sometimes bloody) and vomiting. Symptoms may include fever and cold, headache, nausea, abdominal pain and cramps, distress, and weakness which in some instances may lead to respiratory arrest; other symptoms include signs of shock which include weak or rapid pulse or shallow breathing, confusion **(Mead *et al.*, 1999).** In developing countries, an estimated 70% of diarrheal episodes are associated with the ingestion of contaminated foods **(WHO, 2008).** Approximately 10 to 20% of foodborne disease outbreaks result from contamination of foods by the food handlers **(Gizaw *et al.*, 2014; Zain and Naing, 2002).**

The surge in urbanization has increased the frequency of eating of meals outside the home and despite this growth, there is little or no effective education or training for the food handlers or hygienic control of the food sold in many restaurants. The contamination of food with pathogens and its persistence, growth, multiplication and/or toxin production is now a significant public health concern. Despite this, only a fraction of all food-borne infections is ever diagnosed and officially reported or can be traced to a definite vehicle **(Lukinmaa *et al.*, 2004).**

The problems of food safety are considerably different between the industrialized and developing countries. The hygienic standard of food is usually assessed by the analysis of indicator microorganisms **(Forsythe and Hayes, 1988).** Some of the major microorganism groups are used alone or together to verify the microbiological characteristics and the hygienic condition of the food. Faecal coliforms like *Escherichia coli* are used as an indicator of the sanitary condition. It is a typical component of the faecal microbiota and its detection may show the potential occurrence of other microorganisms which could be even more pathogenic **(Sousa, 2005).** Several *E. coli* pathotypes have been implicated with the diarrheal illness, a major public health problem worldwide, with over two million deaths occurring each year **(WHO, 2002).**

Cross-contamination during food preparation is identified as a critical factor associated with the foodborne illness **(Wanyenya *et al.*, 2004).** Food handlers also play a role in ensuring food safety in food preparation, processing and storage chain. Complete or partial disregard for the hygiene measures by food handlers may result in food contamination and its attendant consequences. A sizeable proportion of foodborne illnesses could be prevented by the actions taken by the food handlers and there is the need to reduce these risks by taking steps to train the people involved in food handling **(Gilling *et al.*, 2001).** The cleaning procedures for the food contact surfaces should be evaluated with special attention given to the utensils used during the processing (Souza *et al.*, 2003). The U.S. Food and Drug Administration (FDA) recommends that food be

prepared with the least possible manual contact, with suitable utensils, and on the surfaces that prior to use have been cleaned, rinsed and sanitized to prevent cross-contamination **(FDA, 1978).**

Staphylococcus aureus is a major human pathogen with capabilities of causing a wide range of infections. Staphylococcal food poisoning is caused by the enterotoxigenic producing *S. aureus* and an important foodborne disease throughout the world. Many staphylococcal strains produce enterotoxin, the cause of the staphylococcal food poisoning. Enterotoxigenic strains have a high possibility of being implicated in food poisoning **(Bergdoll, 2000).**

Salmonella is a leading cause of human foodborne infections. The risk factors regarding the cross contamination of food include inapt cleaning and disinfection, manipulation of contaminated materials as such and (re)contaminated surfaces **(Berends *et al.*, 1998).** The isolation of microorganisms and subsequent cultivation on culture medium is the first basic step in more detailed investigations of the nature, source, and potential risk of the microbial contamination of the food **(Allen *et al.*, 2004**). This research was therefore carried out to evaluate the prevalence of the foodborne pathogens recovered from ready-to-eat food ad their contact surfaces from restaurants within and around Okada, Edo state, Nigeria.

MATERIALS AND METHODS

Study sites

Six restaurants selected for this study are all located in the university town of Okada headquarters to Ovia North-East Local Government Area of Edo State, Nigeria. It has an area of 2,301 km² and a population of 153,849 at the 2006 census.

Sampling

Ready-to-eat cooked foods were collected in sterile containers from the selected restaurants in Okada and were transported to the Laboratory of Igbinedion University, Okada for analysis within 6 hours of sample collection. One-gram portion of the food samples was used to prepare a 10-fold serial dilution to 10^{-5} in 1.5 % W/V sterile peptone water, after which 0.1 ml of each diluted suspension was subsequently spread on triplicate petri-plates containing sterile solidified media.

Collection of samples

The samples were collected from washed ready-to-use serving plates and the hands of food handlers in the sampled restaurants. Sampling was done by swabbing 25 cm^2 area of the contact surfaces with 5 sterile swab sticks according to the method specified by ISO 18593:2004 **(ISO, 2004).** The area of sampling was delimited by sterile templates (an improvised wire that was used to properly define the area of sampling). After swabbing, the swab sticks were put into a sterile container containing 10 ml of 2 % W/V sterile peptone water and was then transported to the Microbiology Laboratory in Igbinedion University, Okada, Edo State, Nigeria where they were analyzed within 6 hours of sampling. 0.1 ml of each suspension was subsequently spread on triplicate petri-plates containing sterile solidified media.

Isolation of microbes from food samples and contact surfaces of foods was performed by spread plating method **(APHA, 1998),** using both general purpose medium (nutrient agar) and selective/differential media (MacConkey agar complemented with crystal violet, and mannitol salt agar). After incubation of agar plates at 37ºC for 18 to 24 hours, distinct colonies seen on the plates were then enumerated and identified.

Identification and characterization of microbes

Phenotypic identification of microbes was performed according to standard methods **(Barrow and Feltham, 2003).** Expressed microbial morphological traits examined include the orientation, size, and pigmentation which were performed by visual inspection of microbial isolates on petri-plates, as well as cell wall characteristics which was performed by Gram staining of the isolates. Expressed biochemical traits examined include: the production of coagulase enzyme (coagulase test); the production of catalase enzyme (catalase test); the production of urease enzyme (urease test); biodegradation of tryptophan to produce indole (indole test); utilization of citrate as a sole carbon source (citrate test); production of stable acids from glucose fermentation (methyl red test); production of acetoin as the main end product with small quantities of mixed acids from glucose metabolism (Voges Proskauer test); and production of haemolysins (hydrolysis test)

Exposure assessment

Exposure assessment was used to quantitatively evaluate the impact of hygiene practices by restaurants situated in Igbinedion University and environs **(Cassini *et al.*, 2016).** The probability of exposure of consumers to pathogenic microbial species isolated from ready-to-eat food samples and food contact surfaces such as ready-to-use serving plates and hands of food handlers was deduced from the prevalence of pathogenic species present in these samples, while the concentration of the microbial isolates in the examined samples was used to deduce the extent of contamination in the restaurants. Parameters such as total aerobic viable counts, total coliform counts, and total *Staphylococcus* counts were also determined.

The microbial counts were performed using the spread plate method **(APHA, 1998).** Total aerobic viable counts (TAVC), total coliform counts (TCC), and total halophilic counts (THC) were carried out by spread plating on sterile nutrient agar, MacConkey agar supplemented with crystal violet, and mannitol salt agar respectively.

The microbial counts and prevalence (the presence of a single microbial isolate in a given sample) of the food samples were then calculated using the following equations:

$$Microbial\ counts = \frac{number\ of\ colonies\ counted}{volume\ of\ sample\ inoculated} \times dilution\ factor \quad (1)$$

Where:

Microbial counts were expressed as colony forming units per gram of food samples (cfu/g); Dilution factor (*d*) is expressed as the reciprocal of specific dilution counted ($\frac{1}{d}$).

$$Microbial\ prevalence = \frac{Number\ of\ a\ specific\ microbe\ isolated\ from\ the\ food\ samples}{Total\ number\ of\ all\ microbes\ isolated\ from\ the\ food\ samples} \quad (2)$$

The microbial counts and prevalence in the food contact surfaces were deduced using the following equations:

$$Microbial\ count = \frac{Number\ of\ colonies\ counted}{Area\ equivalent\ of\ the\ volume\ of\ inoculated\ sample} \quad (3)$$

Where:

Microbial count was expressed as colony forming units per square centimeter of contact surface (cfu/cm^2).

$$Area\ equivalent\ of\ the\ volume\ of\ inoculated\ sample = \frac{Volume\ of\ inoculated\ sample\ x\ Total\ Area\ of\ sampled\ surfaces}{Total\ volume\ equivalent\ of\ swabbed\ area} \quad (4)$$

Given that:

Volume of inoculated sample = 0.1 ml; Total area of sampled surface = 25 cm^2; Total volume equivalent of swabbed area = 10 ml

$$Microbial\ prevalence = \frac{Number\ of\ a\ specific\ microbe\ isolated\ from\ the\ food\ contact\ surface}{Total\ number\ of\ all\ microbes\ isolated\ from\ the\ food\ contact\ surface} \quad (5)$$

RESULTS

Table 1 Concentration of microbes on Contact surfaces (hands of ready- to - eat food servers and ready to use serving plates) in selected restaurants.

Sources of Microbes	Restaurant	Concentration of Microflora			*Hm	Confirmed Isolated Microorganism
		Mean TAVC (cfu/cm^2)	Mean TCC (cfu/cm^2)	Mean TSC (cfu/cm^2)		
Hands of ready- to - eat food servers	1	12.33 ± 0.33	1.33 ± 0.33	5.00 ± 0.58	γ β	*Enterobacter* species *Streptococcus* species
	2	3.33 ± 0.33	1.00 ± 0.00	3.00 ± 0.00	β γ	*Staphylococcus aureus* *Enterobacter* species
	3	22.67 ± 0.33	0.00 ± 0.00	20.33 ± 0.33	γ β	*Enterobacter* species *Staphylococcus aureus*
	4	10.67 ± 0.33	0.00 ± 0.00	8.00 ± 0.00	β γ	*Staphylococcus aureus* *Enterobacter* species
	5	6.67 ± 0.33	0.00 ± 0.00	5.00 ± 0.00	γ γ	*Enterobacter* species *Micrococcus* species
	6	14.33 ± 0.33	0.00 ± 0.00	12.33 ± 0.33	β β	*Streptococcus* species *Staphylococcus aureus*
Ready-to-Use serving plates	1	14.67 ± 0.33	0.67 ± 0.33	0.00 ± 0.00	γ	*Enterobacter* species
	2	5.67 ± 0.33	1.00 ± 0.00	2.33 ± 0.33	γ	*Enterobacter* species
	3	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	γ	*Enterobacter* species
	4	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	γ	*Saccharomyces* species
	5	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	β	*Streptococcus* species
	6	12.67 ± 0.33	1.33 ± 0.33	10.33 ± 0.33	β	*Streptococcus* species

TAVC: Total aerobic viable counts; TCC: Total coliform counts; TSC: Total *Staphylococcus* counts; SE: Standard error; cfu: colony forming units; cm^2: square centimeter. Hm: Haemolysis test; β represents complete haemolysis; γ represents no haemolysis.

Table 2 Prevalence (%) of each microbe found in the Six Restaurants sampled in the Area

Source	Isolated Microbes	Prevalence
Ready to Eat Food	*Enterobacter* sp.	4/8 (50.00 %)
	Bacillus sp	1/8 (12.50 %)
	Streptococcus sp.	1/8 (12.50 %)
	Micrococcus sp.	1/8 (12.50 %)
	Staphylococcus aureus	1/8 (12.50 %)
Hands of ready – to - eat Food Servers	*Enterobacter* sp.	4/11(36.36 %)
	Streptococcus sp.	1/11(9.09 %)
	Micrococcus sp.	1/11(9.09 %)
	Staphylococcus aureus	5/11(45.46 %)
Ready-to -use Serving Plates	*Streptococcus* sp.	2/6(33.33 %)
	Enterobacter sp.	3/6(50.00 %)
	Saccharomyces sp.	1/6(16.67 %)

DISCUSSION

Exposure assessment was used to evaluate the level of hygiene practices by the restaurants sampled. The probability of exposure of consumers to pathogenic microbial species isolated from ready-to-eat food samples and food contact surfaces such as ready-to-use serving plates and hands of food handlers was calculated from the prevalence of pathogenic species present in these samples. The concentration of the microbial isolates reflected the degree of contamination in the restaurants. The total aerobic viable counts (TAVC) is an indicator of the overall hygiene status in the restaurants ranged from 5.67 cfu/cm^2 – 22.67 cfu/cm^2 and represented in Table 1. The total coliform counts (TCC) is an indicator of potential faecal contamination of ready-to-eat and contact surfaces in the restaurants ranged from 0.67 cfu/cm^2 – 1.33 cfu/cm^2. The total *Staphylococcus* counts (TSC) indicated the unsanitary food handling in the restaurants. These parameters are extensive measures of the degree of contamination in these restaurants. The ready to eat food sampled across the six restaurants were fried rice, soup and jollof rice. These menus were picked because they were high in demand in these restaurants as well being available in almost the restaurants. These ready-to-eat foods yielded a high mean concentration of TAVC and TCC, with only three of these restaurants having no TSC. The organisms isolated from the ready to eat foods in the restaurants and their prevalence were *Bacillus* species (12.50%), *Enterobacter* species (50%), *Streptococcus* species (12.50%), *Micrococcus* species (12.50%) and *Staphylococcus aureus* (12.50%) and presented in Table 2. *Bacillus* sp and *Staphylococcus* sp have also been isolated from similarly prepared ready- to- eat-foods in previous studies **(Nichols *et al*., 1999; Mensah *et al*., 2002; Idowu, 2006; Taulo *et al*., 2008 and Oranusi *et al*., 2013).**

The food contact surfaces (Hands of ready-to-eat food servers and ready-to-use serving plates) also had high microbial loads. The prevalence of the organism isolated from the hands of ready to eat food servers were *Enterobacter* sp. (36.1%), *Streptococcus* sp. (9.09%), *Micrococcus* sp. (9.09%) and *Staphylococcus aureus* (45.46%). This was in agreement with the result of **Almeida *et al*., (1995)** who worked on the microbiological analyses of food workers' hands and revealed a high possibility of cross contamination of food pathogens by food handlers. In their study, the food workers' hands showed aerobic mesophilic plate counts of up to 10^7CFU/hand and the presence of *S. aureus and Clostridium perfringens*. The ready-to-use serving plates had the following isolates *Streptococcus* (33.3%), *Enterobacter* (50%) and

Saccharomyces (16.1%). *Enterobacter* sp. and *Streptococcus* were isolated from all the three-sample source while *Staphylococcus* and *Micrococcus* were isolated from only the food and the hands of the food servers and handlers. The haemolysis test was employed as a phenotypic marker of pathogenicity. The prevalence of isolated organism positive for pathogenicity in the ready-to-eat food were *Staphylococcus aureus* (12.50%), *Streptococcus* spp. (12.50%), hands of the food handlers were *Streptococcus* sp (9.09%) and *Staphylococcus sp.* (45.46%) and the one isolated from the serving plate was *Streptococcus* sp. (33.33 %). The isolation *Enterobacter* an enteric is an indication of a possible faecal contamination of the food, their food contact surfaces and poor hygiene practices **(Little *et al*., 1998; Tambekar *et al*., 2007).** High concentrations of coliforms in food is often associated with food poisoning **(WHO, 1993).** Ready-to-eat foods cooked food must be free from microorgaisms as well as other contaminants as much as possible. The presence of any of these isolates establishes a potential health risk as these organisms are pathogenic and are often implicated in foodborne diseases **(Granum, 2005; Wagner, 2009; CFIA, 2009).**

CONCLUSION

The obtained results in this study reveal a high possibility of cross contamination of food from food handlers and contact surfaces sampled. Organisms like *Enterobacter* sp., *Bacillus* sp, *Streptococcus* sp., *Micrococcus* sp., *Staphylococcus aureus*, *Streptococcus* sp. were isolated from the food and contact surfaces. It is recommended that some preventive measures be adopted to avoid contamination of cooked food like the routine examination of cooked food in restaurants by the relevant authorities and consequent sanctions if minimum standard are violated, hygiene awareness for personnel who prepare and handle ready to eat cooked food, training on the adoption on the integration of Hazard Analysis Critical Control Point (HACCP) procedures into the food preparation and production process.

REFERENCES

ALLEN, M. J., EDBERG, S.C., REASONER, D. J. 2004. Heterotrophic plate count bacteria-what is their significance in drinking water? Int. J. Food Microbiol., 92, 265-274.

ALMEIDA, R.C., KUAYE, A.Y., SERRANO, A. M., ALMEIDA, P.F., 1995. Evaluation and control of the microbiological quality of hands in food handlers. Rev. Saude Publica, 29, 290-294.

APHA, 1998. Standard Methods for the Enumeration of Water and Wastewater. 20th ed., Washington DC., United States. 38 p.

BARROW, G.I., FELTHAM, R.K.A. 2003. Cowan and Steel's Manual of Medical Bacteria. 3rd ed. Cambridge University Press. 352 p.

BERENDS, B.R., VAN KNAPEN, F., MOSSEL, D.A., BURT, A.S., SNIJDERS, J.M. 1998. Salmonella spp. on pork at cutting plants and at the retail level and the influence of particular risk factors. Int. J. Food Microbiol., 44, 207-217.

BERGDOLL, M.S. 2000. Staphylococcus até onde sua importância em alimentos? Hig. Alim., 14, 32-40.

CASSINI, A., HATHAWAY, S., HAVELAAR, A., KOOPMANS, M., KOUTSOUMANIS, K., MESSENS, W., SCHEUTZ, F. 2016. Microbiological risk assessment. EFSA Journal 14, 1-10.

CFIA. 2009. Canadian Food Inspection Agency, *Salmonella* Food Safety Facts. Preventing foodborne illness.

FDA, 1978. Food Service Manual 1978. *U.S. Department of Health, Education and Welfare, Public Health Service*. DHEW Publ. Nº. (FDA), 78-2081, Washington, DC.

FORSYTHE S. J.; HAYES, P. R. 1988. Food Hygiene, Microbiology and HACCP. A Chapman and Hall Food Science Book. Aspen Publishers, Gaithersburg.

GILLING, S., TAYLOR, E. A., KANE, K., TAYLOR, J. Z. 2001. Successful hazard analysis critical control point implementation in the United Kingdom: understanding the barriers through the use of a behavioral adherence model. J. Food Protect., 64, 710-715.

GIZAW, Z, GEBREHIWOT, M, TEKA, Z, 2014. Food Safety Practice and Associated Factors of Food Handlers Working in Substandard Food Establishments in Gondar Town, Northwest Ethiopia. Int. J. Food Sci. Nutr. Diet., 3(7), 138-146.

GRANUM, P.E. 2005. *Bacillus cereus* as a Food-borne Pathogen. Microbiology and Molecular Biology. pp. 409 - 420. Great Britain. Caister Academic Press.

GRIFFITH, C. J., COOPER, R. A., GILMORE, J., DAVIS, C., LEWIS, M. 2000. An evaluation of hospital cleaning regimes and standards. Journal of Hospital Infection, 45, 19-28.

IDOWU, O. A., 2006. Oral faecal parasites and personal hygiene of food handlers in Abeokuta, Nigeria. Africa Health Science, 6**,** 160 - 164.

ISO, 2004. Horizontal methods for sampling techniques from surfaces using contact plates and swabs. *Microbiology of Food and Animal Feeding Stuffs*. (ISO 18593:2004). British Standards Institution, London. 14 p.

LITTLE, C. L. 1998. Monsey, H. A., Nichols, G. L. and de Louvois, J. The microbiological quality of ready-to-eat dried and fermented meat and meat products. International Journal of Environmental Health Research, 8, 277-284.

LUKINMAA, S., NAKARI, U., EKLUND, M., SIITONEN, A. 2004. Application of molecular genetic methods in diagnostics and epidemiology of food-borne bacterial pathogens. APMIS, 12, 908-929.

MEAD, P.S., SLUTSKER, L., DIETZ, V., MCCAIG, J.S., SHAPIRO, C., GRIFFIN, P.M., TAUXE, R.V. 1999. Food related illness and death in the United States. J. Emerg. Infect. Dis., 5:607-622.

MENSAH, P., YEBOAH-MANU, D., OWUSU-DARKO, K., AND ABLORDEY, A. 2002. Street foods in Accra, Ghana: How safe are they? Bulletin: World Health Organization, 80(7), 546 - 554.

MOORE, G., GRIFFITH, C. 2002. A comparison of traditional and recently developed methods for monitoring surface hygiene within the food industry: an industry trial. International Journal of Environmental Health Research, 12, 317-329.

NICHOLS, S. L., LITTLE, C. L., MITHANI, V. AND DE LOUVOIS, J. 1999. The microbiological quality of cooked rice from restaurants and take-away premises in the United Kingdom. Journal of Food Protection, 62: 877 - 882.

ORANUSI, S. U., OGUOMA, O. I., AGUSI, E. 2013. Microbiological quality assessment of foods sold in student's cafeterias. Global Research Journal of Microbiology, 3(1), 1 - 7.

SCHARFF R.L., 2012. Economic Burden from Health Losses Due to Foodborne Illness in the United States.J Food Prot, 75(1),123-31.

SOUSA, C. P. (2005), The strategies of Escherichia coli pathotypes and health surveillance. Revisa, 1, 65-70.

SUDERSHAN, R.V., NAVEEN, K.R., KASHINATH, L., BHASKAR, V., POLASA, K. 2014. Foodborne Infections and Intoxications in Hyderabad India. Epidemiol. Res. Int., 1, 5.

TAMBEKAR, D. H., SHIRSAT, S. D., SURADKAR, S. B., RAJANKAR, P. N., BANGIN WAR, Y. S. 2007. Prevention of transmission of infectious disease: Studies on hand hygiene in health-care among students. Continental Journal of Biomedical Sciences, 1, 6 -10.

TAULO, S., WETLESEN, A., ABRAHAMSEN, R., MKAKOSYA R., KULULANGA, G. 2008. Microbiological quality of water, associated management practices and risks at source, transport and storage points in a rural community of Lungwena, Malawi. Africa Journal of Microbiological Research, **7**(2), 131-137.

WAGNER, A. B. 2009. Epidemiology of food poisoning outbreaks in Singapore, 2001- 2005. Epidemiological News Bulletin, Journal of Microbiology, 31, 68 -72.

WANYENYA, I., MUYANJA, C., NASINYAMA, G.W. 2004. Kitchen practices used in handling broiler chickens and survival of Campylobacter spp. on cutting surfaces in Kampala, Uganda. J. Food Protect., 67, 1957-1960.

WHO, 1993. World Health Organization Food Safety Unit. Contaminated food: a major cause of diarrhoea and associated malnutrition among infants and young children. Facts Infant Feed, 1, 1–4.

WHO, 2008. WHO Initiative to Estimate the Global Burden of Foodborne Diseases. Geneva, Switzerland. pp. 1-11

WHO, 2014. Burden of foodborne diseases.

ZAIN, M.M., NAING, N.N. 2002. Socio-demographic characteristics of food handlers and their knowledge, attitude and practice towards food sanitation: A Preliminary report South-East Asian J. trop. Med. Public Health, 33(2),410-417.

OPEN ACCESS

BACTERIAL EMPIRE

2019, VOL. 2, NO. 2, 41-45

SCICELL

REGULAR ARTICLE

OPTIMAL CONDITIONS FOR β-MANNANASE PRODUCTION BY *BACILLUS CIRCULANS* NT 6.7

Jurairat Rungruangsaphakun[1] and Suttipun Keawsompong[1,2]*

Address (es): Mr. Adamu Almustapha Aliero,
[1] Department of Biotechnology, Faculty of Agro-Industry, Kasetsart University, Bangkok 10900, Thailand.
[2] Special Research Unit: Probiotic and Prebiotics for Health, Center for Advanced Studies for Agriculture and Food)CASAF(, Institute for Advanced Studies, Kasetsart University, Bangkok, Thailand.

*Corresponding author: fagisuk@ku.ac.th

ABSTRACT

Optimum medium for mannanase production is very important and producing medium has been shown effective for mannanase production from *Bacillus circulans* NT 6.7 but expensive. This work aim to find low cost medium and optimum condition for mannanase production from *Bacillus circulans* NT 6.7. 1.5% concentration of defatted copra meal as carbon source in shake flask resulted in highest growth and enzyme activities of 10.51±0.53 Log CFU/ml and 12.84±4.38 U/ml at 18 hours accordingly. Also, 2% defatted copra meal in fermenter produced highest growth of 7.94±0.41 Log CFU/ml and enzyme activities of 32.07±2.94 U/ml at 15 hours. Using taguchi design, optimal conditions for inoculum, agitation and aeration were 1%, 600 rpm and 0.75 vvm, respectively. Under this optimized condition, the mannanase experimental yield (29.57±4.81U/ml) closely matched the yield predicted by the statistical model (24.15U/ml) with $R^2 = 0.75$. Using mineral salts medium, slightly increased in mannanase product was observed compare to previously used producing medium. Also, the cost of using mineral salts medium (cost per liter) is 27 time lower than the cost of producing medium. Suggesting that, mineral salt medium provides an economically feasible medium for producing mannanase from *Bacillus circulans* NT 6.7.

Keywords: *Bacillus circulans* NT 6.7, optimal condition, mannanase production, defatted copra meal, mineral salt medium

INTRODUCTION

Copra meal or coconut meal is a by-product from coconut milk and coconut oil processing. After pressing or extracting the oil, the remaining residue is called copra meal. Copra meal structure is galactomannan that consists of beta-D-1, 4-mannopyranose backbone with branch points from their 6-positions linked to alpha-D-galactose **(Burhanudin and Dingle, 2006)**. **Lin and Chen) 2004(** reported the proximate analysis of copra meal and defatted copra meal. Copra meal and defatted copra meal had protein content of 4.7 and 19.2%)w/w(, carbohydrate content was 25.3 and 80.1%)w/w(, fat content of 72.1 and 0%)w/w(, ash content of 1.8 and 1.6%)w/w(and moisture content of 2.7 and 2.8%)w/w(, respectively. Copra meal residue after the coconut oil processing had fat content in ranges from 1% to 22% **Göhl (1982(**. Copra meal is good substrate for produce the mannanase because the copra meal contains 5-30% mannan of pure mannan and galactomannan. Residue cake from coconut frequently discharged as a by-product in the process of oil extraction from copra, which contains large amount of mannose in the form of copra mannan **)Hossain *et al.*, 1996(**.

Beta-mannanase)Endo-1, 4-β-mannanase, EC.3.2.1.78(is hydrolytic enzymes which catalyze randomly β-1,4 mannopyranosyl linkages within the backbones of mannan, glucomannan, galactomannan and galactoglucomannan **)Stoll *et al.*, 1999(**. This enzyme is produced by several living organisms and it has been widely used in the industrial application. *Bacillus circulans* NT 6.7 was isolated from soil of coconut industry from Nakornpathom province. Beta-mannanases from *Bacillus circulans* NT 6.7 has high enzyme activity for locust bean gum) LBG(, 0.306 unit/ ml with appropriate optimum temperature and pH for industrial applications **)Phothichito *et al.*, 2006(**. Defatted copra meal used for mannanase production in a producing medium in 5-l fermenter cultivation: 600 rpm, 0.75 vvm and 45 °C. *Bacillus circulans* NT 6.7 exhibited the highest cell growth and mannanase activity with 9.39 Log CFU/ml and 27.70 units/ml at 6 hours, respectively. **) Pangsri *et al.*, 2015(** . These results were similar to the results of **Phothichitto *et al.*) 2006(** , who reported the optimum pH and temperature of crude enzymes from *Bacillus circulans* NT 6.7 were pH 6.0 and 50 °C, respectively.

Beta-mannanases are useful in many fields including in oil drilling operations **) You *et al.*, 2016(** , bioconversion of biomass wastes to fermentable sugars **)Chandrakant and Bisaria, 1998(**, upgrading of animal feed stuff **)Ray *et al.*, 1982(**, and reducing the viscosity of coffee extracts and fruit juice clarification **)Chauhan *et al.*, 2014(**. They also have potential application in the production of mano-oligosaccharides that are selectively utilized by intestinal *Bifidobacterium spp*. and also as valuable food sweetener or additive **)Tomotari, 1990(**.

Media composition play important role in the growth and overall activities of microorganisms. Different ingredients of media such as carbon sources, nitrogen sources, minerals, metal ions, polyols and additives have different effects on growth and activity of microorganisms. Thus, these parameters needs to be optimized to obtain optimal growth condition for microorganism. Optimum condition for better production yields of desired enzyme at low cost is a significant parameter for making the production process industrially viable.

The aims of this study was to develop a low-cost medium and find the optimal condition for mannanase production by *Bacillus circulans* NT 6.7. Preliminary data from the study showed that mineral salts medium has a lower price than other medium. Hence, was used in the study to produce mannanase.

MATERIAL AND METHODS

Materials

The copra meal was dried at 60 ºC for 4 hours. After that, the copra meal were blended and milled by Hammer mill to obtain the product with the particle size of 0.5 mm. The defatted copra meal was prepared by oil extraction in Soxhlet apparatus for about 4-6 hours.

Development of culture medium

Bacillus circulans NT 6.7 was used in the enzyme production. The bacteria was kept -20 ºC in Nutrient broth)NB(containing 20%)w/v(glycerol solution, and propagated twice at 45 ºC before use. NB medium was used to cultivation of inoculum for produce enzyme. The mineral salts medium) MSM(for fermentation study was prepared by adding 1% defatted copra meal and producing enzyme medium)PM(adding 1% defatted copra meal used as control. The MSM was chosen to study the effects of carbon source using defatted copra meal at varying concentrations)0.5% , 1% , 1.5% and 2% (w/v)(. The best concentration for highest activity was used to study in a fermenter.

Bacillus circulans NT 6.7 was used in the fermentation experiments followed the condition for enzyme production by **Pangsri *et al.*) 2015(**. The nutrient broth medium was used for cultivation of inoculum for the fermentation in the fermenter. The MSM for fermentation study was prepared by adding)1%, 1.5%, 2% (w/v)(defatted copra meal, NH_4NO_3 0.03% , $MgSO_4.7H_2O$ 0.02 % $FeSO_4.7H_2O$ 0.001 % $CaCl_2.2H_2O$ 0.005 % K_2HPO_4 0.754 %, KH_2PO_4 0.232 %, adapted from **Mabrouk and Ahwany (2008**(and was autoclaved at 121°C for 30 min.

Determination of growth temperatures on mannanase production

Determination of growth temperatures

The effective isolate was cultivated in 5 ml of NB medium under aerobic conditions with shaking at 150 rpm for 24 hours at 45 ˚C. Following that, 3% culture broth was transferred into 300 ml of PM with shaking at 150 rpm in varying temperature of 30, 35, 37, 45 and 50˚C. Sample were collected at 0, 4, 8, 12, 16, 20 and 24 hours of incubation. Upon that, the culture media were diluted and spread-plated for cells counting, whiles the supernatant or filtrate culture was assayed for mannanase activity.

Determination of growth pH on mannanase production

The strain NT6. 7 was cultivated in 5 ml of NB medium producing enzyme medium under aerobic conditions with shaking at 150 rpm for 24 hours at 45 ˚C. After 3% culture broth was transferred in 300 ml of PM and pH adjusted to; 4, 5, 6, 7 and 8. Samples were collecting at 12, 16, 20 and 24 hours. Also, the culture broth were diluted and spread-plated for cells count, while the supernatant or filtrate culture was assayed for mannanase activity.

Optimization culture condition for mannanase production

Experimental design

Taguchi techniques are mathematical modeling based on orthogonal array)OA(. Using OA to reduce the number of experiment and to identify significant factors. Taguchi method was applied to determine the parameters, which significantly influence the mannanase production by *Bacillus circulans* NT 6. 7 in a batch fermenter. The parameters of mannanase production were optimized using the standard orthogonal L9 arrays to determine culture condition as mannanase values to investigate three independent variables of inoculum concentration, agitation rate and aeration rate for three different levels)1, 2, 3(as shown in Table 1. The Taguchi with the creation of a series of experiments utilizing Minitab 16 Software **) Minitab Inc., USA(** . In this study, the condition for mannanase production was based on the L9 array when, L is Latin square array and 9 is the number of experiment run flowed in Table 2.

Table 1 Culture condition parameters and assigned levels selected for optimization

Parameter	Level		
	1	**2**	**3**
Inoculum (%); A	0.1	0.5	1.0
Agitation (rpm); B	200	400	600
Aeration (vvm); C	0.5	0.75	1.00

Table 2 Taguchi L9 orthogonal array of experimental design.

Experiment number	Control factor			Parameter value		
	A	**B**	**C**	**Inoculum (%)**	**Agitation (rpm)**	**Aeration (vvm)**
1	1	1	1	0.1	200	0.50
2	1	2	2	0.1	400	0.75
3	1	3	3	0.1	600	1.00
4	2	1	2	0.5	200	0.75
5	2	2	3	0.5	400	1.00
6	2	3	1	0.5	600	0.50
7	3	1	3	1.0	200	1.00
8	3	2	1	1.0	400	0.50
9	3	3	2	1.0	600	0.75

Mannanase production

Fermentation experiments were performed for mannanase production by *Bacillus circulans* NT 6.7 employing the selected 9 experimental trials)Table 2(. All experiments were conducted in 5-l fermenter) Biostat-B(containing 2 L of mineral salt medium and control temperature at 45 ºC.

Determination of mannanase activity

Mannanase was assayed by measuring the reducing sugars using dinitrosalicylic acid)DNS(method, adapted from **Miller (1959(**. The mannanase assay mixture contained 0.1 ml of 1%)w/v(locust bean gum prepared in 50 mM potassium phosphate buffer, pH 6 and 0.1 ml of diluted culture broth. The reaction mixture was maintained at 50 ºC for 60 min. After incubation, 0.2 ml of DNS reagent was added and boiled for 5 min, cooled and diluted to 2 ml. The absorbance was measured at 540 nm. One unit of enzyme activity was defined as the amount of enzyme to liberate 1 µmol of mannose per minute under the assay conditions. Controls were routinely included in which enzyme preparation or substrate was omitted.

RESULTS AND DISCUSSION

Development of culture medium

A preliminary study of mannanase production by *Bacillus circulans* NT 6.7 used producing enzyme medium showed very higher cost than mineral salt medium. Mannanase was produced by *Bacillus circulans* NT 6.7 using copra meal and defatted copra meal for carbon source. It was suggested that, mannanase activity at 24 hours of producing medium supplement with copra meal and defatted copra meal were 0.24 U/ml and 4.79 U/ml, respectively **)Pangsri, 2014(**. In addition, test in mineral salt medium supplement with copra meal and defatted copra meal were 0.002 U/ml and 7.26 U/ml, respectively. The results indicate that, defatted copra meal was a good carbon source for mannanase enzyme production. This is because defatted copra meal contained high amount of carbohydrate but, low fat content. Fat might be the cause of cell growth inhibition observing in copra meal medium. This result related with previous findings by **Lin and Chen)2004(**, who suggested that the presence of coconut oil actually inhibited mannanase production from *Aspergillus niger* NCH-189. Consequently, the defatted copra meal was selected for mannanase enzyme production in fermenter cultivation.
Moreover, mineral salts medium supplemented with defatted copra meal (1%) showed culture growth and the enzyme activity higher than in the producing medium supplemented with defatted copra meal (1%) (9.44 and 8.88 Log CFU/ ml) and (13.88, 5.56) U/ml at; 45ºC, 18 hours, respectively. Upon which the activity of the enzyme curve determined as shown (Figure 1).

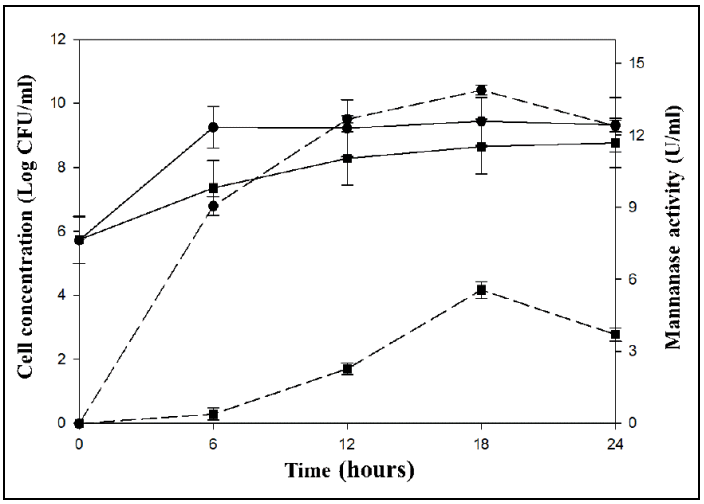

Figure 1 Relationship between microorganism growth and enzyme activity over time in producing medium)■—■;▲—▲(and mineral salts medium)●—●,▼—▼(.

The effect of defatted copra meal on culture and activity vary depending on the concentration (Table 3). Concentration of 1.5% showed the highest growth and enzyme activity (10.51±0.53 Log CFU/ml and 12.84±4.38 U/ml), followed by 2.0% (9.77±0.52 Log CFU/ml and 11.50±2.63 U/ml), 1.0% (9.80±0.71 Log CFU/ml and 10.33±3.17 U/ml) and 0.5% (9.65±0.78 Log CFU/ml and 7.20±1.43 U/ml) showing the lowest, respectively. The effect was more toward enzyme activity than the culture growth. Thus, 1.5% concentration of defatted copra meal was chosen to study in fermenter for enzyme production.

Microbial growth and enzyme production in fermenter using mineral salts medium supplement with defatted copra meal was compared with that of the shake flask to appreciate the different patterns. This is because the conditions for growth and enzyme production in fermenter could be controlled aeration rate and more control agitation rate. The results shows that, 2.0% defatted copra meal gave the highest activity (32 U/ml) (Figure 2) and therefore, selected for the enzymes production. The defatted copra meal at concentration 1.5% also shows

higher enzyme activity than 1.0% but, the enzyme activity was found slightly different from 2.0% concentration. The concentration of defatted copra meal affected the enzyme production because defatted copra meal was inducer of the enzyme activity.

Table 3 Microorganism growth, enzyme activity and yield in mineral salts medium using defatted copra meal at concentrations of 0.5%, 1.0%, 1.5% and 2.0% as carbon source compared with 1% defatted copra meal in producing medium (control).

Defatted copra meal (%)	Time (hr.(	Growth (Log CFU/ml)	Activity (U/ml)	$Y_{p/x}$ (U/mg)
0.5	0	4.64±1.10	0.05±0.00	
	6	8.45±0.72	6.23±3.21	22.67±13.23
	12	8.91±0.15	7.34±2.91	24.08±12.11
	18	9.65±0.78	7.20±1.43	20.48±10.30
	24	8.69±0.05	6.25±1.97	24.71±16.13
1.0	0	4.66±1.10	0.04±0.01	
	6	9.09±0.17	7.16±3.87	22.70±14.57
	12	9.44±0.41	8.87±4.42	27.63±20.32
	18	9.80±0.71	10.33±3.17	29.57±18.91
	24	9.31±0.02	10.26±3.00	33.72±19.30
1.5	0	4.66±1.10	0.05±0.00	
	6	8.98±0.22	6.10±4.12	19.09±12.70
	12	9.73±0.30	11.49±4.80	33.19±21.30
	18	10.51±0.53	12.84±4.38	31.26±18.14
	24	9.72±0.87	11.46±0.02	31.58±2.99
2.0	0	4.65±1.10	0.05±0.00	
	6	8.06±0.13	3.38±2.13	13.52±7.51
	12	9.07±0.09	11.39±4.67	36.75±20.40
	18	9.77±0.52	11.50±2.63	31.04±13.30
	24	9.12±0.00	12.09±2.06	41.09±19.70
1% (control)	0	4.97±1.37	0.06±0.01	
	6	7.74±0.18	0.35±0.05	1.56±0.83
	12	8.22±0.46	1.32±1.34	6.52±6.77
	18	8.50±0.55	4.16±1.97	16.44±11.06
	24	9.07±0.00	3.70±0.00	15.10±0.00

Note: $Y_{p/x}$ was expressed as yield equals to product value divided by cell dry weight.

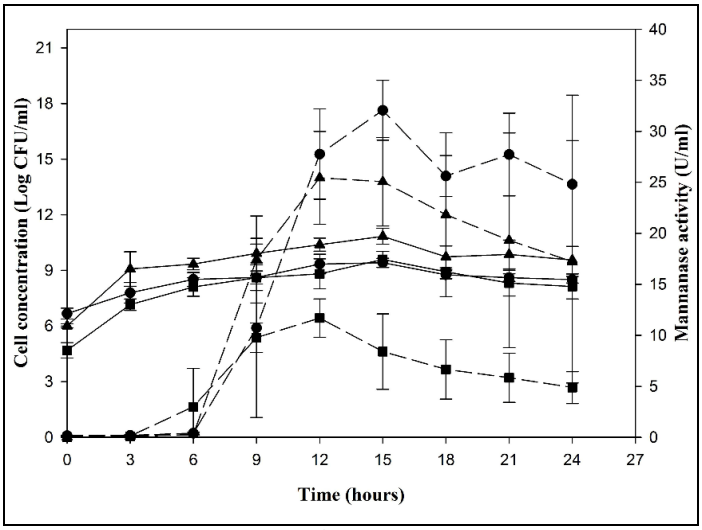

Figure 2 Microorganism growth in fermentor and enzyme activity with time in mineral salts medium using defatted copra meal as carbon source at varying concentrations)—■—,- -■- -) 1.0%,)—▼—,- -▼- -(1.5%, and)—●—,- -●- -) 2.0% inoculum, 600 rpm and 0.75 vvm **)Pangsri *et al.*, 2015(**.

Effect of temperature and pH on mannanase production

A preliminary study of mannanase production by *Bacillus circulans* NT 6.7 was cultivated on locust bean gum medium, pH 7.0 at different temperatures of 40, 45 and 50ºC, respectively. The result showed the highest enzyme activity at 45°C)2.35 U/ml(, the yield of production from cell was 0.62 U/CFU and the specific growth rate was 0.23 hr^{-1})calculated by viable cell count(. The result of *Bacillus circulans* NT 6.7 was similar to *Bacillus subtilis* 5H which produced mannanase at 45°C **) Khanongnuch *et al.*, 1999(** , but it was higher than mannanase production from *Bacillus sp*. KK01 at 30°C **) Hossain *et al.*, 1996(** and *Aspergillus niger* NCH- 189 at 30°C **)Lin and Chen, 2004(**.

The *Bacillus circulans* NT 6.7 was grown on locust bean gum medium at 45ºC in varying pH of 4.0, 5.0, 6.0, 7.0 and 8.0. The *Bacillus circulans* NT 6.7 could not grow at pH 4.0 and 5.0. At 20 hours, the highest mannanase activity was 2.41 unit/ml and the cell count was 7.93 Log CFU/ml at pH 6.0)data not shown(. The pH 6.0 was the best pH for mannanase production from *Bacillus circulans* NT 6.7. The result was similar to mannanase production from *Sclerotium rolfsii*, pH 6.0 **)Gübitz *et al.*, 1996(**, but lower than that reported for mannanase production from *Aspergillus niger*, pH 7.0 **)Ademark *et al.*, 1998(** and *Bacillus subtilis* KU-1, pH 7.0 **)Zakaria *et al.*, 1998(**. This study used locus bean gum because it's a common substrate used in mannanase industrial production. The structure of locus bean gum is galactomannan same with copra meal but the copra meal is complex structure it's difficult to hydrolyze more than locus bean gum. However, Next study change to used defatted copra meal to produce mannanase because want to decrease cost of medium.

Optimization culture condition for mannanase production

The experiment was to assess the fermentation conditions in fermenter using three) 3(factors; inoculum, stirring speed and aeration rate at 45ºC. This is because using different temperatures, preliminary study showed the highest enzyme activity and highest growth at 45ºC. Moreover, pH during fermentation was not control because it falls within pH 6-7. At this range, the bacteria growth and the release of product are not significantly affected. Using the experimental design Taguchi L9, it was found that, the main factor affecting fermentation was the concentration of *Bacillus circulans* NT 6.7, followed by the stirring speed and aeration rate. It was found that the best conditions to obtained higher enzyme production in the fermenter was the concentration of 1.0% inoculum, agitation rate of 600 rpm and aeration rate of 0.75 vvm at 15 hours. Thus, the maximum enzyme activity obtained at this condition was 29.57 U/ml)Table 4(, which is higher than the prediction value.

Table 4 Optimal conditions at varying cultivation time using Minitab 16 program with Taguchi L9 experimental design

Optimum condition					
Hr.	**Factor**	**Level**	**Expect value**	**Experimental value**	**Error (%)**
12	inoculum STIRR Aeration	1% 600 rpm 0.75 vvm	19.13 U/ml	24.41 U/ml	27.60
15	inoculum STIRR Aeration	1% 600 rpm 0.75 vvm	24.15 U/ml	29.57 U/ml	22.44
18	inoculum STIRR Aeration	1% 600 rpm 0.50 vvm	27.86 U/ml	24.66 U/ml	-11.49
21	inoculum STIRR Aeration	1% 600 rpm 0.50 vvm	33.90 U/ml	27.71 U/ml	-18.26
24	inoculum STIRR Aeration	1% 400 rpm 0.50 vvm	33.37 U/ml	27.41 U/ml	-17.86

The program predicted 3 conditions to give the highest activity within the period of 12-24 hours. The results showed the optimum point of the enzyme at different time intervals) Figure 3(, period where difference were obtained to allow comparison. The condition at the 15 h time only showed higher activity values than predictive values, which approve the maximum product value. This is because the relationship between time and mannanase activity shown in figure 3 indicate a trend similar to the observation attained in the experimental results according the program)Table 4(.

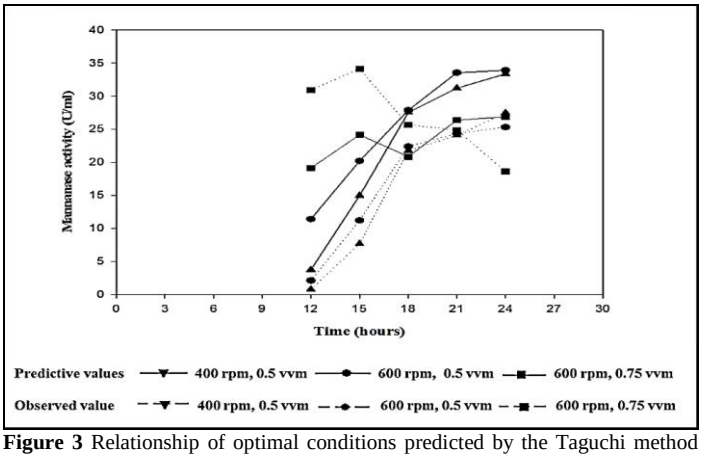

Figure 3 Relationship of optimal conditions predicted by the Taguchi method compared with actual values of enzyme activity in a fermenter at 12-24 hours.

The enzyme activity of the experiment and predicted values evaluated were dependable at the confidence interval of 95% based on the experiment data. This was used to build a linear regression model to describe the relationship between the study variables that affects the activity of enzymes. The correlation coefficient)R^2(was 0.75 as shown in the equation.

$$Y = -13.809 + 19.715X_1 + 0.021X_2 + 4.240X_3$$

Where X_1 is a concentration of inoculum)%(, X_2 is the rate of stirring)rpm(and X_3 is the rate of aeration)vvm(.

A regression model was used to calculate predicted values of mannanase activity compared with experimental values. Statistical analysis showed that regression was not significant)P<0.05(. The model may not be good theoretically, because the correlation coefficient indicates the goodness of fit for the model. This equation showed the lowest value of correlation coefficient may be have another model its better. Conversely, this study suggested the model as best, because the condition provided maximum yield at surface constraints. Inoculum was not increased more than 1% which was the concentration limitation due to the high rate of exponential phase. Agitation rate were not increased more than 600 rpm respectively because the limitation of fermenter in the large scale cannot working. However, the factor of time it important for enzyme production but can't know the maximum activity range. This study don't designated time in Taguchi.

The results of this experiment indicated that the bacteria increased in number at 15 h, thus resulted in high growth of *Bacillus circulans* NT 6.7 with desire mixing and aeration. Higher population led to more products liberation.

Table of regression Analysis at 15 hours

Predictor	**Coef**	**SE Coef**	**T**	**P(<0.05)**
Constant	-13.809	9.867	-1.40	0.221
Inoculum	19.715	5.637	3.50	0.017
Agitation rate	0.02062	0.01271	1.62	0.166
Aeration rate	4.24	10.17	0.42	0.694

Following Taguchi design, 2% defatted copra meal concentration in the fermenter resulted in 32.07±2.94 U/ml of mannanase activity under the optimum condition in 9 run experiment)table 2(. Upon repeating)3 times(under the same optimized condition in the fermenter, the mean of activity was 29.57±4.81 U/ml, which was not significantly different. The concentration)1.0%(of inoculum, 600 rpm agitation, and 0.75 vvm aeration rate at 45ºC for 15 hours gave the highest enzyme activity of 29. 57±4. 81U/ ml) Figure 4(, which corresponds to the observation by **Pangsri)2014(** and **Feng *et al.*)2003(** . It was found that, the maximum enzymes production by *Bacillus circulans* NT 6.7 was attained using producing medium containing 1% defatted copra meal as a carbon source.

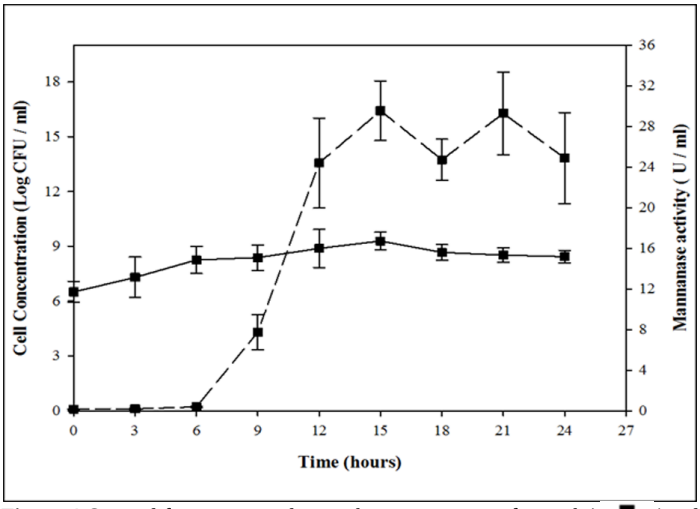

Figure 4 Optimal fermenter condition showing pattern of growth)—■—(and enzyme activity)▪ ■ ▪(.

In the fermenter containing mineral salts medium, maximum enzyme activity of 27.70 U/ml was obtained at 6 h. This was higher than the activity value observed using the Producing medium. Though, using the producing medium maximum enzymes activities can be achieved 9 hours lower than in the current study. This possibly because of the presence of poly peptone and corn steep liquor, which is easily used by the bacteria for their growth and activity. Thus, this could have resulted in maximum activity at shorter time than in current study. However, using producing medium for enzyme production come with challenges such as enzyme activity in each production is fixed depending on corn steep liquor used, and high price medium composition.

The study compared optimum condition in the enzyme production and beta-mannanase activity to previous work by **Feng *et al.*)2003(**, which used *Bacillus licheniformis* NK-27. From the former study, optimal conditions to achieved maximum enzyme yield were 600 rpm stirring rate and 0.75 vvm aeration rate at 30ºC for 36 hours. However, in current study, using mineral salts medium, maximum enzyme production can achieved similar stirring rate and aeration by *Bacillus circulans* NT 6.7 at less time)15 h(. Hence, current finding could be considered as best for enzyme production in the industrial scale.

CONCLUSION

Mineral salts medium supplemented with defatted copra meal as carbon source can be used to produce the enzyme beta-mannanase from *Bacillus circulans* NT 6.7. In the shaking flask, 1.5% concentration was suitable to obtain the maximum bacteria growth of 10.51±0.53 Log CFU/ml and enzymes activities of 12.84±4.38 U/ml at 18 hours. Meanwhile, using fermenter, 2% concentration showed the highest bacteria growth of 7.94±0.41 Log CFU/ml and the highest enzyme activities of 32.07±2.94 U/ml at 15 hours. Considering this, optimization of the enzymes production in a reaction tank 5 liters)fermenter(using working volume of 2 liters at 45ºC was determined. It was found that, the optimum condition to achieved higher enzyme activity) 29.57±4.81U/ml(were 1.0% inoculum concentration of bacteria, 600 rpm agitation and 0.75 vvm aeration rate at cultivation time at 15 hours. The study suggest that, mineral salts medium might be good to achieve higher enzyme production at low cost. Therefore, this could be considered to be used in the industrial scale.

Acknowledgement: This research was financially supported by the Graduate School Kasetsart University and the National Research Council of Thailand.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest in the publication.

REFERENCES

Ademark, P., Varga, A., Medve, J., Harjunpaa, V., Drakenberg, T., Tjerneld, F., Stalbrand, H. 1998. Softwood hemicellulose-degrading enzymes from *Aspergillus niger*. Purification and properties of a β-mannanase. *Journal of Biotechnology*. 63, 199-210. https://doi.org/10.1016/S0168-1656(98)00086-8

Burhanudin, S., Dingle, J. 2006. Title of subordinate document. *In: Proceedings Queensland Poultry Science Symposium Australia*. Available via DIALOG. http://www.idp.com. Cited 1 Feb 2006.

Chandrakant, P., Bisaria, V. S. 1998. Simultaneous bioconversion of cellulose and hemicellulose to ethanol. *Critical Reviews in Biotechnology*, 18, 295-331. https://doi.org/10.1080/0738-859891224185.

Chauhan, PS., Sharma, P., Puri, N., Gupta, N. 2014. Purification and characterization of an alkali-thermostable β-mannanase from *Bacillus nealsonii* PN-11 and its application in mannooligosaccharides preparation having prebiotic potential. *European Food Research and Technology*, 238, 927-936. https://doi.org/10.1007/s00217-014-2170-7.

Feng, Y., He, Z., Ong, SL., Hu, J., Zhang, Z., Ng, W. J. 2003. Optimization of agitation, aeration, and temperature conditions for maximum beta-mannanase production. *Enzyme and Microbial Technology*, 32, 282-289. https://doi.org/10.1016/S0141-0229(02)00287-9.

Göhl, B. 1982. Les aliments du bétail sous les tropiques. FAO, Division de Production et Santé Animale, Roma, Italy.

Gübitz, G. M., Hayn, M., Urbanz, G., Steiner, W. 1996. Purification and properties of an acidic β-mannanase from *Sclerotium rolfsii*. Institute of Biotechnology, SFB Biokatalyse, Technical University Graz, Austria. *Journal of Biotechnology*, 45, 165-172. https://doi.org/10.1016/0168-1656(95)00158-1.

Hossain, M., Abe, J., Hizukuri, S. 1996. Multiple from of β-mannanase from *Bacillus sp.* KK01. *Enzyme and Microbial Technology*, 18, 95-98. https://doi.org/10.1016/0141-0229(95)00071-2.

Khanongnuch, C., Ooi, T., Kinoshita, S. 1999. Cloning and nucleotide sequence of β-mannanase and cellulose genes from *Bacillus sp.* 5H. *World Journal of Microbiology and Biotechnology*, 15, 221-228.

Lin, T. C., Chen, C. 2004. Enhanced mannanase production by submerged culture of *Aspergillus niger* NCH-189 using defatted copra based media. *Process Biochemistry*, 39, 1103-1109. https://doi.org/10.1016/S0032-9592(03)00218-8.

Miller, G. L. 1959. Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Analytical Chemistry*, 31, 426-428. https://doi.org/10.1021/ac60147a030.

Mabrouk, M. E. M., Ahwany, A. M. D. E. 2008. Production of β-mannanase by *Bacillus amylolequifaciens* 10A1 cultured on potato peels. *African Journal of Biotechnology*, 7)8(, 1123-1128. https://doi.org/10.5897/AJB08.047.

Pangsri, P. 2014. Characterization of mannanase from *Bacillus circulans* NT 6.7 and its application in mannooligosacchrides preparation. Ph. D. thesis Kasetsart University.

Pangsri, P., Piwpankaew, Y., Ingkakul, A., Nitisinprasert, S., Keawsompong, S. 2015. Characterization of mannanase from *Bacillus circulans* NT 6.7 and its application in mannooligosaccharides preparation as prebiotic. *springerplus*, 4, 771. https://doi.org/10.1186/s40064-015-1565-7.

Phothichito, K., Nitisinprasert, S., Keawsompong, S. 2006. Isolation, Screening and Identification of Mannanase Producing Microorganisms. Kasetsart Journal)Natural Science(40)Suppl), 26-38.

Ray, S., Pubols, M.H., Mgginnis, J. 1982. The effect of a purified guar degrading enzyme on chicken growth. *Poultry Science*. 61, 488-494. https://doi.org/10.3382/ps.0610488.

Stoll, D., Stalbrand, H., Warren, R. A. J. 1999. Mannan-degrading enzymes from *Cellulomonas fimi*. *Applied and Environmental Microbiology*, 65)6(, 2598-2605.

Tomotari, M. 1990. Bifidobacteria and their role in human health. *Journal of Industrial Microbiology*, 6(4), 263-267. https://doi.org/10.1007/BF01575871.

Wootton, A.N., Luker-Brown, R.J., Cheetham, P.S.J. 1993. The extraction of a glucomannan polysaccharide from konjac corms)elephant yam, *Amorphophallus rivierii*(. *Journal of the Science of Food and Agriculture*, 61, 429-33. https://doi.org/10.1002/jsfa.2740610408.

You, J., Liu, J., Yang, S., Mu, B. 2016. Low-temperature-active and salt-tolerant β-mannanase from a newly isolated Enterobacter sp. strain N18. *Journal of Bioscience and Bioengineering*, 121) 2(, 140-146. https://doi.org/10.1016/j.jbiosc.2015.06.001.

Zakaria, M. M., Yamamoto, S., Yagi, T. 1998. Purification and characterization of an endo-1,4-β-mannanase from *Bacillus subtilis* KU-1. *FEMS Microbiology Letters*, Japan, 158, 25-31. https://doi.org/10.1111/j.1574-6968.1998.tb12795.x

BACTERIAL EMPIRE
2019, VOL. 2, NO. 2, 46-48

OPEN ACCESS
SCICELL

REGULAR ARTICLE

OCCURRENCE OF *Yersinia enterocolitica* SEROTYPE O:9, AND *Citrobacter freundii*, TWO POTENTIAL HUMAN PATHOGENS IN THE THROATS OF TROPICAL PIGS OF GRENADA ORIGIN

Victor A. Amadi[*], *Harry Hariharan, Vanessa Matthew-Belmar, Roxanne Nicholas-Thomas, Claude DeAllie, Ravindra Sharma*

Address (es): Victor A. Amadi,
[1]Department of Pathobiology, School of Veterinary Medicine, St. George's University, St. George's, Grenada, West Indies.

*Corresponding author: vamadi@sgu.edu

ABSTRACT

A study to determine the occurrence of *Yersinia enterocolitica* and *Citrobacter freundii*, two potential human pathogens in the throats of tropical pigs of Grenada origin and the antimicrobial pattern of the isolates was carried out. During a period of 30 days (15 September 2017 to 15 October 2017), throat swabs from a total of 97 weaned pigs were sampled to isolate and characterize *Y. enterocolitica* by serotyping and resistance testing, and to isolates *C. freundii*. Of the pigs tested, four (4.1%) pigs were positive for *Y. enterocolitica*. The four *Yersinia*-positive pigs included one (2.4%) of 41 male pigs and three (5.4%) of 56 female pigs. There were no significant differences between the proportions of *Yersinia*-positive male and female pigs (p = 0.8437). Of these four *Yersinia*-positive pigs, two were mixed with *C. freundii*, one had slight contamination, and the other was pure. All belonged to serotype 0:9. There were 31 pigs positive for *C. freundii*, and all showed mixed growth. Antimicrobial susceptibility tests against 14 drugs indicated that all isolates of *Y. enterocolitica* were susceptible to third-generation cephalosporins and fluoroquinolones, two classes of antimicrobials recommended for the treatment of *Y. enterocolitica* infection in humans.

Keywords: *Yersinia enterocolitica*, *Citrobacter freundii*, tropical pigs, human pathogens, throats

INTRODUCTION

Enteropathogenic *Y. enterocolitica* is a zoonotic pathogen, causing human disease worldwide with symptoms ranging from gastroenteritis to severe complications of mesenteric lymphadenitis, liver abscesses and postinfectious extraintestinal sequelae (**Batzilla *et al.*, 2011; Valentin-Weigand *et al.*, 2014**). It has also been isolated from cases of diarrhea in dogs in Canada (**Hariharan and Bryenton, 1990**). Recently, an unusual increase of *Y. enterocolitica* in humans occurred in Creuse, central France (**Martin *et al.*, 2015**). Human infections are directly or indirectly derived from animal sources and may be contracted through the ingestion of contaminated food (**Oxoid 2018**). Pigs are considered to be the major reservoir of this pathogen. They may carry this organism in throats, tonsils, tongues, and to a lesser extent in feces (**Hariharan *et al.*, 1995; Schiemann and Fleming, 1981; Singh *et al.*, 2003**). Although the re-emergence and importance of this organism in temperate areas of the world is well documented as indicated by recent publications (**Arrausi-Subiza *et al.*, 2016; Bonardi *et al.*, 2014; Fondrevez *et al.*, 2014; Van Damme *et al.*, 2015; von Altrock *et al.*, 2015**), there is very little information from tropical and subtropical countries. There is no data on the occurrence or properties of this pathogen in animals, including swine raised in the Caribbean. It is known that *C. freundii* an opportunistic human pathogen, may have a colony morphology resembling *Y. enterocolitica* on the selective medium used for isolation of *Y. enterocolitica* (**Oxoid Ltd, 2018**). Published information on *C. freundii*, in pigs is minimal, although it is known that some strains can cause gastroenteritis and hemolytic uremic syndrome (**Tschape *et al.*, 1995**). The objectives of this study were to determine the carriage rates of *Y. enterocolitica* in pigs raised for food, and to characterize the isolates with regard to serotype and antimicrobial susceptibility. We also ascertained the occurrence of *C. freundii* in the throats of the pigs studied.
This study will help determine the possible role of tropical pigs as reservoirs of human pathogenic *Y. enterocolitica* strains and identify drugs which are likely to be effective for treatment, in the event of disease in humans originating from pigs. Slaughter procedures and storage of meat may need modifications to prevent contamination of meat and subsequent multiplication of this psychrophilic organism which can multiply in refrigeration temperatures.

MATERIAL AND METHODS

Study design and sample collection

This study had the approval of the St. George's University Institutional Animal Care and Use Committee (IACUC 16007-R). Young weaned pigs of approximately 16 weeks of age were randomly selected from pig farms in Grenada. For each sampled pig, the gender and age were recorded. All sampling was done in a period of 30 days (15 September 2017 to 15 October 2017). Long guarded body cavity culture swabs (Santa Cruz Animal Health, Dallas, Texas) designed for collection throat samples from animals were used.

Isolation and identification of *Yersinia enterocolitica* and *Citrobacter freundii*

For the isolation of *Y. enterocolitica* and *C. freundii*, each swab was placed in a tube containing 5 ml of peptone sorbitol bile broth (Sigma-Aldrich, St. Louis, USA). The tube was vortexed, and 1 ml of the suspension was added to 9 ml of Irgasan-Ticarcillin-potassium Chlorate (ITC) broth (Bio-Rad, Marnes La Coquette, France), and incubated for 48 h at 25°C. Then, 10 µl of the culture was streaked on Cefsulodin-Irgasan-Novobiocin (CIN) agar plates (*Yersinia* selective agar base and supplement (Oxoid, Basingstoke, UK), and incubated at 30°C for 24 hours. Suspected *Y. enterocolitica* and *C. freundii* colonies were identified using the API20E (Analytical Profile Index; BioM6rieux, Hazelwood, MO) bacterial identification strips.

Serotyping of *Yersinia enterocolitica*

For the serotyping of the *Y. enterocolitica*, the *Y. enterocolitica* serotyping kit (Denka Seiken Co. Ltd., Tokyo, Japan), which contained one polyvalent antiserum (groups O1 and O2), and 4 types of monovalent antisera for groups O3, O5, O8, and O9) was used.

Antimicrobial susceptibility test

Antimicrobial susceptibility tests was done using the standard disk diffusion method on Mueller Hinton agar (Difco/BD) following recommendation of the Clinical and Laboratory Standard Institute (**CLSI, 2015**). All the Y. enterocolitica were tested for susceptibility to 14 antimicrobials. The antimicrobial disks used were: amoxicillin-clavulanic acid (AmC-30), ampicillin (Am-10), aztreonam (ATM-30), ceftriaxone (CRO 30), ceftazidime (CAZ-30), cefoxitin (FOX-30), cephalothin (CF-30), chloramphenicol (C-30), ciprofloxacin (CIP-5), gentamicin (GM-10), imipenem IPM-10), trimethoprim-sulfamethoxazole (SXT-1.25/23.75), streptomycin (S-10), and tetracycline (TE-30). The inhibition zone sizes were interpreted based on CLSI guidelines. *Escherichia coli* ATCC 25922 was used as quality control strain **(Eguale *et al.*, 2015**).

Statistical analysis

An online data analysis software: http://www.openepi.com/Menu/OE_Menu.htm was used for all the statistical analysis. The OpenEpi-Two by Two table (chi-squared (χ^2) analysis) was used to compare the differences in the proportions of female vs male pigs. The level of statistical significance was set at alpha equal to 0.05 ($\alpha = 0.05$). A value of $P < 0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

Ninety seven weaned pigs were sampled. By gender, they comprised of 41 (42.3%) male pigs, and the remaining 56 (57.7%) were females. Throat swabs from four (4.1%) pigs were positive for *Y. enterocolitica*. The four *Yersinia*-positive pigs included one (2.4%) of 41 male pigs and three (5.4%) of 56 female pigs. There were no significant differences between the proportions of *Yersinia*-positive male and female pigs ($p = 0.8437$). Of these four *Yersinia*-positive pigs, two were mixed with *C. freundii*, one had slight contamination, and the other was pure. All belonged to serotype 0:9. There were 31 pigs positive for *C. freundii*, and all showed mixed growth. The other isolates included species of no pathogenic significance or poor identifications according to API strips. The United States Centers for Disease Control and Prevention (**CDC, 2016**) estimates *Y. enterocolitica* causes almost 117,000 illnesses, with 35 deaths in the U.S. every year. Raw or undercooked pork contaminated with *Y. enterocolitica* is the most common source of human infection worldwide. *Y. enterocolitica* strains found in pigs and pork are indistinguishable from strains found in humans, further supporting the association between yersiniosis and consumption of pork (**Fredriksson-Ahomaa and Korkeala, 2003**). There are over 70 serotypes of Y. pathogenic serotypes of y. enterocolitica. Most human infections involve serotypes O:3, O:5, O:8, and O:9 (**Schriefer and Petersen, 2011**). Although, O:3 is the leading serotype, human infections due to *Y. enterocolitica*, serotype 09 have been reported from the UK (**Wale *et al.*, 1991**) Germany (**Luedde *et al.*, 2004**), Japan (**Moriki *et al.*, 2010**), and Poland **(Kamińska and Sadkowska-Todys, 2016**). Children are more prone to infection, and serotypes O3 and O:9 predominated in the Netherlands (**Hoogkamp-Korstanje and Stolk-Engelaar, 1995**). In Poland, of 244 cases in 2014, 5.2% were due to O:9 serotype (**Kamińska and Sadkowska-Todys, 2016**).

Although only 4% of the pigs in the present study were positive for *Y. enterocolitica*, the fact that all belonged to the same serotype O:9 is noteworthy. This is in contrast to a study conducted by one of the authors on slaughter hogs in Canada (**Hariharan, *et al.*, 1995**), where majority of the isolates from the tonsils belonged to serotype O:3. The isolation method used in the present study was similar to that used in the Canadian study. The low isolation rates in natural samples may be due to the limited sensitivity of cultural methods (**Fredriksson-Ahomaa and Korkeala, 2003**). On the other hand it may be noted that studies on tropical pigs are lacking, and it is impossible to make comparisons at present.

Citrobacter spp. are opportunistic pathogens that are commensal inhabitants of the intestines on humans and animals. *C. freundii* is a potential foodborne pathogen which has been implicated in human gastroenteritis, hemolytic uremic syndrome, and pigs could be a source (**Bai *et al.*, 2012; Liu *et al.*, 2017; Nimri *et al.*, 2014; Tschape, *et al.*, 1995**).

Table 1. shows the antimicrobial susceptibility (mean zone diameters and interpretation) of four *Y. enterocolitica* serotype O:9 isolates and control strain *Escherichia coli*. Antimicrobial drugs traditionally used to treat human infection, includes cotrimoxazole, doxycycline and chloramphenicol (**Stolk-Engelaar *et al.*, 1995**). Our isolates were susceptible to these drugs, although one isolate showed only intermediate susceptibility to tetracycline. Strains resistant to ampicillin and cephalothin like ours have been found in slaughter pigs in other parts of the world (**Terentjeva and Berzins, 2010**). All four strains in our study were susceptible to third-generation cephalosporins and fluoroquinolones (ciprofloxacin), two classes of drugs recommended for *Y. enterocolitica* infection in humans (**von Altrock, *et al.*, 2015**).

Table 1 Antimicrobial susceptibility of four *Yersinia enterocolitica* serotype O:9 isolates and control strain *Escherichia coli*

Antimicrobial drug and disk potency	Pig # 25	Pig # 31	Pig # 32	Pig # 36	Control strain: *E. coli* ATCC 25922
Amoxicillin/clavulanic acid (AMC-30)	9 (R)	14 (I)	12 (R)	20 (S)	20 (S)
Ampicillin ((AM-10)	0 (R)	10 (R)	0 (R)	12 (R)	21 (S)
Aztreonam (ATM-30)	35 (S)	40 (S)	39 (S)	25 (S)	32 (S)
Ceftriaxone (CRO-30)	37 (S)	39 (S)	39 (S)	27 (S)	33 (S)
Ceftazidime (CAZ-30)	35 (S)	37 (S)	39 (S)	30 (S)	26 (S)
Cefoxitin (FOX-30)	14 (R)	16 (I)	16 (I)	27 (S)	33 (S)
Cephalothin (CF-30)	0 (R)	0 (R)	0 (R)	14 (R)	17 (I)
Chloramphenicol (C-30)	30 (S)	35 (S)	34 (S)	25 (S)	25 (S)
Ciprofloxacin (CIP-5)	39 (S)	39 (S)	43 (S)	31 (S)	33 (S)
Gentamicin (GM-10)	27 (S)	31 (S)	31 (S)	23 (S)	20 (S)
Imipenem (IPM-10)	34 (S)	39 (S)	41 (S)	25 (S)	30 (S)
Trimethoprim-sulfamethoxazole (SXT-1.25/23.75)	31 (S)	34 (S)	36 (S)	24 (S)	22 (S)
Streptomycin (S-10)	25 (S)	27 (S)	25 (S)	17 (S)	17 (S)
Tetracycline (TE-30)	19 (S)	27 (S)	27 (S)	18 (I)	21 (S)

CONCLUSION

Our study showed that presently, tropical pigs of Grenada origin are not major reservoirs for the pathogenic *Y. enterocolitica* serotype O:9, we estimated the occurrence rate in the throats of pigs in Grenada to be 4.1%. This current study also showed that tropical pigs harbor *C. freundii* another potential pathogen that has been associated with human gastroenteritis. Antimicrobial resistance profiles indicated that all isolates of *Y. enterocolitica* were susceptible to third-generation cephalosporins and fluoroquinolones, two classes of antimicrobials recommended for the treatment of *Y. enterocolitica* infection in humans.

Acknowledgements: The authors are thankful to the Board of Trustees of St. George's University for providing funds for the research.

REFERENCES

ARRAUSI-SUBIZA, M., GERRIKAGOITIA, X., ALVAREZ, V., IBABE, J. C., & BARRAL, M. 2016. Prevalence of *Yersinia enterocolitica* and *Yersinia pseudotuberculosis* in wild boars in the Basque Country, northern Spain. *Acta Vet Scand*, 58, 4.

BAI, L., XIA, S., LAN, R., LIU, L., YE, C., WANG, Y., JIN, D., CUI, Z., JING, H., XIONG, Y., BAI, X., SUN, H., ZHANG, J., WANG, L., & XU, J. 2012. Isolation and characterization of cytotoxic, aggregative *Citrobacter freundii*. *PLoS One*, 7(3), e33054.

BATZILLA, J., ANTONENKA, U., HÖPER, D., HEESEMANN, J., & RAKIN, A. 2011. *Yersinia enterocolitica palearctica* serobiotype O:3/4 - a successful group of emerging zoonotic pathogens. *BMC Genomics*, 12(1), 348.

BONARDI, S., ALPIGIANI, I., PONGOLINI, S., MORGANTI, M., TAGLIABUE, S., BACCI, C., & BRINDANI, F. 2014. Detection, enumeration and characterization of *Yersinia enterocolitica* 4/O:3 in pig tonsils at slaughter in Northern Italy. *Int J Food Microbiol*, 177, 9-15.

CDC, 2016. *Yersinia enterocolitica* (Yersiniosis). https://www.cdc.gov/yersinia/index.html (accessed 10/16/2018).

CLSI. (2015). Performance standards for antimicrobial disk and dilution susceptibility tests for bacteria isolated from animals; document VET01-S2 (3rd ed.). Wayne, PA: Second information supplement, Clinical and Laboratory Standards Institute.

EGUALE, T., GEBREYES, W. A., ASRAT, D., ALEMAYEHU, H., GUNN, J. S., & ENGIDAWORK, E. 2015. Non-typhoidal *Salmonella* serotypes, antimicrobial resistance and co-infection with parasites among patients with diarrhea and other gastrointestinal complaints in Addis Ababa, Ethiopia. *BMC Infect Dis*, 15, 497.

FONDREVEZ, M., MINVIELLE, B., LABBE, A., HOUDAYER, C., ROSE, N., ESNAULT, E., & DENIS, M. 2014. Prevalence of pathogenic *Yersinia*

enterocolitica in slaughter-aged pigs during a one-year survey, 2010-2011, France. *Int J Food Microbiol*, 174, 56-62.
FREDRIKSSON-AHOMAA, M., & KORKEALA, H. 2003. Low occurrence of pathogenic *Yersinia enterocolitica* in clinical, food, and environmental samples: a methodological problem. *Clin Microbiol Rev*, 16(2), 220-229.
HARIHARAN, H., & BRYENTON, J. 1990. Prince Edward Island. Isolation of *Yersinia* spp. from cases of diarrhea. *Can Vet J*, 31(11), 779.
HARIHARAN, H., GILES, J. S., HEANEY, S. B., LECLERC, S. M., & SCHURMAN, R. D. 1995. Isolation, serotypes, and virulence-associated properties of *Yersinia enterocolitica* from the tonsils of slaughter hogs. *Can J Vet Res*, 59(3), 161-166.
HOOGKAMP-KORSTANJE, J. A., & STOLK-ENGELAAR, V. M. 1995. *Yersinia enterocolitica* infection in children. *Pediatr Infect Dis J*, 14(9), 771-775.
KAMIŃSKA, S., & SADKOWSKA-TODYS, M. 2016. Yersiniosis in Poland in 2014. *Przegl Epidemiol.* , 70(3), 367-374.
LIU, L., LAN, R., WANG, Y., ZHANG, Y., & XU, J. 2017. Antimicrobial resistance and cytotoxicity of *Citrobacter* spp. in Maanshan Anhui Province, China. *Front Microbiol*, 8, 1357
LUEDDE, T., TACKE, F., CHAVAN, A., LANGER, F., KLEMPNAUER, J., & MANNS, M. P. 2004. *Yersinia* infection mimicking recurrence of gastrointestinal stromal tumor. *Scand J Gastroenterol*, 39(6), 609-612.
MARTIN, L., CABANEL, N., LESOILLE, C., MÉNARD, T., & CARNIEL, E. 2015. Investigation of an unusual increase in human yersinioses in Creuse, France. *International Journal of Infectious Diseases*, 34, 76-78.
MORIKI, S., NOBATA, A., SHIBATA, H., NAGAI, A., MINAMI, N., TAKETANI, T., & FUKUSHIMA, H. 2010. Familial outbreak of *Yersinia enterocolitica* serotype O9 biotype 2. *J Infect Chemother*, 16(1), 56-58.
NIMRI, L., ABU AL-DAHAB, F., & BATCHOUN, R. 2014. Foodborne bacterial pathogens recovered from contaminated shawarma meat in northern Jordan. *J Infect Dev Ctries*, 8(11), 1407-1414.
OXOID LIMITED, 2018. Yersinia selective agar base CM0653 and Yersinia selective supplement SR0109. http://www.oxoid.com/UK/blue/prod_detail/prod_detail.asp?pr=CM0653&c=UK&lang=EN (Accessed 10/22/2018).
SCHIEMANN, D. A., & FLEMING, C. A. 1981. *Yersinia enterocolitica* isolated from throats of swine in eastern and western Canada. *Can J Microbiol*, 27(12), 1326-1333.
SCHRIEFER, M. E., PETERSEN, J. M. 2011. *Yersinia*. Manual of Clinical Microbiology 10th edition Volume. ASM Press, Washington DC. Pp 627-638.
SINGH, I., BHATNAGAR, S., & VIRDI, J. S. 2003. Isolation and characterization of *Yersinia enterocolitica* from diarrhoeic human subjects and other sources. *Current Science*, 84(10), 1353-1355.
STOLK-ENGELAAR, V. M., MEIS, J. F., MULDER, J. A., LOEFFEN, F. L., & HOOGKAMP-KORSTANJE, J. A. 1995. In-vitro antimicrobial susceptibility of *Yersinia enterocolitica* isolates from stools of patients in The Netherlands from 1982-1991. *J Antimicrob Chemother*, 36(5), 839-843.
TERENTJEVA, M., & BERZINS, A. 2010. Prevalence and antimicrobial resistance of *Yersinia enterocolitica* and *Yersinia pseudotuberculosis* in slaughter pigs in Latvia. *J Food Prot*, 73(7), 1335-1338.
TSCHAPE, H., PRAGER, R., STRECKEL, W., FRUTH, A., TIETZE, E., & BÖHME, G. 1995. Verotoxinogenic *Citrobacter freundii* associated with severe gastroenteritis and cases of haemolytic uraemic syndrome in a nursery school: green butter as the infection source. *Epidemiology and infection*, 114(3), 441-450.
VALENTIN-WEIGAND, P., HEESEMANN, J., & DERSCH, P. 2014. Unique virulence properties of *Yersinia enterocolitica* O:3 – An emerging zoonotic pathogen using pigs as preferred reservoir host. *International Journal of Medical Microbiology*, 304(7), 824-834.
VAN DAMME, I., BERKVENS, D., VANANTWERPEN, G., BARÉ, J., HOUF, K., WAUTERS, G., & DE ZUTTER, L. 2015. Contamination of freshly slaughtered pig carcasses with enteropathogenic *Yersinia* spp.: Distribution, quantification and identification of risk factors. *International Journal of Food Microbiology*, 204, 33-40.
VON ALTROCK, A., SEINIGE, D., & KEHRENBERG, C. 2015. *Yersinia enterocolitica* Isolates from Wild Boars Hunted in Lower Saxony, Germany. *Appl Environ Microbiol*, 81(14), 4835-4840.
WALE, M. C. J., LIDDICOAT, A. J., & PETHER, J. V. S. 1991. *Yersinia enterocolitica* biotype 2 serotype O9 septicaemia in a previously fit man, raw goats' milk having been the apparent vehicle of infection: A cautionary tale. *Journal of Infection*, 23(1), 69-72.

BACTERIAL EMPIRE
2019, VOL. 2, NO. 2, 49-53

REGULAR ARTICLE

CYTOTOXIC POTENTIAL OF L-ASPARAGINASE FROM *BACILLUS* SP. *IN VITRO*

Renita Maria D'Souza[1], *Asha Abraham**[2]

Address (es): Victor A. Amadi,
[1] Department of Biotechnology, St.Aloysius College (Autonomous) Mangalore, Karnataka, India.
[2] Father George Albuquerque Pai Cell and Molecular Biology Laboratory, Department of Post Graduate Studies & Research in Biotechnology, St Aloysius College (Autonomous).

*Corresponding author: abraham.asha@gmail.com

ABSTRACT

This study reports the cytotoxic potential of L-Asparaginase isolated from *Bacillus* sp. *Bacillus* sp. was isolated from local soil/water samples and identified by rapid plate assay and further confirmed by phenotypic characterization. Extracellular L- Asparaginase was isolated from broth culture of *Bacillus* sp. and purified by ammonium sulfate precipitation, followed by dialysis and ion exchange and gel filtration chromatography techniques. The purified enzyme was used to study the *in vitro* cytotoxic potential. Varying concentrations (31.25, 62.5, 125, 250, 500 µg/mL) of purified L-Asparaginase was tested on MCF7, HeLa, HepG2 and 3T3L1cell lines by MTT assay. Curcumin was maintained as a positive control. The results revealed that the enzyme showed a significant cytotoxic activity and a dose dependent effect. The minimum inhibitions exhibited at a concentration of 32.25 µg/mL was 19.44% (MCF-7), 10.04% (HeLa), 7.45% (HepG2) and 4.4% (3T3L1) , while the maximum inhibition at a concentration of 500 µg/mL was 71.14% (MCF-7), 68.92% (HeLa), 68.28% (HepG2) and 47.4% (3T3L1). The positive control, curcumin (5 µM) showed an inhibition of 48.13% (MCF-7), 54.42 (HeLa), 64.94% (HepG2) and 44.5% (3T3L1).

Keywords: L-Asparaginase, MCF-7, HeLa, HepG2, 3T3L1, ion exchange, gel filtration

INTRODUCTION

L-asparaginase (L-asparagine amidohydrolase EC 3.5.1.1) is a hydrolase that plays a major role in the metabolism of all living organisms specifically catalyzing the hydrolysis of L-asparagine to L-aspartic acid and ammonia **(Lincoln and More, 2014**). The reaction is irreversible when maintained at physiological conditions. The enzyme is found widespread in nature and has been isolated from various sources such as plant tissues, bacteria, fungi and the serum of rodents (**Batool *et al.*, 2016**). L-asparaginases havebeen isolated and purified from several different bacteria such as *E. coli* **(Roberts *et al.*, 1966**), *Serratia marcescens* **(Whelan and Wriston, 1974)**, *Vibrio succinogens* **(Saxena and Sinha, 1981)**, *Pseudomonas acidovorans* **(Davidson *et al.*, 1977)** *Pseudomonas geniculata* **(Kitto *et al.*, 1979)**, *Corynebacterium glutamicum* **(Mesas *et al.*, 1990)**, *Pseudomonas stutzeri* MB-405 **(Manna *et al.*, 1995**), and *Pseudomonas aeruginosa* **(El-bessoumy *et al.*, 2004).**
The enzyme is proven to have cytotoxic activity and is being used in the treatment of acute lymphoblastic leukemia. This is due to the fact that the leukemic cells do not have the property to produce L-asparagine, which is a non-essential amino acid, whereas the normal cells can produce their own. Therefore, the leukemic cells are deprived of L-asparagine which is their source of nutrition and they are prevented from malignant growth **(El-Naggar *et al.*, 2018).** ELSPAR, ONCASPAR, ERWINASE and KIDROLASE are some of the brands of L-Asparaginase approved by FDA for the treatment of acute lymphoblastic leukemia and lymphosarcoma **(Noura El-Ahmady *et al.*, 2014)**
With reports suggesting difficulties in the bulk preparation of L-asparaginase from pig serum, focus has been shifted to alternate sources where sufficient amount of enzyme can be extracted **(Verma *et al*., 2007**). The objective of our research was to screen local soil and water isolates for L Asparaginase producers, purify and study the cytotoxic potential of the L-Asparaginase isolated.

MATERIAL AND METHODS

Screening and isolation of L-Asparaginase producing organisms

The screening and isolation of L-Asparaginase producing organisms was carried out as described earlier **(Gulati *et al.*, 1997**); (**D'Souza *et al*., 2018**) with some modifications. The media employed was modified M-9 media. The organism giving maximum yield of L-Asparaginase was identified by enzyme assay **(Imada *et al.*, 1973**).

Identification of the L-Asparaginase producing bacterial isolate

All the isolates positive for L-Asparaginase in the screening were subjected to primary identification according to Bergey's Manual of Systematic Bacteriology **(Sneath *et al.*, 1986)**
The isolate giving maximum enzyme activity $B10^{-1}C_2$ (B represents Bandhar sample, 10^{-1}– represents the dilution and C_2- represents the colony number was sent for phenotypic characterization to Microbial Type Culture Collection, IMTECH, Chandigarh, India.

Scale up of the *Bacillus* sp. by suspension culture

***Bacillus* sp.** positive for L-asparaginase by plate assay technique **(Gulati *et al.*, 1997)** and maintained on M-9 agar slant pH 7 at 4ºC, revived at regular intervals, was used for L-asparaginase production from bacteria.The bacterial isolate was transferred to a freshly prepared M-9 agar plate with a sterile loop and incubated at 37ºC for 24 hours. A loop full of culture was aseptically inoculated to 50 mL of M-9 broth in a 100 mL Erlenmeyer flask and incubated at 37ºC in an incubator for 24 hours. This was used as a starter culture for large scale production of the enzyme. M-9 media (1 litre) was taken in a 2000 mL conical flask and 100 mL of 24 hours old inoculum was inoculated into the media and left in static conditions in the incubator at 37ºC for 3 days.

Purification of L-Asparaginase

The purification of the crude extract was carried out at 4°C according to the method of **(Distasio *et al*., 1976).** Finely powdered ammonium sulfate was added to 70% saturation. The mixture was left overnight for 12 hours at 4°C, followed by centrifugation at 8000 rpm for 20 min at 4°C. The precipitate was dissolved in 0.05M Tris HCl buffer pH 8.5, and dialyzed overnight against the same buffer at 4°C. The dialyzed sample was further purified by passing through a column of DEAE cellulose previously equilibrated with 0.05M Tris HCl buffer, pH 8.5. A total of 55 fractions were collected at the flow rate of 3 mL/15min. Fractions showing high activity were pooled together and dialysed against the same buffer. The dialysed sample was lyophilized and concentrated. Lyophilized sample (1 mL)was loaded on to the Sephacryl S-200 gel filtration column and was eluted by using the Tris HCl buffer pH 8.5. A total of 45 fractions were collected at the rate of 3mL/15min.Fractions showing high activity were pooled together dialyzed, lyophilized and used for further studies.

Evaluation of Cytotoxic potential of L-Asparaginase

The cytotoxic potential of L-Asparaginase was carried out according to the procedure described by **(Mosmann, 1983**) . Human Cervical Cancer cell line,

Human Breast Cancer Cell line, Human Liver Cancer Cell line and Mouse Embryo Fibroblast Cell lines were obtained from NCCS, Pune. The cells were maintained in DMEM medium supplemented with 10% FBS and Penicillin (100U/ mL) in a humidified atmosphere of 50µg/ mL CO_2 at 37°C. The cytotoxicity of the sample on the cell lines was determined by MTT assay. Cell suspension (200µL) was seeded in a 96 well plates at required cell density (20,000 cells per well), without the test agent. The cells were allowed to grow for about 24 hours. The various concentrations of the sample (31.25, 62.5, 125, 250, 500 µg/mL) were added. The plates were incubated at 37°C for 48 hours in a 5% CO_2 atmosphere.

After the incubation period, the spent media was removed and MTT reagent was added to a final concentration of 0.5 mg/mL of the total volume. The plates were returned to the incubator and incubated for 3 hours. The MTT was removed and 100 µl of solubilisation solution (DMSO) was added. The absorbance was read on an ELISA reader (ELX800, bioteck) at 570 nm and 630 nm was used as reference wavelength. The IC_{50} value was determined graphically. All experiments were performed in triplicates.

RESULTS AND DISCUSSION

Screening and isolation of L-Asparaginase producing organisms

Strains with pink colour zones around the colonies were considered as positive result for L-asparaginase producing strains

Figure 1 *Bacillus* sp. Colonies showing pink zone on M-9 media supplemented with 0.09% phenol red.

The morphological and cultural characteristics of the isolate $B10^{-1}C_2$ (B represents Bandhar sample , 10^{-1}– represents the dilution and C_2- represents the colony number) are appended herewith. The colonies of isolate were circular, with entire margin, flat elevation and smooth surface. The isolate was found to be gram positive bacilli. It was motile, sporulating bacteria (Table 1). It was exposed to different temperatures 10-55ºC (Table 2), pH range 4-9 (Table 3) and varying concentrations of sodium chloride 2-9% (Table 4), biochemical tests (Table 5) and acid production from various carbohydrates (Table 6). It exhibited growth at pH range of 5-9 and tolerated sodium chloride concentrations of 2-9%, respectively. Phenotypic characterization of the isolate $B10^{-1}C_2$ identified it as *Bacillus* sp.

Table 1 Colony Morphology of the isolate

Colony Morphology	
Character	Observed feature
Configuration	Circular
Margin	Entire
Elevation	Flat
Surface	Smooth
Density	opaque
Gram reaction	+ ve Rods
Spore	+
position	Central
Shape	Oval
Motility	positive

Table 2 Effect of temperature on the growth of the isolate

Temperature	Growth
10ºC	-
15 ºC	-
25 ºC	+
30 ºC	+
37 ºC	+
42 ºC	+
50 ºC	+
55 ºC	-

'+' indicates presence of growth;'-' indicates absence of growth

Table 3 Effect of pH on the growth of the isolate

pH	Growth of the isolate
pH 4	-
pH 5	+
pH 6	+
pH 7	+
pH 8	+
pH 9	+

'+' indicates presence of growth;'-' indicates absence of growth

Table 4 Growth of the isolate at different NaCl (%) concentration

Concentration of NaCl (%)	Growth of the isolate
2.0	+
3.0	+
4.0	+
5.0	+
6.0	+
7.0	+
8.0	+
9.0	+

'+' indicates presence of growth;'-' indicates absence of growth

Table 5 Biochemical tests of the isolate

Biochemical tests	
Growth on Mac Conkey agar medium	-
Indole test	-
Methyl red test	+
Voges Proskauer test	-
Citrate test	+
Casein test	+
Starch hydrolysis	-
Gelatin hydrolysis	-
Nitrate reduction	-
catalase	+
oxidase	+
Esculine hydrolysis	+
H2S gas production	-
Urease test	-

'+' indicates positive result for the test;'-' indicates negative result for the test

Table 6 Acid production from various carbohydrates by the isolate

Acid production from carbohydrates	
Glucose	+
Fructose	+
Salicin	+
Mannitol	+
Raffinose	-
Sucrose	+
Rhamnose	-
Galactose	-
Inositol	-
Xylose	-

'+' indicates production of acid;'-' indicates no production of acids

Purification of L-Asparaginase

Table 7 represents the purification profile of L-Asparaginase from *Bacillus* sp., which shows that there was 14.82 fold purification of the enzyme. The purified enzyme had a specific activity of 124.52 IU/mL/mg.

Table 7 Purification profile of L-Asparaginase from *Bacillus* sp.

Steps	Total activity (IU/mL)	Total protein Conc. (mg)	Specific activity (IU/mL/mg)	Fold purification	Yield
Crude enzyme	19,219.6	2285.76	8.40	1	100
Ammonium sulphate (80%) Precipitation	10,271.9	729.3	14.08	1.676	53.44
Dialysis	5,291	148.10	35.72	4.252	27.52
DEAE cellulose chromatography	1150	16.12	71.33	8.49	5.98
Sephacryl S-200 gel filtration chromatography	327.5	2.63	124.52	14.82	1.70

Evaluation of Cytotoxic potential of L-Asparaginase

Table 8 represents the cytotoxic potential of L-Asparaginase against various cell lines. The IC_{50} value of L-Asparaginase for HeLa cells was found to be 263.18 µg/mL, MCF-7 it was 277.40 µg/mL, for HepG2 it was found to be 273.16 µg/mL and showed resistance against 3T3L1 normal cell line.

Table 8 Cytotoxic effect of *Bacillus* sp. L-Asparaginase on HeLa, MCF-7, HepG2 and 3T3L1 cell lines by MTT assay

Cell lines	Concentration (µg/mL)	cell death (%)	cell viability (%)	IC_{50} (µg/mL)
HeLa	31.25	10.04 ± 0.02	89.96 ± 0.02	263.18
	62.5	22.95 ± 0.00	77.05 ± 0.00	
	125	31.71 ± 0.01	68.29 ± 0.01	
	250	43.51 ± 0.00	56.49 ± 0.00	
	500	68.92 ± 0.01	31.08 ± 0.01	
MCF-7	31.25	19.44 ± 0.02	80.56 ± 0.02	277.40
	62.5	24.44 ± 0.0	75.56 ± 0.0	
	125	37.80 ± 0.0	62.20 ± 0.0	
	250	52.52 ± 0.0	47.48 ± 0.0	
	500	71.14 ± 0.01	28.86 ± 0.01	
HepG2	31.25	7.45 ± 0.00	92.55 ± 0.00	273.16
	62.5	15.86 ± 0.00	84.14 ± 0.00	
	125	28.23 ± 0.00	71.77 ± 0.00	
	250	45.52 ± 0.01	54.48 ± 0.01	
	500	68.28 ± 0.01	31.72 ± 0.01	
3T3L1	31.25	4.40 ± 0.02	95.60 ± 0.02	> 500
	62.5	11.2 ± 0.01	88.80 ± 0.01	
	125	14.3 ± 0.02	85.70 ± 0.02	
	250	30.2 ± 0.00	69.80 ± 0.00	
	500	47.4 ± 0.01	52.60 ± 0.01	

Table 9 Cytotoxic effect of Curcumin (positive control) on HeLa, MCF-7, HepG2 and 3T3L1 cell lines by MTT assay

cell lines	Curcumin concentration (µM)	cell death (%)	cell viability (%)
HeLa	5	54.42	45.58
MCF-7	5	48.13	51.87
HepG2	5	64.94	35.06
3T3L1	5	44.5	55.5

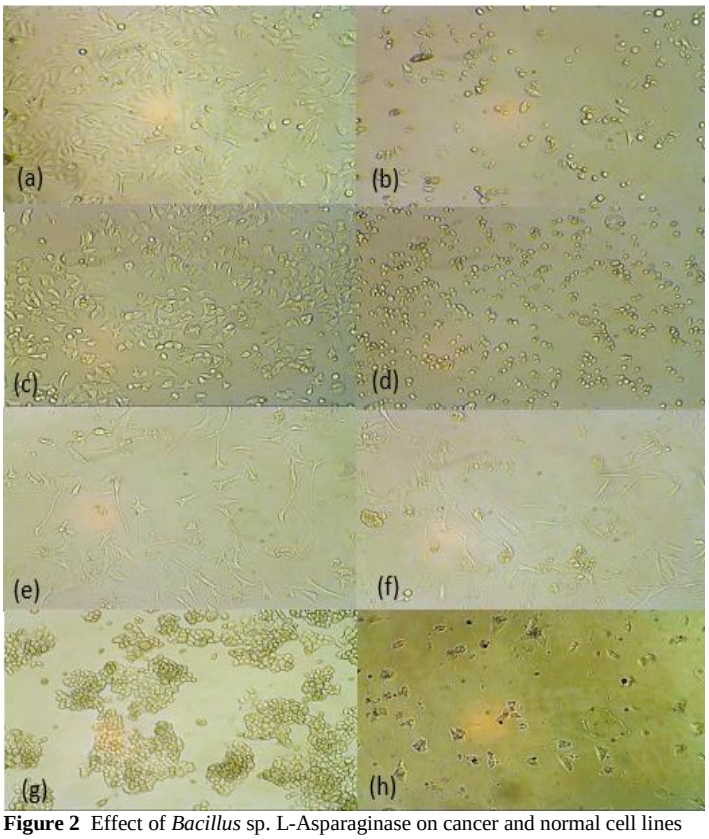

Figure 2 Effect of *Bacillus* sp. L-Asparaginase on cancer and normal cell lines HeLa cell lines (a) untreated (b) treated ; MCF cell lines (c) untreated (d) treated; 3T3L1 cell lines (e) untreated (f) treated ; HepG2 cell lines (g) untreated (h) treated .

Morphological analysis revealed that the number of cells were reduced significantly upon treatment and the treated cells undergoing apoptosis were characterized by cellular rounding up, shrinkage, membrane blebbing and loss of cell adhesion. However 3T3L1 cells showed less resistance to the enzyme treatment.

DISCUSSION

In the present study we observed that the L-Asparaginase from *Bacillus* sp. showed varied levels of inhibition of HeLa, MCF-7, HepG2 cancer cell lines and resistance towards 3T3L1 normal cell lines. The purified L-asparaginase from *Bacillus licheniformis* showed cytotoxic effect against Jurkat clone E6-1, MCF-7 and K-562 **(Mahajan *et al.*, 2014)** with an IC_{50} value of 0.78 IU against MCF-7 cell lines.

Antitumor activity of L-Asparaginase from *Erwinia caratovora* has been studied on Jurkat, Molt 4, human chronic myloid leukemia K562 cells, HL 60 and other human soild tumors. L-Asparaginase significantly increased the number of apoptotic cells to 40% (Jurkat cells) and 99% (HL60 cells) suggesting that the enzyme cytoxicity is associated with only L- asparagine deficiency **(Abakumova *et al.*, 2012)**. *In vitro* cytotoxicity of L-Asparaginase from pathogenic strain *Helicobacter pylori* against different cell lines reported that AGS and MKN 28 gastric epithelial cells being the most affected **(Scotti *et al.*, 2010)**. L-asparaginase is reported to show sensitivity against fibrosarcoma and liposarcoma **(Tardito *et al.*, 2007)**. L-Asparaginase from *Salinicoccus* KJ997975 also exhibited cytotoxicity against Jurkat and HeLa cell lines with an IC_{50} value of 0.171 IU/mL and 0.096 IU/mL respectively **(Bhat and Marar, 2015)**. L-Asparaginase also causes selective death of asparagine dependent tumor cells and induces apoptosis in tumor cells **(Kelo *et al.*, 2009)**. Human Asparaginase enzyme inhibits the growth of Leukemic cells. Lymphoid cells (NALM-6 and MOLT-4) are more sensitive than myeloid cells (K562) to L-Asparaginase treatment (**Belviso *et al.*, 2017**). L-Asparaginase showed cytotoxicity and high selectivity to three leukemic cell lines with a IC_{50} value of 1.16 µg/mL against Daudi cell line, 1.38 µg/mL against Jurkat cell line and 1.08 µg/mL against Molt 4 cell line **(Aljewari *et al.*, 2010).**The differences in the IC_{50} values might be due to the differences in the level of purity of the enzymes. We could achieve 14 .84 fold purity in the present study. Further fold purification of L-Asparaginase might prove to be useful.

CONCLUSION

L-Asparaginase producing bacterial isolate was screened and purified. The *in vitro* cytotoxic effect of L-Asparaginase isolated from *Bacillus* sp. was tested on human cancer cell lines, namely, MCF-7, HeLa, HepG2, and normal cell line 3T3L1. In this study it showed cytotoxic effect on MCF-7, HeLa and HepG2 cell lines. Purified enzyme did not show any effect on normal 3T3L1 cell lines indicating that it may be used for chemotheraputic purpose.

Acknowledgments: The authors thank UGC South western Office, Bangalore, for the minor research project grant (No. MRP(S)-0128/12-13/KAMA002/UGC-SWRO) to carry out part of this work.The authors also thank the Principal and Management of St Aloysius College (Autonomous) for providing the laboratory facilities to carry out this work. Renita Maria D'souza acknowledges the support in terms of FIP granted to her by the Management of St Aloysius College (Autonomous) to carry out this work.

REFERENCES

Abakumova, O.Yu., Podobed, O.V., Karalkin, P.A., Kondakova, L.I., Sokolov, N.N., 2012. Antitumor activity of L-asparaginase from Erwinia carotovora against different human and animal leukemic and solid tumor cell lines. Biochemistry (Moscow) Supplement Series B: Biomedical Chemistry 6, 307–316. https://doi.org/10.1134/S1990750812040026

Aljewari, H.S., Nader, M.I., Alfaisal, A.H.M., Natthida, W., Sahapat, 2010. High Efficiency, Selectivity against Cancer Cell Line of Purified L-Asparaginase from Pathogenic Escherichia coli. International Journal of Medical, Health, Biomedical, Bioengineering and Pharmaceutical Engineering 4.

Batool, T., Makky, E.A., Jalal, M., Yusoff, M.M., 2016. A Comprehensive Review on l-Asparaginase and Its Applications. Applied Biochemistry and Biotechnology 178, 900–923. https://doi.org/10.1007/s12010-015-1917-3

Belviso, S., Iuliano, R., Amato, R., Perrotti, N., Menniti, M., 2017. The human asparaginase enzyme (ASPG) inhibits growth in leukemic cells. PLOS ONE 12, e0178174. https://doi.org/10.1371/journal.pone.0178174

Bhat, M., Marar, T., 2015. Cytotoxic Effect of Purified L-asparaginase from Salinicoccus sp. M KJ997975. International Journal of Current Microbiology and Applied Sciences 4, 701–712.

Davidson, L., Brear, D.R., Wingard, P., Hawkins, J., Kitto, G.B., 1977. Purification and Properties of an L-Glutaminase-L- Asparaginase from Pseudomonas acidovorans. J. BACTERIOL. 129, 1379–1386.

Distasio, J.A., Niederman, R.A., Kafkewitz, D., Goodman, D., 1976. Purification and characterization of L-asparaginase with anti-lymphoma activity from Vibrio succinogenes. J. Biol. Chem. 251, 6929–6933.

D'Souza, R.M., Pinto, S.K., Abraham, A., 2018. Identification of a Novel Strain of Extracellular L- Asparaginase Producing Scopulariopsisbrevicaulis (Sacc.) Bainier using Molecular Techniques. International Journal of Scientific Research in Computer Science Applications and Management Studies 7, 1–5.

El-bessoumy, A.A., Sarhan, M., Mansour, J., 2004. Isolaton, and Purification of L-Asparaginase from Pseudomonas Aeruginosa 50071 Using Solid-state Fermentation. Journal of Biochemistry and Molecular Biology 387–393.

El-Naggar, N.E.-A., Deraz, S.F., El-Ewasy, S.M., Suddek, G.M., 2018. Purification, characterization and immunogenicity assessment of glutaminase free L-asparaginase from Streptomyces brollosae NEAE-115. BMC Pharmacol Toxicol 19. https://doi.org/10.1186/s40360-018-0242-1

Gulati, R., Saxena, R.K., Gupta, R., 1997. A rapid plate assay for screening l-asparaginase producing micro-organisms. Letters in Applied Microbiology 24, 23–26. https://doi.org/10.1046/j.1472-765X.1997.00331.x

Imada, A., Igarasi, S., Nakahama, K., Isono, M., 1973. Asparaginase and Glutaminase Activities of Micro-organisms. Journal of General Microbiology 76, 85–99. https://doi.org/10.1099/00221287-76-1-85

Kelo, E., Noronkoski, T., Mononen, I., 2009. Depletion of L-asparagine supply and apoptosis of leukemia cells induced by human glycosylasparaginase. Leukemia 23, 1167–1171. https://doi.org/10.1038/leu.2008.387

Kitto, G.B., Smith, G., Thiet, T.-Q., Mason, M., Davidson, L., 1979. Tumor Inhibitory and Non-Tumor Inhibitory L-Asparaginases from Pseudomonas geniculata. Journal of Bacteriology 137, 204–212.

Lincoln, L., More, S.S., 2014. Isolation and production of clinical and food grade L- Asparaginase enzyme from fungi. Journal of Pharmacognosy and Phytochemistry 3, 177–183.

Mahajan, R.V., Kumar, V., Rajendran, V., Saran, S., Ghosh, P.C., Saxena, R.K., 2014. Purification and Characterization of a Novel and Robust L-Asparaginase Having Low-Glutaminase Activity from Bacillus licheniformis: In Vitro Evaluation of Anti-Cancerous Properties. PLoS ONE 1–8, e99037. https://doi.org/10.1371/journal.pone.0099037

Manna, S., Sinha, A., Sadhukhan, R., Chakrabarty, S.L., 1995. Purification, characterization and antitumor activity of l-asparaginase isolated from Pseudomonas stutzeri MB-405. Current Microbiology 30, 291–298. https://doi.org/10.1007/BF00295504

Mesas, J.M., Gil, J.A., Martin, J.F., 1990. Characterization and Partial Purification of L-Asparaginase from Corynebacterium Glutamicum. Journal of General Microbiology 136, 515–519. https://doi.org/10.1099/00221287-136-3-515

Mosmann, T., 1983. Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. Journal of Immunological Methods 65, 55–63. https://doi.org/10.1016/0022-1759(83)90303-4

Noura El-Ahmady, E.-N., El-Ewasy, S.M., El-Shweihy, N.M., 2014. Microbial L-asparaginase as a Potential Therapeutic Agent for the Treatment of Acute Lymphoblastic Leukemia: The Pros and Cons. International Journal of Pharmacology 10, 182–199. https://doi.org/10.3923/ijp.2014.182.199

Roberts, J., Prager, M.D., Bachynsky, N., 1966. The Antitumor Activity of Escherichia coli L-Asparaginase. Cancer Research 26, 2213–2217.

Saxena, R.K., Sinha, U., 1981. L-asparaginase and glutaminase activities in the culture filtrates of *Aspergillus nidulans*. Current Science 50, 218–219.

Scotti, C., Sommi, P., Pasquetto, M.V., Cappelletti, D., Stivala, S., Mignosi, P., Savio, M., Chiarelli, L.R., Valentini, G., Bolanos-Garcia, V.M., Merrell, D.S., Franchini, S., Verona, M.L., Bolis, C., Solcia, E., Manca, R., Franciotta, D., Casasco, A., Filipazzi, P., Zardini, E., Vannini, V., 2010. Cell-Cycle Inhibition by Helicobacter pylori L-Asparaginase. PLoS ONE 5, e13892. https://doi.org/10.1371/journal.pone.0013892

Sneath, P.H.A., Mair, N.S., Sharpe, M.E., Holt, J.G., 1986. Bergey's manual of systematic bacteriology. Volume 2. Bergey's manual of systematic bacteriology. Volume 2.

Tardito, S., Uggeri, J., Bozzetti, C., Bianchi, M.G., Rotoli, B.M., Franchi-Gazzola, R., Gazzola, G.C., Gatti, R., Bussolati, O., 2007. The inhibition of glutamine synthetase sensitizes human sarcoma cells to l-asparaginase. Cancer Chemotherapy and Pharmacology 60, 751–758. https://doi.org/10.1007/s00280-007-0421-z

Verma, N., Kumar, K., Kaur, G., Anand, S., 2007. L-Asparaginase: A Promising Chemotherapeutic Agent. Critical Reviews in Biotechnology 27, 45–62. https://doi.org/10.1080/07388550601173926

Whelan, H.A., Wriston, J.C., 1974. Purification and properties of l-asparaginase from Serratia marcescens. Biochimica et Biophysica Acta (BBA) - Protein Structure 365, 212–222. https://doi.org/10.1016/0005-2795(74)90266-9

OPEN ACCESS

BACTERIAL EMPIRE

2019, VOL. 2, NO. 2, 54-57

SCICELL

REGULAR ARTICLE

VIABILITY OF *Lactobacillus acidophilus* AND SYNERESIS OF PROBIOTIC YOGHURT PRODUCED FROM RECONSTITUTED SKIM AND WHOLE MILK POWDER DURING 35 DAYS REFRIGERATED STORAGE AT 4±2 ºC

Obi T.E[1] and Akpoka A.O.[2]*

Address (es): Victor A. Amadi,
[1] Department of Chemistry, College of Natural and Applied Sciences, Igbinedion University Okada, Edo State, Nigeria.
[2] Department of Biological Sciences, College of Natural and Applied Sciences, Igbinedion University Okada, Edo State, Nigeria.

*Corresponding author: succobi@yahoo.com

ABSTRACT

Currently, the food industry wants to expand the range of probiotic yogurts but each probiotic bacteria offers different and specific health benefits. This study examined the viable counts of *Lactobacillus acidophilus* and percentage syneresis of probiotic yoghurt produced from reconstituted skim and whole milk powder stored for 35 days at 4±2 ºC. Skim milk probiotic yoghurt (SMPY) and Whole milk probiotic yoghurt (WMPY) were produced by reconstituting dry milk powder (130 g/900 ml w/v), which was pasteurized at 85 ºC for 15 mins, cooled to 43 ºC and inoculated with freeze dried probiotic yoghurt mixed starter culture containing *Streptococcus thermophilus* (ST), *Lactobacillus bulgaricus* (LB) and *Lactobacillus acidophilus* (LA). The yoghurt samples were analyzed for viability of ST, LB, LA and also syneresis. During storage, the viable counts of ST in SMPY decreased from $5.43x10^8$ to $5.18x10^6$ cfu/ml, LB ($2.47x10^8$ to $8.10x10^5$ cfu/ml) and LA ($1.83x10^8$ to $5.78x10^5$ cfu/ml). Similarly, the viable counts of ST in WMPY decreased significantly from $5.40x10^8$ to $5.15x10^6$ cfu/ml, LB ($2.43x10^8$ to $7.82x10^5$ cfu/ml) and LA ($1.80x10^8$ to $5.84x10^5$ cfu/ml). Although the mean viable counts of the LA decreased during storage, both SMPY and WMPY still contained an average of $1.48x10^6$ cfu/ml of probiotic cells up to 28 days of storage, which is above the "therapeutic minimum" of 10^6 cfu/ml. The percentage syneresis of SMPY and WMPY increased significantly during the 35 days of storage, from 24.4-32.0 % and 24.8-32.7 % respectively. There was a positive correlation between storage time and syneresis thus affecting the texture. In conclusion, yoghurt made from either skim or whole milk powder can be used as an adequate carrier of LA (probiotic bacteria) up to a period of 28 days at 4±2 ºC and a stabilizer should be used to reduce the separation of whey and thus maintain the texture.

Keywords: Lactic acid bacteria, probiotic bacteria, plain stirred yoghurt, syneresis, storage time

INTRODUCTION

Lactic acid bacteria (LAB) that are basically used in the dairy industry for the fermentation of a wide variety of food products are used primarily for their preservative and therapeutic effects (**Gourama and Bullerman, 1995**). However, during the past two decades, there has been renewed interest in the study of the nutritional and therapeutic aspects of dairy products (**Sinha *et al.*, 1989**). *Streptococcus thermophilus* and *Lactobacillus delbruekii* ssp. *bulgaricus* are used for manufacturing of yogurt. *Bifidobacteria spp*, *Lactobacillus acidophilus* and *Lactobacillus casei* are widely used as probiotic bacteria in human and animal health (**Gilliland, 1990**). They are called probiotic bacteria and are defined as living microorganisms, which upon ingestion in sufficient quantity exert health benefits beyond inherent basic nutrition (**Grosso and Trindade, 2004**). Also, with the advent of functional foods (foods with additional health benefits), the trend towards healthier eating has continued to grow (**Farooq and Haque, 1992**) and emphasis has been placed on developing new fermented milks containing these microorganisms called probiotics (**Banon and Hardy, 1991**). *Lactobacillus spp*. (probiotic microorganisms) constitutes a major part of the human intestinal microflora and plays an important role in maintaining good health (**Tamime and Robinson, 1985**). *Lactobacillus spp*. are increasingly being incorporated into fermented dairy products such as yoghurt and acidophilus milk. However, they grow slowly in milk because they lack proteolytic activities and the usual practice is to add yoghurt starter bacteria culture to enhance the fermentation process in the production of probiotic yoghurt (**Dave and Shah 1996**). In order for *Lactobacillus spp*. to provide therapeutic benefits, it has been recommended that they be viable and ingested in numbers greater or equal to 1 million cells per gram of yoghurt (**Kailasapathy and Chin**). But, several factors such as pH, hydrogen peroxide, oxygen content, lactic and acetic acids concentration and temperature of storage have been postulated to affect the viability of probiotic bacteria in yoghurt (**Shah *et al.*, 1995**). Thus, maintaining the viability of *Lactobacillus spp.* until yoghurts are consumed in order to ensure the delivery of live organisms has been of much interest. Foods containing probiotics are sold in many countries, although their survival rate in foods is doubtful since some of the strains are extremely sensitive to a series of factors (**Tamime and Robinson, 1985**), and one of the requirements for microorganisms to be used as dietary adjuncts is the need to maintain viability and activity in the carrier food before consumption (**Fuller, 1999**).

Syneresis occurs when whey separates from yoghurt due to contraction of the coagulum. (**SAS, 2004**) reported that the cause of whey separation is not clear. In addition to the protein network formed as a result of the acidification in yoghurt production, yoghurt culture bacteria such as *Streptococcus thermophilus* are known to produce a polysaccharide slime. Which is thought to have a stabilizing effect on the protein gel that helps to prevent syneresis and responsible for enhancing the rheological properties of yoghurt (**Farooq and Haque, 1992**). Therefore, in the commercial manufacture of yoghurt, particularly stirred type, it is common practice to add additional stabilizers like gelatin, pregelatinized starch, agar, guar gum, pectin and carrageenan. Such additives are used to prevent syneresis, improve viscosity and body, as well as playing a cost-saving role in reducing the amount of extra milk solids required and enhanced mouth-feel in low-fat-reduced varieties (**Farooq and Haque, 1992**).

Although a lot of research work has been done on probiotic yoghurt made from fresh milk and skim milk fortified fresh milk, literature is scanty on the viability of *Lactobacillus acidophilus* in probiotic yoghurt produced from reconstituted whole or skim milk powder. Hence, a practical approach towards creating awareness among manufacturers, food regulatory bodies and other consumers of probiotic yoghurt is to study the percentage syneresis and viability of *Lactobacillus acidophilus* in probiotic yoghurt produced from reconstituted whole and skim milk powder.

Therefore, the objective of this study was to assess the effect of storage on percentage syneresis and viability of *Lactobacillus acidophilus* in probiotic yoghurt produced from reconstituted whole and skim milk powder in a mixed culture of *Streptococcus thermophilus* and *Lactobacillus delbrueckii* ssp. *bulgaricus* stored at 4±2 ºC for 35 days to ascertain the extent to which the probiotic activity can be sustained in both types of milk .

MATERIALS AND METHODS

Substrates and Starter Culture

The substrates used for the study were reconstituted whole and skim milk powder. Freeze-dry mixed yoghurt starter culture containing *Streptococcus thermophilus*, *Lactobacillus delbrueckii* ssp. *bulgaricus* and *Lactobacillus acidophilus* bacteria was purchased from Geomex Industries Ltd. Canada.

Production of Whole and Skim Milk Probiotic Yoghurt

This was done according to the method of (Shah *et al*., 1995) and as described by (**Lee and Lucey, 2004**) in Fig 1. After production, the yoghurt samples were subjected to determination of viable counts of *Streptococcus thermophilus*, *Lactobacillus delbrueckii* ssp. *bulgaricus* and *Lactobacillus acidophilus* bacteria and examined for syneresis (Day 0) and weekly for a period of 35 days. All analyses were done in triplicates**.**

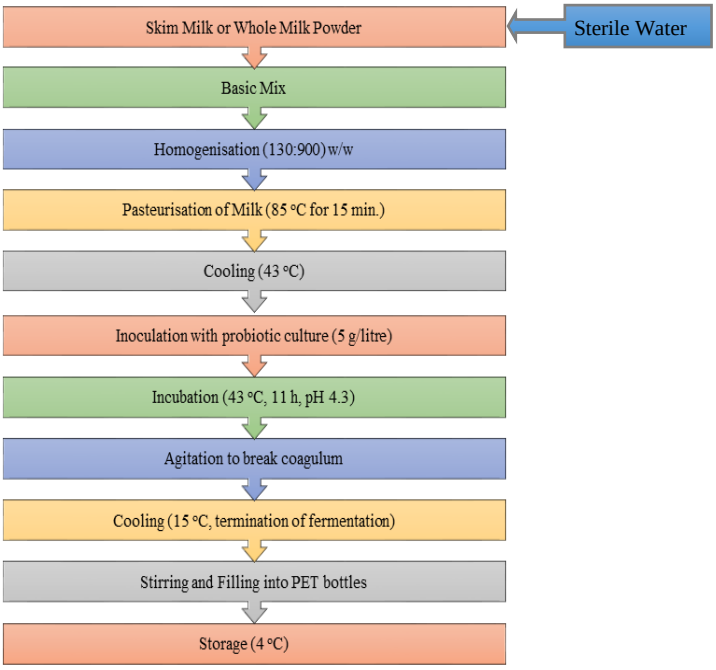

Figure 1 Flow Chart for the Production of Probiotic Yoghurt
Source: (Shah *et al.*, 1995)

Microbiological Analyses

Approximately 1 ml of each yoghurt sample (SMPY AND WMPY) was diluted with 9 ml of sterile 0.1 % (w/v) peptone water and mixed uniformly with a vortex mixer. Subsequent serial dilutions were made and viable counts of *Streptococcus thermophilus*, *Lactobacillus delbrueckii* ssp. *bulgaricus* and *Lactobacillus acidophilus* enumerated using the pour plate technique.

Enumeration of *Streptococcus thermophilus* bacteria

The counts of *Streptococcus thermophilus* were enumerated on *Streptococcus thermophilus* (ST) agar after incubating the plates aerobically at 37 ºC for 24 h. The colonies of *Streptococcus thermophilus* on the plates were counted using a colony counter and recorded as colony forming units per ml of sample (**Dannenberg and Kessler, 1988**).

Enumeration of *Lactobacillus delbrueckii* ssp. *bulgaricus* bacteria

The counts of *Lactobacillus bulgaricus* were differentially enumerated on MRS agar adjusted to pH 5.2 and incubated anaerobically (using the BBL Gas Pak system) at 37 ºC for 72 h. The colonies of *Lactobacillus bulgaricus* were counted using a colony counter and recorded as colony forming units per ml of sample (**Dannenberg and Kessler, 1988**).

Enumeration of *Lactobacillus acidophilus* bacteria

The counts of *Lactobacillus acidophilus* were selectively enumerated on MRS-Maltose agar with pH adjusted to 6.2 and incubated anaerobically (using the BBL Gas Pak system) at 37 ºC for 72 h. Plates containing the colonies of *Lactobacillus acidophilus* were counted using a colony counter and recorded as colony forming units per ml of sample (**Dannenberg and Kessler, 1988**).

Determination of percentage syneresis

Syneresis was determined using the modified method of **Gilliland (1990)**. Approximately 50 ml of yoghurt was filtered with a funnel for 2 h at 10 ºC. The resultant whey was collected in 100 ml graduated cylinder and used as an index of syneresis. The % syneresis was thus calculated from the formula:

$$\%\ Syneresis = \frac{Vol.of\ Separated\ Whey}{Vol.of\ Sample} \times 100$$

Statistical analyses

All data were subjected to Analysis of variance (ANOVA) to determine any significant difference at 5% level using the method of **Shah et al., (1995)** and was reported as means of three replicates. Means were separated by Duncan's multiple range tests to establish if there were significant differences between the samples (**SAS, 2004**).

RESULTS

Table 1 Changes in Viable Counts (cfu/ml) of *Streptococcus thermophilus* during refrigerated storage at 4±2 ºC for 35 days

Storage Time (days)	*Streptococcus thermophilus*	
	SMPY	**WMPY**
0	$5.43x10^{8}{}_{a}$	$5.40x10^{8}{}_{a}$
7	$5.23x10^{8}{}_{a}$	$5.19x10^{8}{}_{a}$
14	$4.90\ x10^{8}{}_{b}$	$4.88\ x10^{8}{}_{b}$
21	$8.42x10^{7}{}_{c}$	$8.40x10^{7}{}_{c}$
28	$7.63x10^{7}{}_{d}$	$7.60x10^{7}{}_{d}$
35	$5.18x10^{6}{}_{e}$	$5.15x10^{6}{}_{e}$

Legend: SMPY – Skim milk probiotic yoghurt; WMPY- Whole milk probiotic yoghurt

Values in the same column and type of yoghurt with different subscripts are significantly different at ($p<0.05$)

Table 2 Changes in Viable Counts (cfu/ml) of *Lactobacillus bulgaricus* during refrigerated storage at 4±2 ºC for 35 days

Storage Time (days)	*Lactobacillus bulgaricus*	
	SMPY	**WMPY**
0	$2.47x10^{8}{}_{a}$	$2.43x10^{8}{}_{a}$
7	$2.13x10^{8}{}_{b}$	$2.07x10^{8}{}_{b}$
14	$6.01x10^{7}{}_{c}$	$5.98x10^{7}{}_{c}$
21	$9.40x10^{6}{}_{d}$	$9.37x10^{6}{}_{d}$
28	$3.52x10^{6}{}_{e}$	$3.50x10^{6}{}_{e}$
35	$8.10x10^{5}{}_{f}$	$7.82x10^{6}{}_{f}$

Legend: SMPY – Skim milk probiotic yoghurt; WMPY- Whole milk probiotic yoghurt

Values in the same column and type of yoghurt with different subscripts are significantly different at ($p<0.05$)

Table 3 Changes in Viable Counts (cfu/ml) of *Lactobacillus acidophilus* during refrigerated storage at 4±2 ºC for 35 days

Storage Time (days)	*Lactobacillus acidophilus*	
	SMPY	WMPY
0	$1.83x10^{8}{}_{a}$	$1.80x10^{8}{}_{a}$
7	$1.57x10^{8}{}_{b}$	$1.50x10^{8}{}_{b}$
14	$4.73\ x10^{7}{}_{c}$	$4.68\ x10^{7}{}_{c}$
21	$3.60x10^{6}{}_{d}$	$3.53x10^{6}{}_{d}$
28	$1.50x10^{6}{}_{e}$	$1.47x10^{6}{}_{e}$
35	$5.78x10^{5}{}_{f}$	$5.84x10^{5}{}_{f}$

Legend: SMPY – Skim milk probiotic yoghurt; WMPY- Whole milk probiotic yoghurt

Values in the same column and type of yoghurt with different subscripts are significantly different at ($p<0.05$)

Syneresis and Storage Time

Syneresis values for both samples of probiotic yoghurt stored at 4º±C for thirty-five days are shown in Fig 2. There was a significant increase in syneresis ($P< 0.05$) for both samples of probiotic yoghurts during the storage period. The percentage syneresis of SMPY and WMPY increased significantly during the 35 days of storage, from 24.4% to 32.0% and 24.8% to 32.7% respectively This finding is in agreement with the report of (**Dave and Shah, 1996**). Furthermore, there was a good correlation between syneresis and storage time. The increase in syneresis values could be due to the probable absence of stabilizers which could have made the yoghurt samples firm and whey separation minimal. The stabilizers would have in addition improved the water binding capacity of the yoghurt samples. The increase could also be due to the denaturation of beta-lactoglubin in the processed milk and the aging process of the curd as storage progressed (**Collier, 2004**). Moreover, the slower rate of syneresis in skim milk probiotic yoghurt (SMPY) could be as a result of higher total solids as reported by (**Varga *et al*., 2002**) who observed that increases in total solids reduce the rate of syneresis.

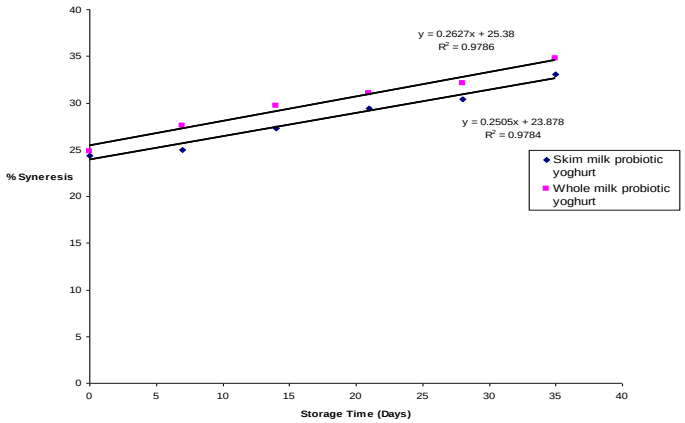

Figure 2 Changes in Syneresis of Skim and Whole Milk Probiotic Yoghurt during storage

DISCUSSION

Tables 1, 2 and 3 show the changes in the viable counts of *Streptococcus thermophilus* (ST) *Lactobacillus* ssp. *bulgaricus* (LB) and *Lactobacillus acidophilus* (LA) both samples of probiotic yoghurt (SMPY and WMPY). The viable counts of *S. thermophilus, L. bulgaricus* and *L. acidophilus* decreased throughout the storage period irrespective of the type of milk used. The viable counts of ST in SMPY decreased from $5.43x10^{8}$ to $5.18x10^{6}$ cfu/ml, LB ($2.47x10^{8}$ to $8.10x10^{5}$ cfu/ml) and LA ($1.83x10^{8}$ to $5.78x10^{5}$ cfu/ml). Similarly, the viable counts of ST in WMPY decreased significantly from $5.40x10^{8}$ to $5.15x10^{6}$ cfu/ml, LB ($2.43x10^{8}$ to $7.82x10^{5}$ cfu/ml) and LA ($1.80x10^{8}$ to $5.84x10^{5}$ cfu/ml). However, viable counts of *S. thermophilus* were more numerous than that of *L. bulgaricus* and *L. acidophilus* in both types of yoghurt. The decrease in viable counts was more pronounced in *S. thermophilus* followed by *L. acidophilus* and *L. bulgaricus*. This result is in agreement with the findings of (**Dave and Shah, 1996**). However, the viable counts of LA in both sample of yoghurt remained well above the recommended therapeutic minimum of 1 million cells per ml of yoghurt up to 28 days of storage. But, after 28 days, the decrease in the viable count of LA fell below the recommended therapeutic minimum. The decrease in viability of the lactic acid bacteria especially *L. acidophilus* could be as a result of the decrease in pH of the yoghurt samples. This finding is in agreement with the report of (**Tamime and Robinson, 1995**) who reported one log cycle decrease in viable count of LA with decrease in pH. Also, (**Sakai *et al*., 1987**) reported that the final pH of yoghurt can also affect the viability of *L. acidophilus* and *Bifidobacteria* spp. It has also been reported that pH values of 4.5 or lower can jeopardize the viability of probiotic organisms in yoghurt stored at 5 ºC (**Banon and Hardy, 1991**). Furthermore, (**Yeganehzad *et al*., 2007**) reported that the pH of fermented milks may decrease considerably during storage, which can affect the growth and viability of *L. acidophilus* and *Bifidobacterium* spp. Furthermore, (**Kurman and Rasic, 1991**) reported that the most important factor in probiotic mortality was the low pH of the yoghurt and any drop in pH below 4.3 greatly affected their viability.

CONCLUSION

This study showed that the viability of *L. acidophilus* was sustained above the 'therapeutic minimum' of 10^{6} cfu/ml up to the 28th day of storage and that the changes in the viability of *Lactobacillus acidophilus* in skim and whole milk probiotic yoghurt did not differ significantly. Thus, yoghurt made from either skim or whole milk powder can be used as an adequate carrier of probiotic bacteria like fresh milk. The positive correlation between syneresis and storage time of the yoghurts showed that stabilizers should be used to prevent whey separation during storage.

Acknowledgement: The authors are grateful to the technical staff of the Department of Food Science and Technology of the University of Agriculture, Abeokuta for their technical assistance**.**

REFERENCES

Banon, S. & Hardy, J. (1991). A Colloidal Approach of Milk Acidification by Glucono-Delta-Lactone. *Journal of Dairy Sci.*, 75, 935-941. https://doi.org/10.3168/jds.S0022-0302(92)77833-3

Collier, J. (2004). Promoting bacterial culture: The importance of probiotics and prebiotics to good health. *Cell Science Review*, l, 1

Dannenberg, F. & Kessler, H. G. (1988). Effect of denaturation of ß-lactoglobulin on texture properties of set-style non-fat yoghurt. 1. Syneresis. *Milchwissenschaft*, 43, 632-636.

Dave, R. I. & Shah, N. P. (1996). Evaluation of media for selective enumeration of *Streptococcus thermophilus, Lactobacillus delbrueckii* sp. *bulgaricus, Lactobacillus acidophilus*, and *Bifidobacteria*. *Journal of Dairy Sci.*, **79**, 1529-1536. https://doi.org/10.3168/jds.S0022-0302(96)76513-X

Farooq, H. & Haque, Z. U. (1992). Effect of sugar esters on the textural properties of nonfat low-calorie yoghurt. *Journal of. Diary Sci.* 75, 2676-2680. https://doi.org/10.3168/jds.S0022-0302(92)78029-1

Fuller, R. (1999). Probiotics in man and animals. *Journal of Applied Bacteriology*, 66, 365-378. https://doi.org/10.1111/j.1365-2672.1989.tb05105.x

Gilliland, S. E. (1990). Acidophillus milk products, a review of potential benefits to consumers. *Journal of Diary Sci.*, 72, 2483-2494. https://doi.org/10.3168/jds.S0022-0302(89)79389-9

Gourama, H. & Bullerman, L. B. (1995). Antimycotic and antiaflatoxigenic effect of lactic acid bacteria. A review. *J Food Protection*, 58 (11), 1275-1280. https://doi.org/10.4315/0362-028X-58.11.1275

Grosso, C. F. & Trindade, C. S. (2004). Stability of free and immobilized *Lactobacillus acidophilus* and *Bifidobacterium lactis* in acidified milk and of immobilized *Bifidobacterium lactis* in yoghurt. *Brazilian Journal of Microbiology*, 35, 1-2. http://dx.doi.org/10.1590/S1517-83822004000100025

Kailasapathy, K. & Chin, J. (2000). Survival and therapeutic potential of Probiotic organisms with reference to *Lactobacillus acidophilus* and *Bifidobacterium* spp. *Immune Cell Biology*, 78, 80-88. https://doi.org/10.1046/j.1440-1711.2000.00886.x

Kurman, J. A. & Rasic, J. L. (1991). The health potential of products containing bifidobacterial. In: Robinson RK (ed) *Therapeutic Properties of Fermented Milks*. Elsevier Science *Publishers* Ltd., London.

Lee, W. J. & Lucey, J. A. (2004). Rheological properties, whey separation and microstructure in set-style yoghurt: Effects of heating temperature and incubation temperature. *Journal of Texture Study*, 34, 515-536. https://doi.org/10.1111/j.1745-4603.2003.tb01079.x

Sakai, K., Mishima, C., Tachiki, T., Kumagai, H. & Tochikura, T. (1987). Mortality of *bifidobacterial* in in boiled yoghurt. *Journal Fermentation Technology*, 65, 215-220. https://doi.org/10.1016/0385-6380(87)90167-1

SAS. (2004). *SAS user's guide*. 12.3. Cary, NC, USA: SAS Institute Inc.

Shah, N. P., Lankaputhra, W. E., Britz, M. L. & Kyle, W. S. (1995). Survival of *L. acidophilus* and *Bifidobacterium bifidum* in commercial yoghurt during refrigerated storage. *International Dairy Journal*, 5, 515–21. https://doi.org/10.3168/jds.S0022-0302(92)77895-3

Sinha, R. P., Modler, H. W. & Emmons, D. B. (1989). Changes in acidity and starter bacteria in commercial yoghurts during storage. *Cultured Dairy Products Journal*, 24(2),12-14.

Steel, R. G. & Torrie, J. H. (1980). *Principles and Procedures of Statistics – A biometrical approach.* McGraw-Hill, New York. 2nd edn.

Tamime, A. Y. & Robinson, R. K. (1985). Technology of manufacture of thermophilic fermented milks. *International Dairy Federation Bulletin*, Brussels, Belgium, 277, 82.

Varga, L., Szigeti, J., Kovacs, R., Foldes, T. & Buti, S. (2002). Influence of a *Spirulina platensis* biomass on the microflora of fermented ABT milks during storage (RI*). Journal of Dairy Sci.*, 85, 1031-1038.

Yeganehzad, S., Mazaheri-Tehrani, M. & Shahidi, F. (2007). Studying microbial, physicochemical and sensory properties of directly concentrated probiotic yoghurt. *African Journal of Agricultural Research*, 2(8), 366-369.