MEASUREMENT OF YEAST GROWTH USING SPECTROPHOTOMETER

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MEASUREMENT OF YEAST GROWTH USING SPECTROPHOTOMETER

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1.1 Introduction

Yeasts are unicellular eukaryotic fungi with a particle size of 5 × 10 μm (Montes de Oca *et al*., 2016). They are classified as ascomycetes, a group which also includes species of the genera *Neurospora* and *Sordaria*. Yeasts have simple nutritional needs and require reduced carbon sources such as glucose, sucrose, fructose and maltose for energy production. The composition of the yeast cell is identical to the animal cell and includes organelles like nucleus, endoplasmic reticulum, mitochondrion, golgi apparatus, vacuole, and cytoskeleton with all its three major components (microfilaments, microtubules and intermediate filaments) (de Becze, 1955; Montes de Oca *et al*., 2016).

Yeast cells can indefinitely reproduce both as haploids (n) and diploids (2n). The transition between haploid and diploid phases of the life cycle is accomplished through either sexual reproduction where two haploid cells of opposite mating types (*a* and *ά*) communicate with each other using proteins called pheromones and mate to form a diploid zygote, or meiosis where one diploid cell undergoes premeiotic S-phase and two meiotic divisions, resulting in four haploid cells enclosed in ascospore walls. The haploid cells can also reproduce mitotically as stable haploid cells (de Becze, 1955; Montes de Oca *et al*., 2016).

Yeast population growth studies require inoculation of viable cells of a pure culture into a sterile medium and incubation of the culture under optimally controlled environmental conditions. The cells normally reproduce rapidly and the dynamics of the microbial growth is charted by means of a population growth curve, which is constructed by plotting increase in cell number, metabolic activity or absorbance against time of incubation. The curve can be used to delineate the stages of the growth cycle, and facilitate measurement of cell numbers and growth rate of yeast as expressed by its generation time, thus the time required for a microbial population to double. The stages of a typical growth curve comprises lag phase, log (exponential) phase, stationary phase and death (decline) phase. Each of these phases represents a distinct period of growth that is associated with a typical physiological change in the cell culture.

The lag phase occurs during the cells' adjustment to new environment (culture conditions). It is the transition to the exponential phase after the initial population has doubled (Yates & Smotzer, 2007). During this phase, cellular metabolism accelerates, resulting in rapid biosynthesis of cellular macromolecules (enzymes) in preparation for the log phase. Despite the increase in cell size, there is no cell division and therefore no increase in cell number. The lag phase usually lasts from minutes

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to several hours. The length of the lag phase can be controlled to some extent due to its dependence on the type of medium and the initial inoculum size. For example, if an inoculum is taken from an exponential phase culture, there will be no noticeable lag phase. In contrast, a lag phase will be observed in a growth curve if the inoculum was taken from a stationary phase culture since the stationary phase cells adjust to the new conditions and shift physiologically from stationary phase cells to exponential phase cells (Maier, 2008).

The second phase of growth is the log phase. This phase is characterised by a period of most rapid growth possible under the conditions present in the culture system. During the exponential growth, the physiologically robust cells reproduce at a uniform rate, thus the rate of increase of cells in the culture is proportional to the number of cells present at any particular time. The yeast population doubles regularly until a maximum number of cell is reached. The duration of the log phase varies with regards to the species and the composition of the medium. The generation time of the species is calculated from the log phase (linear portion) of the growth curve.

At the stationary phase, the number of cells undergoing division equal the number of cells dying. There is no further increase in cell number and the population is maintained at its maximum level for a period of time. The basic factors responsible for this phase are depletion of some essential metabolites and accumulation of toxic end products in the medium. The occurrence of endogenous metabolism (growth on dead cells) is best observed during this phase, particularly when growth is measured in terms of oxygen uptake or evolution of carbon dioxide.

The final phase of the growth curve is the death phase, which is characterised by a net loss of culturable cells. This results from continuing depletion of nutrients and build-up of metabolic wastes. Usually, the cells die at a rapid and uniform rate. This decrease in population closely parallels the increase in population during the log phase. Theoretically, the entire population should die during a time interval equal to that of the log phase. However, this does not occur, since a small number of highly resistant yeasts persist for an indeterminate length of time (Maier, 2008).

1.1.1 Research objectives

1.1.1.1 *General objective*

The broad aim of the study was to measure the growth of yeast species using spectrophotometer.

1.1.1.2 *Specific objectives*

The specific objectives of the study were to:

1. Isolate budding and fission yeasts.
2. Study the growth of the species over a period of time.
3. Determine the generation times and growth rates of the species.

1.2 Materials and Methods

1.2.1 Sample collection

Fruits of banana, tomato and orange that were near rotten were obtained from the University of Cape Coast Science market and used for the study.

1.2.2 Culture media

The general media used for the study were yeast peptone dextrose agar (YPDA) and yeast peptone dextrose broth (YPDB).

Table 1: *Recipe for general purpose media*

	Yeast peptone dextrose agar (YPDA)	Yeast peptone dextrose broth (YPDB)
Yeast	5 g	5 g
Peptone	10 g	10 g
Dextrose	20 g	20 g
Agar	15 g	–
Distilled water	1000 mL	1000 mL
pH	5	5

1.2.2.1 Preparation of yeast peptone dextrose agar (YPDA)

This process was carried out using the method described by Brown (2013). Yeast (5 g) was weighed using an analytical balance (Mettler Toledo PG203) and transferred into a conical flask containing 500 mL sterile distilled water. The mixture was vigorously shaken and allowed to stand for 24 hr for separation. The supernatant was filtered off into a 1000 mL measuring cylinder using a filter paper and the filtrate was topped up with distilled water to the 1000 mL mark. The filtrate was then transferred into a flat bottom flask containing dextrose (20 g), peptone (10 g) and agar (15 g). The mixture was transferred unto a heat-stirrer (Stuart SB162) and the pH was adjusted to 5 using a pH metre (DENVER UB-10), and hydrochloric acid (HCl) and sodium hydroxide (NaOH) solutions. After the pH adjustment, equal volumes of the mixture were dispensed into conical flasks, corked with cotton wool, wrapped with aluminium foil and sterilised by autoclaving at 121 ^{o}C and 15 psi for 15 min. The sterile media were then transferred unto a shelf for storage.

1.2.2.2 *Preparation of yeast peptone dextrose broth (YPDB)*

The procedure described in Section 1.2.2.1 was followed for the preparation of yeast peptone dextrose broth (YPDB), except the addition of agar. The mixture was autoclaved at 121 ^{o}C and 15 psi for 15 min, and stored on a shelf.

1.2.3 Isolation of test organisms

One gramme (1 g) each of banana pulp, tomato and orange loculus was weighed and transferred using flame sterile forceps into test tubes containing 10 mL sterile YPD broth amended with amoxicillin (antibiotic). The cultures were then incubated on orbital shaker (IKA®KS 260 basic) at 300 rpm for 72 hr.

The grown cultures were serially diluted with sterile distilled water and 1 mL aliquots from the 10^{-3} and 10^{-4} dilutions were pipetted into sterile Petri dishes. Molten YPD agar amended with antibiotic was poured into the Petri plates, swirled clockwise and anticlockwise for uniform distribution and allowed to solidify. The Petri plates were then incubated at room temperature of 25 ± 2 ^{o}C for 48 hr. Well-isolated colonies were picked using flame sterile inoculation needle, transferred into fresh YPD broth (10 mL) and incubated at room temperature for 48 hr. The cultures were then streaked, using flame sterile inoculation loop, and maintained on fresh YPD agar plates.

1.2.4 Identification and preservation of test organisms

The test organisms were identified based on morphological characteristics and microscopic examinations under the microscope with the aid of identification keys (Khattab *et al*., 2016; Lachance, 2011; Schneiter, 2004). Cells of young actively growing cultures were stained with bromophenol blue and used for the microscopy. Pure cultures of the test organisms were preserved in 10% glycerol and stored at -20 ^{o}C.

1.2.5 Preparation of stock culture

Budding and fission yeasts were selected and used for the preparation of stock cultures. Agar discs (0.8 cm diameter) of plate cultures of budding and fission yeasts were made with flame sterile cork borer and transferred into conical flasks containing 100 mL sterile YPD broth amended with antibiotic. The broth cultures were incubated on orbital shaker at 300 rpm for 48 hr and used as stock for determination of cell concentration and growth curve.

1.2.6 Determination of cell concentration

Serial dilution of 1 mL stock culture was carried out to determine the cell concentration. An aliquot (1 mL) of the stock culture was pipetted into a test tube containing 9 mL sterile distilled water. One millimetre (1 mL) of the mixture was pipetted into fresh test tube containing 9 mL sterile distilled water. The process was repeated till the fourth dilution (10^{-4}). Samples (1 mL) from the 10^{-3} and 10^{-4} dilution factors were pipetted into sterile Petri dishes and molten sterile YPD agar amended with

antibiotic was added to the mixtures. The plates were swirled for uniform distribution and incubated at room temperature for 48 hr. The number of colonies on each plate was counted and the total number of colonies was determined using eqn (1).

No. of viable colonies = ((No. of colonies on plate / Vol. plate) × (1 / Dilution factor)) (1)

1.2.7 Determination of yeast growth curve

This process was carried out by determining the optical density/absorbance (OD) of the yeast species using spectrophotometer (Genova). Five millilitre (5 mL) stock culture was pipetted into 200 mL sterile YPD broth amended with antibiotic and incubated on orbital shaker at 300 rpm. The absorbance of the cultures were determined at 1 hr intervals for 52 hr starting at time zero (0 hr).

1.2.7.1 Spectrophotometric analysis

The wavelength of the spectrophotometer was set to 600 nm and blanked with a cuvette containing 2 mL sterile YPD broth (Friedman, 2010; Bridges Directed Research Program [BDRP], 2009). The culture (1 mL) was pipetted into a fresh cuvette and topped with 1 mL sterile YPD broth. The cell suspension cuvette was placed into the cuvette slot and the cuvette chamber was closed. The absorbance of the cell suspension was recorded and the process was repeated at an hour interval for 52 hr. The optical densities for cell suspensions with absorbance greater than 1 were calculated using eqn (2).

Optical density (absorbance) = 2 – log %T (2)

where %T is percent transmittance.

1.2.8 Determination of growth rates and generation times

The growth rates and generation times of the species were determined from a graphical presentation of the results obtained from Section 1.2.7.1. Equations (3) – (4) were used for the calculations (Garg, 2017; Anonymous, n.d)

Generation time (G) = $t_{(OD\ b)} - t_{(OD\ B)}$ (3)

Growth rate (K) = $\frac{1}{t\ (OD\ b) - t\ (OD\ B)}$ (4)

Where OD b = Optical density (absorbance) representing a point beginning the doubling of a turbidity during the log phase.

OD B = Optical density (absorbance) representing a point ending the doubling of a turbidity during the log phase.

t = corresponding times for OD b and OD B.

1.3 Results

1.3.1 Isolation and identification of test organisms

The test organisms were isolated from near rotten fruits of tomato, orange and banana. Identification was carried out based on cultural and morphological characteristics (Table 2). Plate cultures and microscopic morphologies of budding and fission yeasts isolated from the samples are shown in Figure 1. The budding yeasts were characterised by lemon-shaped and egg-shaped cells. The lemon-shaped cells showed axial mono buds whereas the egg-shaped cells showed radial mono buds. Colonies of the lemon-shaped cells were butyrous, slight glistening, raised and smooth with entire margin. Similar features were observed in colonies of the egg-shaped cells. The egg-shaped cells were however more coherent than the lemon-shaped cells. The fission yeasts were rod shaped with medial fission. Their culture colonies were white, glistening and flat with undulating margins.

Table 2: *Yeast species isolated from samples*

Yeast species	Source
Fission yeast	Orange
	Tomato
Budding yeast	
Lemon-shaped	Orange
	Tomato
	Banana
Egg-shaped	Banana

1.3.2 Growth curve of budding and fission yeasts

The cell concentrations and growth patterns of the fission and budding (lemon-shaped) yeasts were determined (Figures 2 and 3). The fission yeast recorded a cell concentration of 125×10^5 CFU mL^{-1} (7.1 OD) whereas the budding yeast recorded a cell concentration of 352×10^5 CFU mL^{-1} (7.5 OD). The optical densities of the fission yeast and budding yeast at time zero were found to be 0.5 and 0.3 respectively. However, an optical density of 1.4 was observed in both organisms at the 52nd hour. The highest optical density in the fission yeast (2.7) was noticed at 35 and 40 hr. In contrast, the budding yeast recorded a highest optical density of 2.4 at 23, 35, 36, 37 and 38 hr.

1.3.3 Determination of generation times and growth rates

The generation time and growth rate of the fission and budding yeasts were determined from the graph using the coordinates of the extrapolations (Figures 2 and 3). The generation time of the fission yeast was 4 hours 48 minutes whereas the generation time of the budding yeast was 2 hours 48 minutes. Growth rates of 13 doublings per minute and 21 doublings per minute were also recorded for the fission yeast and budding yeast respectively. The procedure is shown below:

Fission yeast

From Figure 2, $t_{(OD\ 2.0)} = 10.4$ hours

$t_{(OD\ 1.0)} = 5.6$ hours

Generation time (G) $= t_{(OD\ b)} - t_{(OD\ B)}$

$= 10.4 - 5.6$

$= 4.8$ hour

G = 4 hour 48 minute

Growth rate (K) $= \frac{1}{t\,(OD\ b) - t\,(OD\ B)}$

$= \frac{1}{10.4 - 5.6}$

$= \frac{1}{4.8}$

$= 0.208$ per hour

K = 13 doublings per minute

Budding yeast

From Figure 3, $t_{(OD\ 1.2)} = 6.4$ hours

$t_{(OD\ 0.6)} = 3.6$ hours

Generation time (G) $= t_{(OD\ b)} - t_{(OD\ B)}$

$= 6.4 - 3.6$

$= 2.8$ hours

G = 2 hours 48 minutes

Growth rate (K) $= \frac{1}{t\,(OD\ b) - t\,(OD\ B)}$

$= \frac{1}{6.4 - 3.6}$

$= \frac{1}{2.8}$

$= 0.357$ per hour

K = 21 doublings per minute

1.4 Discussion

The study sought to measure the growth of yeast species under *in vitro* conditions. Six (6) species of yeast were isolated from near rotten fruits of banana, orange and tomato, and identified as budding (4 species) and fission (2 species) yeasts based on cell morphology. Reproduction in yeast is primarily by budding and occasionally by fission (Montes de Oca *et al*., 2016). Yeasts live principally on sugar-containing liquids such as juices of damaged fruits. Studies have shown that isolation of yeasts from natural resources is the most successful technique to obtain yeast isolates (Brown, 2013; Khattab *et al*., 2016). The surfaces of 100 healthy grape berries was asserted to contain as high as 22 million yeast cells and the surfaces of damaged berries up to 800 million yeast cells (de Becze, 1956). Khattab *et al*. (2016) identified eleven yeast isolates belonging to six genera from food samples including orange and tomato.

The budding yeasts comprised lemon-shaped cells and egg-shaped cells. The cells of the fission yeast were rod shaped and divided by medial fission. Budding and fission yeasts are free-living cells that are easily grown in the laboratory, and have different cell shapes and patterns of division (Forsburg, 2001). Several studies have reported a number of criteria used to identify and characterise

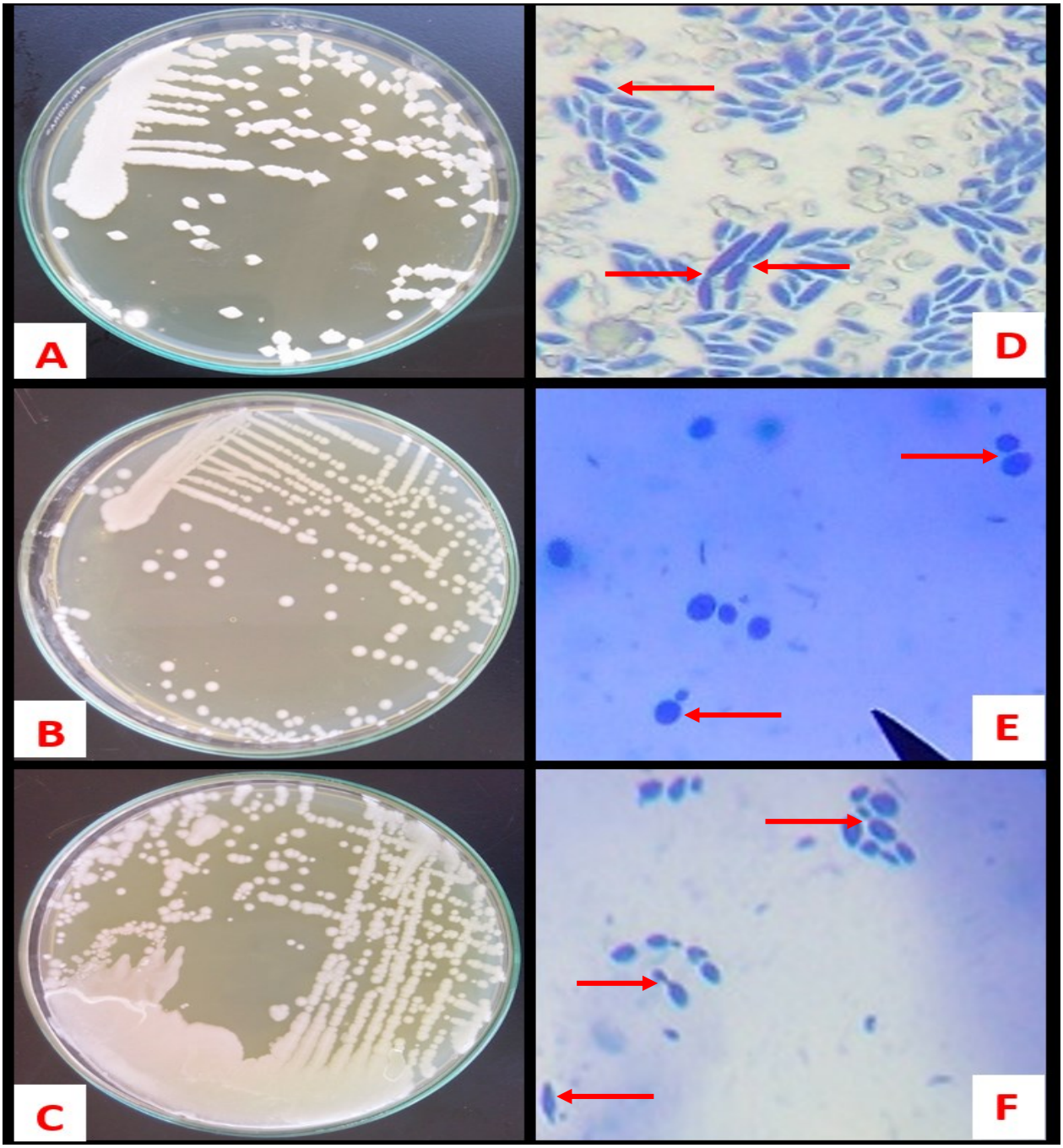

Figure 1: Yeast cultures growing on YPDA. A) Fission yeast, B) Budding yeast, C) Budding yeast, D) Rod shaped cells of fission yeast, E) Lemon-shaped cells of budding yeast, F) Egg-shaped cells of budding yeast. Red arrows indicate yeast cells.

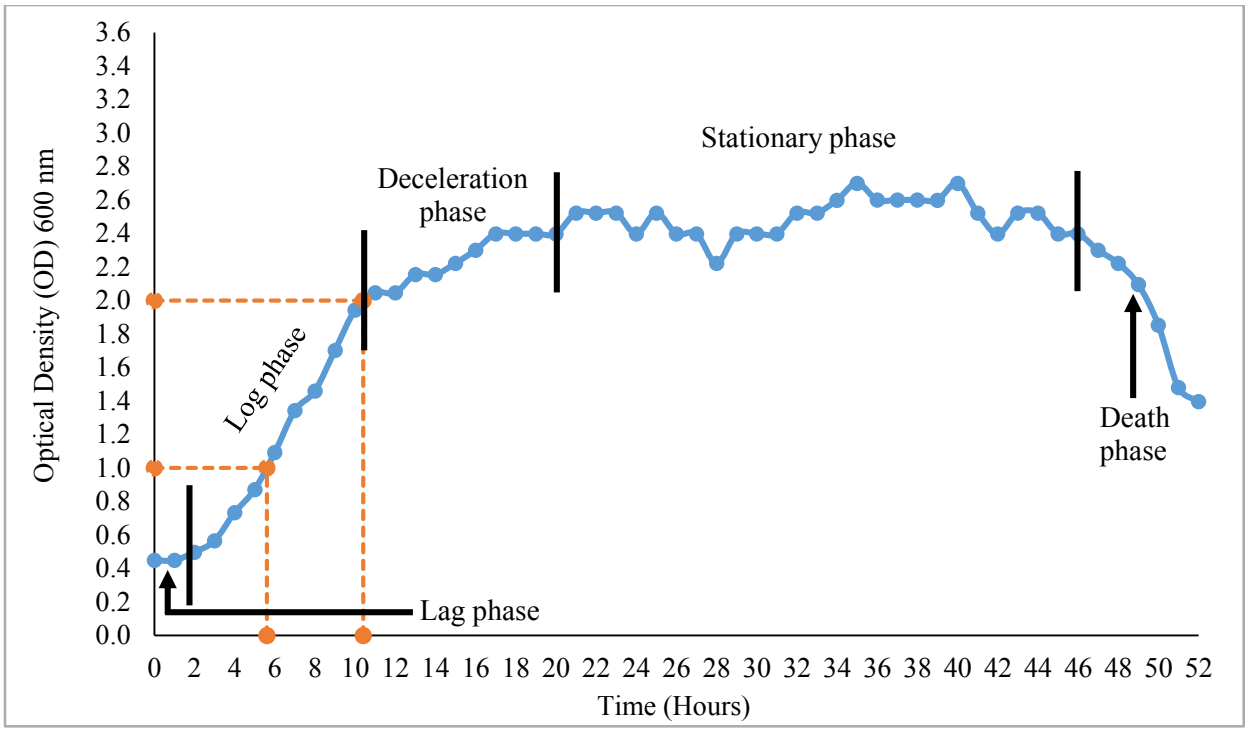

Figure 2: Growth curve of fission yeast.

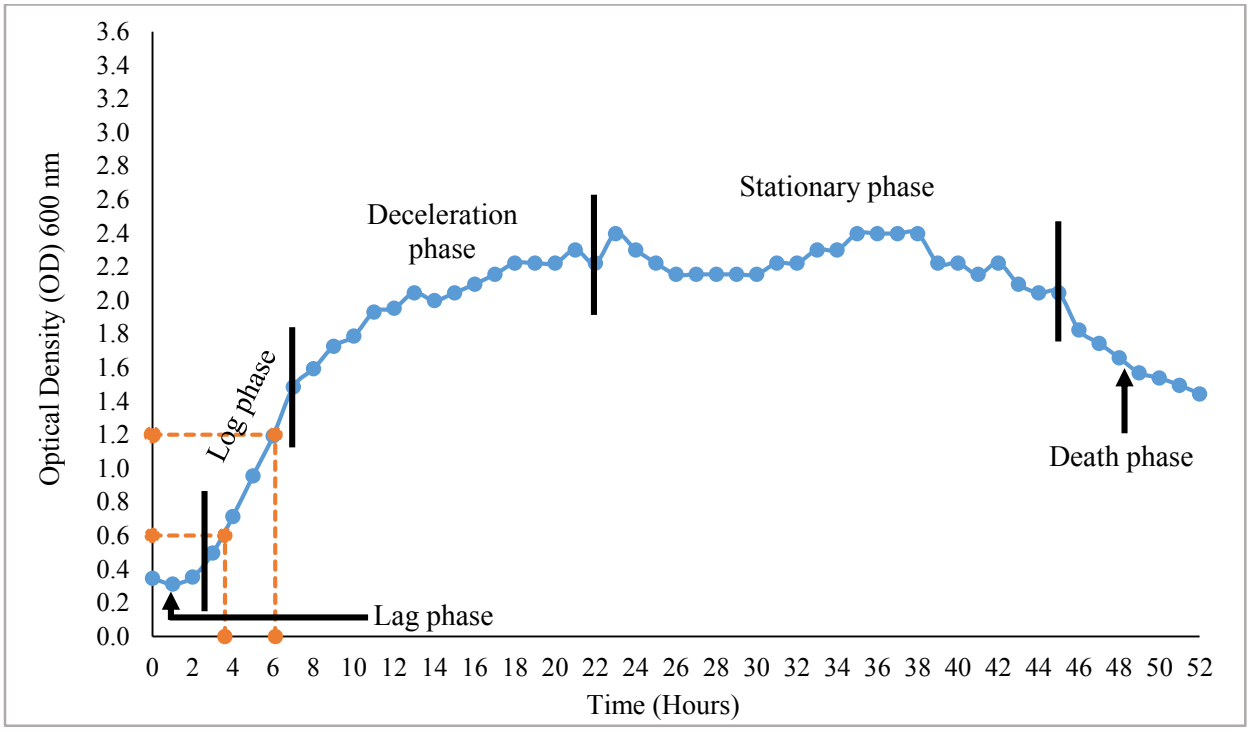

Figure 3: Growth curve of budding yeast.

yeasts species including cell morphology (mode of cell division and spore shape) (de Becze, 1956; Khattab *et al*., 2016; Montes de Oca *et al*., 2016). At the present, molecular sequence analyses are being increasingly used by yeast taxonomists to categorise new species (Walker, 2009). The growth of *Saccharomyces cerevisiae* (most widely used domesticated yeast) is synchronised with the growth of buds (Montes de Oca *et al*., 2016; Zadrag-Tecza *et al*., 2013). The standard vegetative cells of *S. cerevisiae* are egg-shaped but occasionally spherical. Species of *Saccharomycodes*, *Nadsonia*, *Hanseniaspora* and *Kloeckera* produce mainly lemon-shaped budding cells (de Becze, 1956; Forsburg, 2001; Schneiter, 2004). Similarly, *Schizosaccharomyces pombe* has been reported as a fission yeast with rod shape, elongated form of growth and medial fission (Gómez & Forsburg, 2004).

The growth curves of a budding yeast (lemon-shaped cell) and a fission yeast were studied by determining the optical densities of the cells over a 52-hour period at 600 nm using spectrophotometer. Spectrophotometer measures the fraction of light that is absorbed by a solution and report that as absorbance units (optical density) (Hall *et al*., 2013). Optical densities of yeast cells are generally determined at 600 nm (Friedman, 2010; Hall *et al*., 2013; Stevenson *et al*., 2016). The budding yeast recorded a higher cell concentration (352×10^5 CFU mL^{-1} or 7.5 OD) than the fission yeast (125×10^5 CFU mL^{-1} or 7.1 OD). The various stages of yeast growth were clearly observed (sigmoid shape) (Figures 2 and 3). Optical density (OD) measurement of microbial growth is one of the most common techniques used in microbiology, with applications ranging from studies of growth under different nutritional or stress environments to studies of antibiotic efficacy and characterisation of different mutant strains **(**Li & de orduna, 2010; Stevenson *et al*., 2016).

The stationary phases in both curves were undulating but not steady states. This can be attributed to the fact that some cells easily synthesised some components than others for growth. Maier (2008) reported that growth in the stationary phase is unbalanced regardless of the reason why cells enter stationary phase. As some components of the medium become more and more limiting, cells will still keep growing and dividing as long as possible. The optical densities of the cells declined amidst metabolism and cell division as the cells entered the death phase. A study has similarly shown that more viable cells are lost than gained in the death phase although individual cells may metabolise and divide, resulting in a net loss of viable cells (Maier, 2008). The optical density at time zero was higher in the fission yeast (0.5) than in the budding yeast (0.3), and this is in conformation to their spore concentrations determined. Similarly, a higher optical density of 2.7 was recorded in the fission yeast whilst the budding yeast showed an optical density of 2.4 at the stationary phase. However, both yeasts showed an optical density of 1.4 at the end of the growth period.

The stationary and death phases started at 20 hr and 46 hr for the fission yeast and 22 hr and 45 hr for the budding yeast respectively. The generation times (time required for a cell division to occur) differed by 2 hours interval, with the fission yeast recording the higher value of 4 hours 48 minutes than the budding yeast, 2 hours 48 minutes. Besides, the budding yeast showed a higher growth rate (change in the number of cells per unit time) of 21 doublings per minute whilst the fission yeast showed a growth rate of 13 doublings per minute. In contrast, Gómez & Forsburg (2004) reported that fission yeast (*S. pombe*) requires about 2.5–3 hours and a temperature of 30 ^{o}C to complete a cell cycle. The generation time of budding yeast (*S. cerevisiae*) has contrarily been estimated at 1 hour 30 minutes at a temperature of 30 ^{o}C (Minois *et al*., 2004). These variations in the generation times may be due to differences in yeast species, media type and growth conditions, particularly temperature and pH (Petersen & Russell, 2016).

1.5 Conclusion

Fission and budding yeasts were isolated and identified from near rotten fruits of orange, tomato and banana. The growth patterns were determined by measuring the optical densities of the cells over 52 hours, and the growth phases were indicated. The budding yeast grew more rapidly, with a generation time of 2 hours 48 minutes and growth rate of 21 doublings per minute compared with the fission yeast which recorded a generation time of 4 hours 48 minutes and growth rate of 13 doublings per minute. The stationary and death phases in the fission yeast started at 20 hours and 46 hours whilst that of the budding yeast began at 22 hours and 45 hours respectively.

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