Chapter 2

Isolation and characterization of primary cell line from Rice weevil, *Sitophilus oryzae*

2.1 Introduction

The development of insect cell lines has been the subject of strong research since the 1980s which allows the industrial production of proteins, vaccines, and insecticides (Ikonomou *et al.,* 2003; Drugmand *et al.,* 2012). During the last four decades, research about insect cell culture has revealed promising outcomes, leading to the development of high-speed screening technologies as a new insect pest management tools (Smagghe *et al.,* 2009; Airenne *et al.,* 2013). The development of the insect cell culture methods has been an extraordinary technical advance for several biological studies, with an advantage of quick standardization (Hakim *et al.,* 2009). Due to its rapid progress, in the recent years and more than 500 cell lines from over 100 different insect species have been described worldwide and has gained a very rapid expansion into areas of biology, such as immunity, endocrinology, toxicology, biochemistry, and coevolution (Smagghe *et al.,* 2009).

There is a marked interest and usage of insect cell culture (Aljabr *et al.,* 2014), primarily due to its application in recombinant biotechnology for the expression of foreign genes and the potential commercial-scale production of the pesticidal baculoviruses (Zitzmann *et al.,* 2017). While cell lines are now yielding new insights into basic cellular processes, other important processes, such as the mechanisms of cellular resistance to insecticide/ pesticides is still not well explored (Ikonomou *et al.,* 2003; Jarvis, 2009). Furthermore, Insect cell line studies have gained more attention as they reduce the time, they are noninvasive, cost-effective and one can establish cell lines from specific tissues (Gupta *et al.,* 2019). Moreover, the establishment of the insect cell line is an alternative to insect mass rearing and its bioassay for entomopathogens and their toxins, growth regulators or screening and mechanism of chemicals like insecticides (Smagghe *et al.,* 2009; Zhang *et al.,* 2012b; Cruz and Bello *et al.,* 2013).

Most insect cell lines are usually derived from embryos, ovaries, hemocytes, testis, newly hatched larvae (neonate larvae), pupae, fat body, silk glands and epidermis (Mclntosh *et al.* 1983; Su *et al.* 1987; Kolokol'tsova *et al.,* 1995; Shih *et al.,* 1997; Sudeep *et al.,* 2002a, b; Shao *et al.,* 2008; Suganuma *et al.,* 2011; Imanishi *et al.,* 2012; Grasela *et al.,* 2012; Goodman, 2012; Ding *et al,.* 2013; Zhang *et al.,* 2006, 2014; Zheng *et al.,* 2010, 2014; Hu *et al.,* 2014). Apart from these body parts, the alimentary canal is one of the most important tissues playing a crucial role in insect physiology. Study on the insect alimentary canal is important as it is the site of digestion, detoxification, and transport as well as has an important role for semiochemical production, which is an imperative process in the insect life cycle (Hall *et al.,* 2002a, 2002b; Nardi *et al,.* 2002).

The insect gut can be subdivided into three regions based on their origin and functionality. The foregut and hindgut are derived from the embryonic ectoderm and are primarily responsible for ingestion of food (foregut), water absorption, and osmoregulation (hindgut), whereas the primary function of the midgut is to absorb essential nutrients (Corley and Lavine, 2006) and vital for the production, secretion of luminal enzymes and final digestion by microvillar enzymes. According to Haung *et al.,* (2015) insects possess multi-tasking gut epithelium and have opined that the key challenge for future research is to understand how the multiple functions of the insect gut epithelium are integrated by signalling communications among epithelial cells, the gut microbiota, and other insect organs. Histologically, the midgut possesses four cell types in a single epithelial layer that include columnar cells, regenerative cells, goblet cells, and endocrine cells. All cells of the midgut are derived from endodermal tissue and lack the cuticle lining that is present in the foregut and that interferes with absorption. The columnar cells are most numerous, with borders facing the lumen that contain abundant microvilli and numerous folds, increasing the surface area for both absorption and secretion (Smagghe *et al.*, 2005). Largely, nutrients in the gut lumen are absorbed through the columnar cells. They contain extensive networks of the endoplasmic reticulum that are necessary for the production of the digestive enzymes (Li and Xie, 2005). The columnar cells have short life spans, and new ones are continually being formed from the regenerative stem cells present in groups called nidi that may be present as papillae on the hemolymph side of the midgut (Castagnola and Jurat-Fuentes, 2016). A nidus is a stem cell niche, like those of ovary and testis, whose cells divide asymmetrically to give rise to columnar and endocrine cells. Successful culture of midgut cells from Coleoptera and Hymenoptera has been done by well explored by Hakim *et al.*, (2007, 2009) and by Zheng *et al.*, 2014.

The most popular cell lines originated from the agriculture pests are known to be derived from Noctuid species, for instance, SF21 and SF9 from *Spodoptera frugiperda* and high five cells from cabbage looper *Trichoplusia ni* (Yu *et al.*, 2016). The use of cell lines for research and commercial applications is currently dominated by three cell lines. Nevertheless, the continued development of new cell cultures from other species is important for the future growth of insect cell studies. The main sources of these insect cell lines include various tissues from the insects in the orders of Lepidoptera, Diptera, Coleoptera, Blattaria, Hymenoptera, Orthoptera, Hemiptera, etc. Among them, cell lines from Diptera and Lepidoptera are in a large number (Zheng *et al.*, 2013).

Hence, the above literature elucidates the development of cell lines from agricultural pests, while the development of cell lines from stored grain pests remains elusive. Moreover, there is a lacuna in the in-depth studies of insect cell lines derived from Coleoptera in general and store grain pest in particular, and to date cell lines from only eight species of coleopteran insects belonging to the families scarabaeidae (Crawford 1982; Mitsuhashi 1989; Fernon *et al.,* 1996; Mitsuhashi 2003), Chrysomelidae (Lynn and Stoppleworth 1984; Lynn 1995; Charpentier *et al.*, 2002; Long *et al.*, 2002), Curculionidae (Barcenas *et al.,* 1989; Stiles *et al.*, 1992; Aljabr *et al.,* 2014; Zheng *et al.,* 2014), Tenebrionidae (Goodman *et al.,* 2012) and Cerambycidae (Iwabuchi 1999, Hoshino *et al.,* 2009) have been accounted. Taking into consideration the higher degree of species divergence within the order Coleoptera (Hunt *et al.,* 2007), more coleopteran cell lines should be established to enable the study of insects in this order. As discussed in chapter 1, the member of the coleopteran chosen for the study was *S. oryzae* as it is considered being the most destructive pest.

Taking everything into consideration the present study is focused to develop and establish cell line from the midgut tissue of primary insect pest adult Sitophilus oryzae. The study chapter also discusses the techniques and optimum conditions required to maintain the cell culture.

2.2 Materials and Methodology

Experimental Design

The laboratory-reared Adult *S. oryzae* (4.28 ± 0.76 mm) was used for the development and establishment of cell lines. The insects were sacrificed and the midgut cell was Isolated. All procedure was carried out in Laminar hood (Labtach, India).

1. For each explants of midgut cells, approximately 40 to 50 insects were separated and starved for 2 to 3 hours prior to the isolation of cells. Surface sterilization was done by immersing the insects each in a mixer of aqueous solution (20% septisol + 1% antibiotic antimycotic

solution (A002, HiMedia, India) + 0.5% sodium hypochlorite) for 2 minutes.

2. Insects were washed thoroughly 3 times in autoclaved double distilled water and transferred to a sterile Petri dish containing PBS + 1% antibiotic antimycotic aqueous solution.

3. Sterile dissecting tools were used to remove the midgut and were transferred to the modified incubating media as per the method of Hakim *et al.,* 2009.

4. Contents of the midgut lumen were removed; the tissues were mechanically disrupted and dissociated with 1 ml trypsin for 10 min at 37˚C. Dissociation was stopped by adding 2 ml of incubating media.

   The tissue was then transferred through a 70-µm-pore-size cell strainer (TCP025, Himedia, India) and a medium with freed cells was collected in the centrifuge tube and was centrifuged at 600 x g for 10 min (Spinwin MC-1, Tarsons, India). The supernatant was discarded and the pellet was re-suspended in 3 ml of incubating medium (3X Antibiotic antimycotic solution (A002, HiMedia, India)+ 1X gentamicin (A005, HiMedia, India) + 0.1% amphotericin B (A011, HiMedia, India)+ Fetal Bovin Serum (IML1112, HiMedia, India) Vitamin mixture (3mg /ml riboflavin, 1.2 mg/ml vitamin B_{12} + 1.5 mg/ml of folic acid) + Ecdysone (E9004, Sigma, USA) and transferred to a 12.5 cm^2 plastic tissue culture flask (TCG2, HiMedia, India).

5. Cell counts were performed using a hemocytometer and cell viability was checked by trypan blue (T8154, Sigma, India) test and was assessed under an inverted microscope, using a hemocytometer.

6. Primary culture thus establish was named SoMG and subcultured every 6^{th} day in 12.5 cm^2 flask with a 1:2 split ratio till it reached 70% confluence and was transferred to 25 cm^2 flask with 1:1 split ratio

every 8th day. Thereafter, the split ratio was increased gradually to 1:2 to 1:3 ratios on every 8th day and maintained for 17 passages. The incubation temperature was 28˚C, and daily observations like morphological characteristics were performed using an inverted microscope.

Media Optimization

To obtain the maximum cell density optimization of insect cell growth media was carried out. Two Insect growth media (Grace's and TNM-FH medium) were used to conduct this study. Into six-well plates containing 3 ml of insect cell growth media in addition to different concentration of FBS (RM10432, HiMedia, India) and ecdysone (E9004, Sigma, USA) (Table 2.1) with constant concentration of vitamin mixture (3mg /ml riboflavin, 1.2 mg/ml vitamin B_{12} + 1.5 mg/ml of folic acid) were used, and into each well 2×10^4 cells/ml were seeded. Cells were counted in triplicate every 24 hrs for each medium with a Neubauer hemocytometer. Cell density and viable cell percentage were observed up to 96 hrs to find the optimum growth media for SoMG cells. Cell calculations were made as follows:

%Cell viability=Total viable cells (unstained)/Total cells (Viable+dead) x 100

Viable cells/ml= Average viable cell count per square x dilution factor x 10^4

1	15% FBS +5pmol ecdysone	9	25% FBS +5pmol ecdysone
2	15% FBS +10 pmol ecdysone	10	25% FBS +10pmol ecdysone
3	15% FBS +15pmol ecdysone	11	25% FBS +15pmol ecdysone
4	15% FBS +20pmol ecdysone	12	25% FBS +20pmol ecdysone
5	20% FBS +5pmol ecdysone	13	30% FBS +5pmol ecdysone
6	20% FBS +10pmol ecdysone	14	30% FBS +10pmol ecdysone
7	20% FBS +15pmol ecdysone	15	30% FBS +15pmol ecdysone
8	20% FBS +20pmol ecdysone	16	30% FBS +20pmol ecdysone

Table 2.1: Different concentration of FBS and ecdysone in growth media

Characteristics and Cell Growth

The morphology of the cell line was described from visual observations with a phase-contrast inverted microscope (DMRB, Leica) and a catcam130: cc130 microscope camera. Photographs of cell lines were used to characterize the cell type. For growth studies, viable cells assessed with Trypan blue (TCL046, HiMedia, India) where the cells were seeded in the flask at a concentration of 2 x 10^4 cell/ml. After the 24 hrs of time durations, cells were removed from the flask and counted in hemacytometer under a bright-field microscope (METZER M, India). Cells were counted at least 3 times during each period. Doubling time of each cell line was calculated during the exponential growth phase of the cell.

Molecular Characterization

- DNA was extracted from *S. oryzae* adults and SoMG cell line by using Quigen DNA isolation kit (Cat no: 69504).

  1. The appropriate numbers of the cells were centrifuge (maximum 5 x 10^4) for 5 min at 300 x g. The pellet was resuspended in 200 µl PBS. Add 20 µl proteinase K. For RNA-free genomic DNA, we had added 4 µl RNase A (100 mg/ml), mix by vortex, and incubate for 2 min at room temperature.
  2. 200 µl Buffer AL (without added ethanol) was added. Mix thoroughly by vortex, and incubated at 56°C for 10 min.
  3. 200 µl ethanol (96–100%) were added to the sample, and mix thoroughly by vortexing.
  4. The mixture was taken into the DNeasy Mini spin column placed in a 2 ml collection tube (provided in the kit). Centrifuge at 6000 x g (8000 rpm) for 1 min.

5. The DNeasy Mini spin column was placed in a new 2 ml collection tube (provided in the kit), 500 µl Buffer AW1 was added, and centrifuged for 1 min at 6000 x g (8000 rpm). The flow-through in the collection tube was discarded. DNeasy Mini spin column was placed in a new 2 ml collection tube (provided in the kit), 500 µl Buffer AW2 was added, and centrifuged for 3 min at 20,000 x g (14,000 rpm) to dry the DNeasy membrane. The flow-through and collection tube was discarded. It was important to dry the membrane of the DNeasy Mini spin column since residual ethanol might have interfered with subsequent reactions. This centrifugation step ensured that no residual ethanol was carried over during the following elution.

6. Place the DNeasy Mini spin column in a clean 1.5 ml or 2 ml microcentrifuge tube (Abdos, India), and 200 µl buffer AE were added directly onto the DNeasy membrane. Then it was followed by incubation at room temperature for 1 min, and then it was centrifuged for 1 min at 6000 x g (8000 rpm) to elute the DNA.

Procedure

The integrity of DNA was checked by using 0.8% agarose gel electrophoresis. RNA quantification was done by taking the A_{260}/A_{280} ratio using the Perkin Elmer spectrophotometer (Lambda 25). This ratio can reveal the presence of contaminants and give evidence of possible degradation. An A_{260}/A_{280} ratio of 1.8 was considered acceptable for DNA. 5µl of template DNA was aliquot and was added to 1.5ml microcentrifuge tube. To the aliquot, 995µl of nuclease-free water was added and absorbance was measured at the mentioned ratio against the blank having 1000µl of nuclease-free water. The concentration of RNA was done using the following standard formula:

Amount of DNA (µg/mL) (ng/µL) =OD at 260nm × 40 × dilution factor

The DNA product was then amplified for COI and 16s rRNA using PCR (prima-96, HiMedia, India) and primer as shown in Table 2.2.

Name of DNA marker and primer	Primer sequence (5' to 3')	Reference	Amplicon Size
Cytochrome c oxidase subunit 1			
LCO-1490	5'-GGTCAACAAATCATAAAGATATTGG -3'	Folmer *et al.*, 1994	**720bp**
HCO-2198	5'-TAAACTTCAGGGTGACCAAAAAATCA-3'	Folmer *et al.*, 1994	
16s RNA			
16Sar	5'- CCGGTCTGAACTCAGATCACGT-3'	Simon *et al.*, 1994	**800bp**
16Sbr	5'- GCTCAGAACGAACGCTATC-3'	Simon *et al.*, 1994	

Table 2.2: Describe the sequence of primers used to amplify COI and 16sRNA of the midgut cells of *S. oryzae*

PCR Conditions

The DNA product was then amplified in PCR for COI at 94 °C denaturation for 1 min, 5 cycle of 94°C for 1:00 min, 45 °C annealing for 1:30 min, 72°Cfor 1:30 min, followed by 35 cycle of 94°C for 1:00 min, 50 °C for 1:30 min and 72 °C for 1:00 min, and extension was carried out 72 °C for 7 min. A total of 40 cycles were performed using primers given in the Table 2.2. For amplification of 16 sRNA 94^{o}C denaturation for 3 min, 98 °C annealing for 0:10 min and50 °C for 1:30 min, and extension was carried out

72 °C for 7 min. A total of 35 cycles were performed using primers16Sar-5'-CCGGTCTGAACTCAGATCACGT-3', 16Sbr- 5'-GCTCAGAACGAACGCTATC-3' (Simon *et al* 1994).

For COI gene

Stage 1 (1 cycle)	**Stage 1 (5 cycle)**	**Stage 2 (35 cycle)**	**Stage 3 (1 cycle)**
94° C 1:30 min	94°C 1:00 min	94°C 1:00 min	72°C 10:00 min
	45°C 1:30 min	50°C 1:30 min	4.0°C stop for ∞ time
	72°C 1:30 min	72°C 1:00 min	

For 16s gene

Stage 1 (1 cycle)	**Stage 2 (35 cycle)**	**Stage 3 (1 cycle)**
94° C 3.00 min	98°C 0:10 min	72°C 08:00 min
	50°C 0:30 min	4.0°C stop for ∞ time
	72°C 1:30 min	

The amplified product was purified using the thermo-Exosap kit (Cat. no. 78200.200.UL, USA), and sequencing steps were performed according to Yuan *et al.,* (2015a, b).

Sequencing

Sequencing is a method of DNA sequencing first commercialized by Applied Biosystems, based on the selective incorporation of chain-terminating dideoxynucleotides by DNA polymerase during in vitro DNA replication. Developed by Frederick Sanger and colleagues in 1977.

classical chain-termination method requires a:

1. single-stranded DNA template,
2. a DNA primer,
3. a DNA polymerase,
4. normal deoxynucleosidetriphosphates (dNTPs),
5. modified di-deoxynucleotidetriphosphates (ddNTPs),

latter of which terminate DNA strand elongation. These dNTP"s and ddNTP"s lack a 3'-OH group required for the formation of a phosphodiester bond between two nucleotides, causing DNA polymerase to stop the extension of DNA when a modified ddNTP is incorporated. The ddNTPs may be radioactively or fluorescently labeled for detection in automated sequencing machines.

The DNA sample was divided into four separate sequencing reactions, containing all four of the standard deoxynucleotides (dATP, dGTP, dCTP and dTTP) and the DNA polymerase. To each reaction, added only one of the four dideoxynucleotides (dATP, ddGTP, ddCTP, or dTTP), while the other added nucleotides were ordinary ones. The dideoxynucleotide was added to be approximately 100-fold lower in concentration than the corresponding deoxynucleotide, allowing for enough fragments to be produced while still transcribing the complete sequence. Following rounds of template DNA extension from the bound primer, the resulting DNA fragments were heat-denatured and separated by size using gel electrophoresis. This was frequently performed using a denaturing polyacrylamide-urea gel with each of the four reactions run in one of four individual lanes (lanes A, T, G, C). The DNA bands may then be visualized by autoradiography or UV light and the DNA sequence can be directly read off the X-ray film or gel image.

RNA Isolation

Total RNA was isolated by Trizol according to the method of Peterson and Freeman (2009). The cells were harvested and pellet down at 4000 rpm for 10 min. The supernatant was discarded and was re-suspended in 500 µl TRIzol reagent (15596-026, Invitrogen, USA). The homogenate was taken into 2 ml microcentrifuge tubes (Tarsons, India). After successful homogenization, an equal volume of Trizol reagent was added. For complete dissociation of nucleoprotein complexes, homogenized samples were incubated for 5 minutes at room temperature. The incubation was followed by the addition of chloroform and was vigorously shaken for the effective mixing of both the solutions. The samples were kept at room temperature for 5 minutes until the aqueous and organic layers were distinct. Thereafter, the tubes were subjected to centrifugation at 12,000 x g for 15 minutes at 4°C. The mixture got separated into a lower red phenol-chloroform phase, interphase, and a colorless upper aqueous phase. An aliquot of the upper aqueous phase was then transferred into a new 1.5 ml microcentrifuge tube using 1000 µl pipette. Precipitation was done by adding 500 µl of isopropanol to the supernatant that was transferred. The samples were kept at -20°C for 10 minutes, centrifuged at 12,000x g for 15 minutes at 4°C. After precipitation, the supernatant was discarded without disturbing the pellet and was washed in 1000 µl of 75% ethanol and 300 µl of absolute ethanol was added to the pellet. Effective mixing was done by gentle inversion and was further subjected to centrifugation at 7,500 x g for 7 minutes at 4°C. The pellet was resuspended by adding 50 µl of DEPC water (Diethylpyrocarbonate) and was incubated at room temperature for 10 mins.

Quantification of RNA

Prior to quantification, DNAase (Thermo Scientific, USA) treatment was performed. 1 µg of 10X RNA reaction buffer with $MgCl_2$ was added to the tube and was incubated at 37 °C for 30 min. Then after 20 µl chelating agent EDTA was added in 50 mM concentration and was incubated at 65°C for 10 min in a water bath as RNA gets hydrolyzed during heating with divalent cations in the absence of a chelating agent. RNA quantification was done by taking the A_{260}/A_{280} ratio using the Perkin Elmer spectrophotometer (Lambda 25). This ratio can reveal the presence of contaminants and give evidence of possible degradation. An A_{260}/A_{280} ratio of 2.0 is considered acceptable for RNA. 5µl of template RNA was aliquot and was added to a 1.5ml microcentrifuge tube. To the aliquot, 995µl of nuclease free water was added and absorbance was measured at the mentioned ratio against the blank having 1000µl of nuclease-free water.

The concentration of RNA was done using the following standard formula:

Amount of RNA (µg/mL) (ng/µL) =OD at 260nm × 40 × dilution factor

Synthesis

The purity check of RNA that was validated using a spectrophotometer, 1 µg of total RNA was used for reverse transcription reaction using verso cDNA synthesis kit (Thermo Scientific-AB-1453/A). Briefly, fresh nuclease-free PCR tubes (Tarsons, India) were taken, in which 4µl of 5X cDNA synthesis buffer, 2µl dNTP mix, 1µl of RNA primer (oligonucleotides), 1µl of RT enhancer, 1µl of verso enzyme mix, 1-2µl of RNA template (according to the spectroscopic quantification i.e. 1 ng) and the final assay volume was made to 20µl using nuclease-free water. The tubes were effectively mixed by giving a short centrifuge spin for the 30s at around

2000 x g. The tubes containing the kit mixture was PCR amplified by 2 step reaction process. Firstly, the 1 cycle of cDNA synthesis was carried out at 42˚C for 30 mins followed by 1 cycle of inactivation at 95˚C for 2mins.

Amplification

RT-PCR was performed using SYBR Select Master Mix (Applied Biosystems) in Quant Studio 12K (Life technology) real-time PCR machine with primers to detect selected messenger RNA (mRNA) targets. The melting curve of each sample was measured to ensure the specificity of the products. The mean of housekeeping gene β-actin was used as a control to normalize the variability in the expression levels and data was analyzed using $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). Following Primers pairs were used for qPCR

Primer Designing

Primers were designed using NCBI Primer 3 designing software and the optimum conditions that were taken into consideration were:

1. GC content was kept 40-60 %.
2. Tm of both the primers was having ±1°C difference.
3. The sequence homology was kept minimums thus it was not palindrome to each other hence not allowing inter and intramolecular interactions with one own self or to each other.
4. The stability of the primer was also checked by calculating the ΔG value of 5' bases from 3' end.
5. The specific annealing temperature for minimum false product formation.

Opt = 0.3 x(Tm of primer) + 0.7 x(Tm of product) - 14.9

Tm of primer is the melting temperature of the less stable primer-template pair of product is the melting temperature of the final amplified product.

After the successful designing of the primer the amplicon/product length was again validated using the given formula:

length = (Position of antisense primer-Position of sense primer) + 1

Accession No.	**Gene Name**	**Sequence**	**Tm**	**Amplicon size (bp)**
XM_022975529.1	**β –actin F**	**5'-GTATCGTGCTGGACTCCGGT-3'**	**61.0**	**154**
	β–actin R	**5'-GTGAAGGAGTAGCCACGCT-3'**	**59.0**	
EU526836.1	**Cup 1 F**	**5'-GGCAAGAAGGGTTCCGAAGT-3'**	**59.0**	**134**
	Cup 1 R	**5'-TTCTCGTTGGCGACGTACC-3'**	**59.0**	
EU526837.1	**Cup 4 F**	**5'-GAGGGTGGATACCAGTTCGC-3'**	**61.0**	**118**
	Cup 4 R	**5'-CGCGCACAACCACTACTTTG-3'**	**59.0**	
DQ847154.1	**hmg-176 F**	**5'-AAGTTTCCTTGTCATCGCCC-3'**	**57.0**	**105**
	hmg-176 R	**5'-GTCTCCGAACTCTCGGGTA-3'**	**59.0**	

Table 2.3: Depicts the primer sequence with its annealing temperature and product size for the characterization of SoMG cell line

PCR Conditions for Candidate Genes

The amplification was done by adjusting the thermal cycling conditions using the default RT-PCR thermal cycling conditions according to the instrument cycling parameters and melting temperatures of the specific primers. The RT-PCR conditions were 50°C denaturation for 2 min, 95°C 2.30

min and 40cycle of 95°C- 00:15 min, 55°C- 60°C for 00:15 min, 72°C for 1.00 min by using primers given below.

Step	Temperature	Duration (minutes)	Cycle
UDG activation	50°C	2 :00	Hold
Dual-Lock DNA polymerase	95°C	2:00	Hold
Denature	95°C	0:15	40
Anneal	55-60°C	0:15	
Extend	72°C	1:00	

Karyotype analysis

The chromosome numbers per cell were determined using a modification of Mitsuhashi (1995). Cells were grown to near confluence as described above. The spent medium was removed and a medium containing 0.5 mM colchicines (17701, SRL, India) was added to the culture. Cells were incubated at 28°C for 5 h, after which cells were detached by aspiration and pelleted at 1,000 x g for 5 min at 4°C. The supernatant was discarded and the cells were re-suspended and dispersed in 7 ml 0.58 % KCl. Cells were incubated at room temperature for 15 min and re-centrifuged. The pellet was re-suspended in 1 ml (1:1) 0.58 % KCl /fixative solution (3:1 methanol/glacial acetic acid) and was incubated for 15 min. Cells were again gently mixed with 5 ml freshly made fixative solution, incubated at room temperature for 10 min, and was re-centrifuged at 4°C . The step was again repeated, where 1 ml fixative solution was used and then the suspension was added drop wise on to a glass slide. Slides were dried on a heating block, stained with Giemsa (45881, SRL, India) according to manual instruction for 5 min, and was quickly rinsed with water twice followed by an air dry. After thoroughly drying, slides were briefly rinsed in xylene to remove excess lipids and stabilize the stain. For imaging, glycerol was placed on the slides. Cells were imaged using a Carl Zeiss microscope (Axio Scope. A1) using 100X objective and image analysis was done using the Ikaros Karyotyping imaging Metasystem.

Cryopreservation

For freezing and cryopreservation, monolayers' 80% confluents were detached, and the cells were adjusted to 5 x 10^4cells/ml with containing 90% fetal bovine serum (RM10432, HiMedia, India) and 10% DMSO. The suspension was dispersed into sterile cryotubes and refrigerated in decreasing temperature at 4°C to -20°C, put the vial in -80°C freezer overnight and then immediately transfer to liquid nitrogen for permanent storage.

Statistical analysis

The computed data was analyzed using PRISM 6 software. One and two way ANOVA followed by DUNNETs multiple comparison test were used to the test for significant differences among the individual treatment combinations. Statistical significance was accepted at $p<0.05$ for all tests.

2.3 Results

The cell culture was successfully initiated using midgut tissues in two insect growth media i.e Grace's insect media and TNM-FH Medium with 10 % FBS in both media. Initial cell growth was observed at 72 hrs in Grace's insect media and at 96 hrs for TNM-FH media. As shown in Fig 2.1 it was clearly observed that the Grace's insect media was giving more promising results than the TNF-FH. At 72 hrs and 96 hrs the cell density was significantly ($p<0.05$, $p<0.01$) found high in Grace's media compared to TNF-FH. The cell density in grace media was in a range from 3 x 10^4 to 3.7 x 10^4 at

72 hrs and 96 hrs respectively and was named SoMG cells. However, the cell density remained constant till 16 days, and thereafter it declined. Hence, to maintain the cell line the concentration of FBS was changed and vitamins and ecdysone was added.

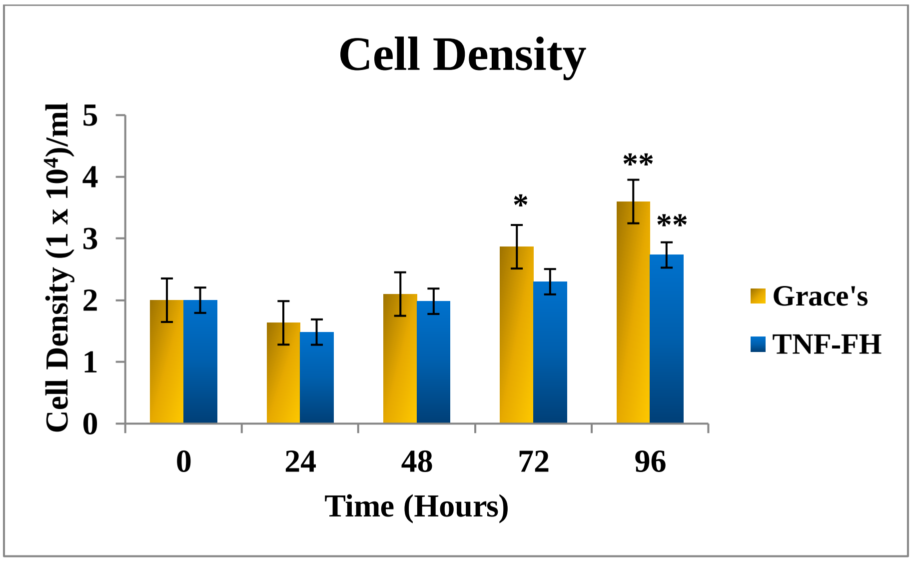


Figure 2.1: Shows the cultured cell density with Grace's and TNF-FH media till 96 hrs of duration

Significant level indicated by *= (p<0.05); **=(p<0.01); *=(p<0.001)**

Optimization of the growth media for the SoMG Cell Culture

Further to achieve the highest cell density combination of different concentrations of vitamin mixture, FBS and ecdysone were added to the incubation media (Table 2.1). The growth media was modified with variable concentrations of ecdysone and FBS keeping vitamin mixture constant, it showed the linner increase in the cell growth in all 16 media (Fig: 2.6). During the study all 16 modified media were assessed to find the optimum insect cell growth medium for SoMG cells. It was observed that the treatment of ecdysone and the concentration of FBS are playing the significant role in Cell growth. The seeding cell density was 2 x10^4 for each. The highest cell density was found in incubation media 10 (Grace's insect media + 25% FBS + 10µM ecdysone + vitamin mixture) at 96 hrs with 93% of Cell viability (Fig 2.5). The first successful subculture was carried out on 6th day after the cells were seeded. Overall the maximum passages were obtained with the Grace's media in compare to TNM-FH media. The maximum passages found with Grace's insect media were over 17 and with TNM-FH media it was not exceed more than 10 passages.

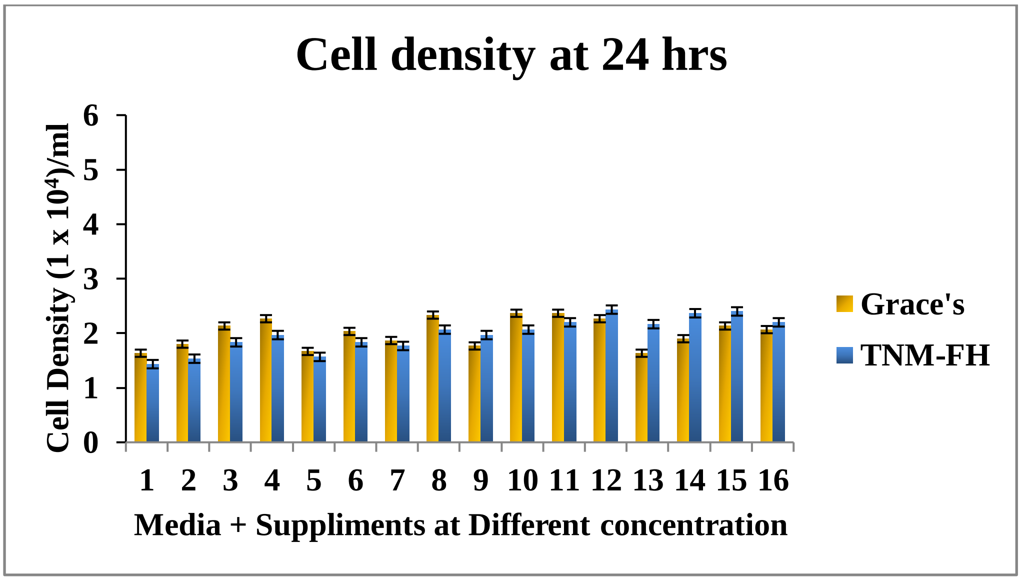


Figure 2.2: Cell density in different incubation media at 24 hrs
Significant level indicated by *= ($p<0.05$); **=($p<0.01$); *=($p<0.001$)**

Media Composition	% Cell growth Grace's media	% Cell growth TNM-FH	% Difference
Media 1	81.66%	71.66%	10%
Media 2	90%	76.66%	13.33%
Media 3	106.66%	91.66%	15%
Media 4	113.33%	98.33%	15%
Media 5	83.33%	78.33%	5%
Media 6	101.66%	91.66%	10%
Media 7	93.33%	88.33%	5%
Media 8	116.66%	103.33%	13.33%
Media 9	88.33%	98.33%	-10%
Media 10	118.33%	103.33%	15%
Media 11	118.33%	110%	8.33%
Media 12	113.33%	121.66%	-8.33%
Media 13	81.66%	108.33%	-26.66%
Media 14	95%	118.33%	-23.33%
Media 15	106.66%	120%	-13.33%
Media 16	103.33%	110%	-6.66%

Table 2.4: Depicts the % cell growth in Grace's media and TNM-FH media with different concentration of FBS and ecdysone with % difference in cell growth at 24 hrs.

Significant level indicated by *= ($p<0.05$); **=($p<0.01$); *=($p<0.001$)**

Table 2.4 shows the percentage rate of cell growth in Grace and TNM-FH media with different concentration of ecdysone and FBS at 24hrs. Among all the media tested, media 10 of Grace's media showed a highest percentage (118%) of cell growth with a difference of 15%compared to TNM-FH respectively. Similarly, the media composition 12-16 showed TNM-FH had more cell viability compared to Grace's media at 24 hrs. However, the growth media composition was further studies was finalized after the 96 hrs.

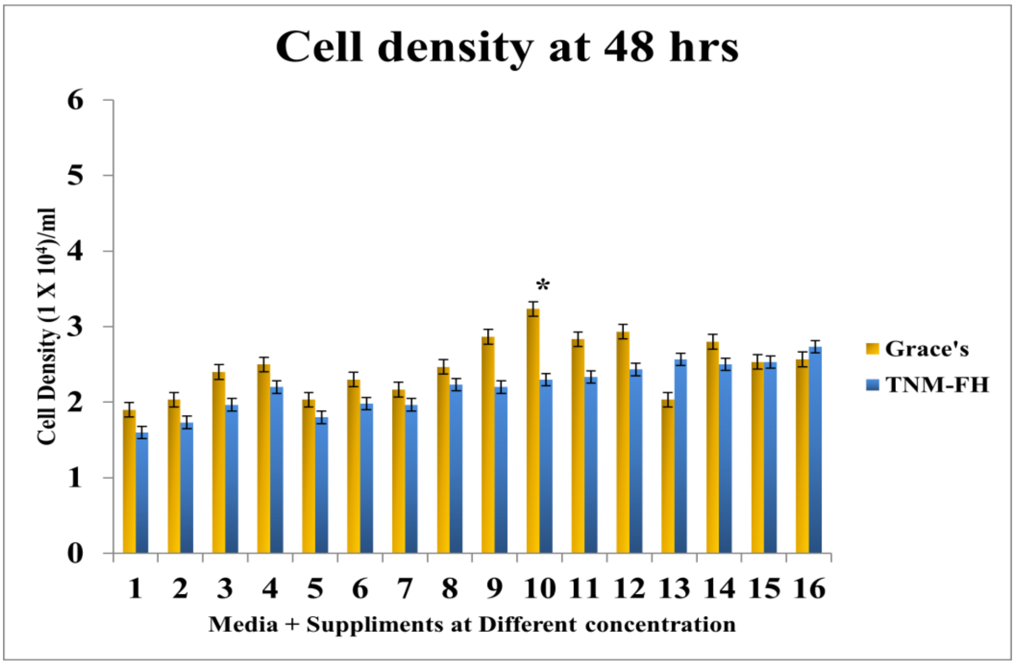


Figure 2.3: Cell density in different incubation media at 48 hrs
Significant level indicated by *= (p<0.05); **=(p<0.01); *=(p<0.001)**

At 48 hrs, media 10 having major component of Grace's media was again found to have the significantly highest percentage (161%) of cell number which was around 46% greater compared to TNM-FH. Hence the media composition was in an order of:

Media 10 (Grace's)> Media 12 (Grace's)> Media 9 (Grace's)> Media 11 (Grace's)

Media Composition	% Cell growth Grace's media	% Cell growth TNM-FH	% Difference
Media 1	95%	80%	15%
Media 2	101.66%	86.66%	15%
Media 3	120%	98.33%	21.66%
Media 4	125%	110%	15%
Media 5	101.66%	90%	11.66%
Media 6	115%	99.16%	15.83%
Media 7	108.33%	98.33%	10%
Media 8	123.33%	111.66%	11.66%
Media 9	143.33%	110%	33.33%
Media 10	161.66%	115%	46.66%
Media 11	141.66%	116.66%	25%
Media 12	146.66%	121.66%	25%
Media 13	101.66%	128.33%	-26.66%
Media 14	140%	125%	15%
Media 15	126.66%	126.66%	0%
Media 16	128.33%	136.66%	-8.33%

Table 2.5: Depicts the % cell growth in Grace's media and TNM-FH media with different Concentration of FBS and ecdysone with % difference in cell growth at 48 hrs.

Significant level indicated by *= ($p<0.05$); **=($p<0.01$); *=($p<0.001$)**

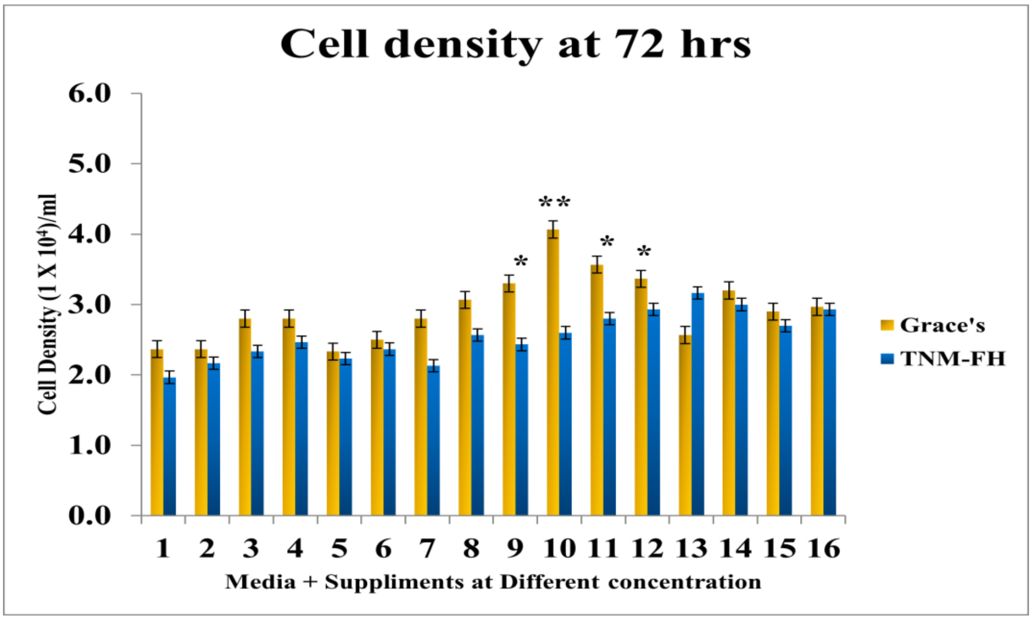


Figure 2.4: Cell density in different incubation media at 72 hrs
Significant level indicated by *= (p<0.05); **=(p<0.01); *=(p<0.001)**

The results of 72 hrs clearly validated Media 10 (Grace's) among all was the best suited for the cell growth with a percentage growth rate of 203.33% and 73.33% difference with respect to number of cells, compared to TNM-FH media.

Media Composition	% Cell growth Grace's media	% Cell growth TNM-FH	% Difference
Media 1	118.33%	98.33%	20.00%
Media 2	118.33%	108.33%	10.00%
Media 3	140%	116.66%	23.34%
Media 4	140%	123.33%	16.67%
Media 5	116.66%	111.66%	5.00%
Media 6	125%	118.33%	6.67%
Media 7	140%	106.66%	33.34%
Media 8	153.33%	128.33%	25%
Media 9	165%	121.66%	43.33%
Media 10	203.33%	130%	73.33%
Media 11	178.33%	140%	38.33%
Media 12	168.33%	146.66%	21.66%
Media 13	128.33%	158.33%	-30%
Media 14	160%	150%	10%
Media 15	145%	135%	10%
Media 16	148.33%	146.66%	1.66%

Table 2.6: Depicts the % cell growth in Grace's media and TNM-FH media with different concentration of FBS and ecdysone with % difference in cell growth at 72 hrs.

Significant level indicated by *= (p<0.05); **=(p<0.01); *=(p<0.001)**

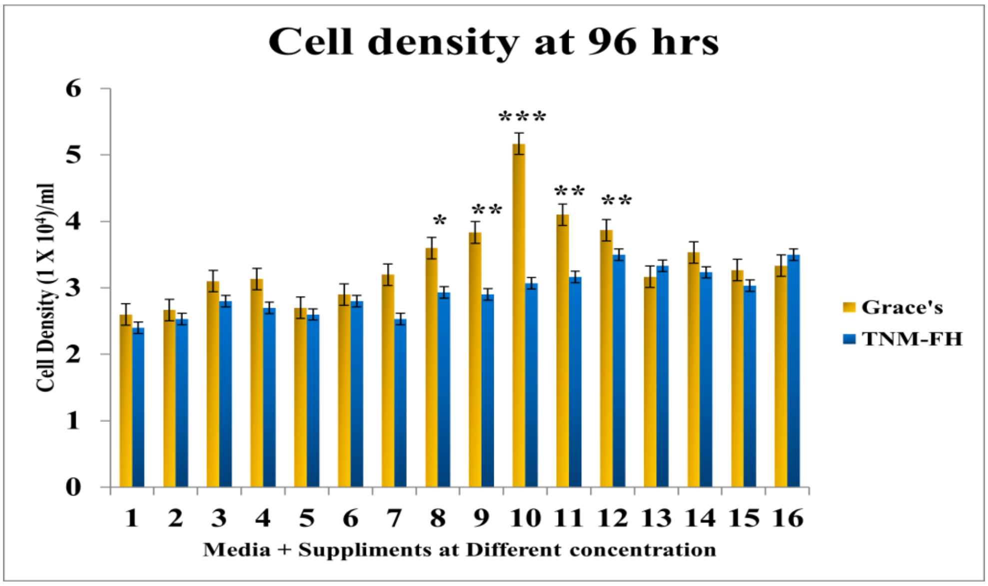


Figure 2.5: Cell density in different incubation media at 96 hrs
Significant level indicated by *= (p<0.05); **=(p<0.01); *=(p<0.001)**

After 96 hrs, the results revealed that there was less mortality of cell in Media 10 and was constantly increasing with a rate of 258.33% which was highest among all the media tested during growth durations (24-96hrs). Moreover, the difference (105%) in the rate of cell growth added that this was the best suited media for SoMG cells. Therefore, this media was preferred for the supplement the growth of cells and further studies were carried out.

Media Composition	% Cell growth Grace's media	% Cell growth TNM-FH	% Difference
Media 1	130%	120%	10%
Media 2	133.33%	126.66%	6.66%
Media 3	155%	140%	15%
Media 4	156.66%	135%	21.66%
Media 5	135%	130%	5%
Media 6	145%	140%	5%
Media 7	160%	126.66%	33.33%
Media 8	180%	146.66%	33.33%
Media 9	191.66%	145%	46.66%
Media 10	258.33%	153.33%	105%
Media 11	205%	158.33%	46.66%
Media 12	193.33%	175%	18.33%
Media 13	158.33%	166.66%	-8.33%
Media 14	176.66%	161.66%	15%
Media 15	163.33%	151.66%	11.66%
Media 16	166.66%	175%	-8.33%

Table 2.7: Depicts the % cell growth in Grace's media and TNM-FH media with different concentration of FBS and ecdysone with % difference in cell growth at 96 hrs.
Significant level indicated by *= (p<0.05); **=(p<0.01); *=(p<0.001)**

Cell growth Curve and cell doubling time

Primary culture cells were sub cultured with a 1:1 split ratio when the cells reached to the 70% confluence; since the time, the cells have been maintained for total of 17 passages. The first two subcultures were done in to 1:1 ratio in 12.5 cm^2 flask with interval of 6 days, from third passage it was transferred to the 25 cm^2 tissue culture flasks with average of 8 day intervals. Thereafter, the split ratio was increased gradually to 1:2 to 1:3 ratios on every 7^{th} to 8^{th} days. Growth curve was measured at the 9^{th} passage. In Fig 2.6, the curve demonstrates that the logarithmic growth phase observed from 72 hrs to 312 hrs after the sub culturing. The saturated cell formed on the day 14 and 15. After which cells aged and died. Saturated cells formed approximately 9.00×10^4 cells in $25 cm^2$ flask, which denoted a 4.5 –fold increase over the initial population (2×10^4 cell/flask) on the 13^{th} day. Population doubling time during logarimatic growth was calculated at 72.3 hrs using the Hayflick formula (Hayflick, 1961).

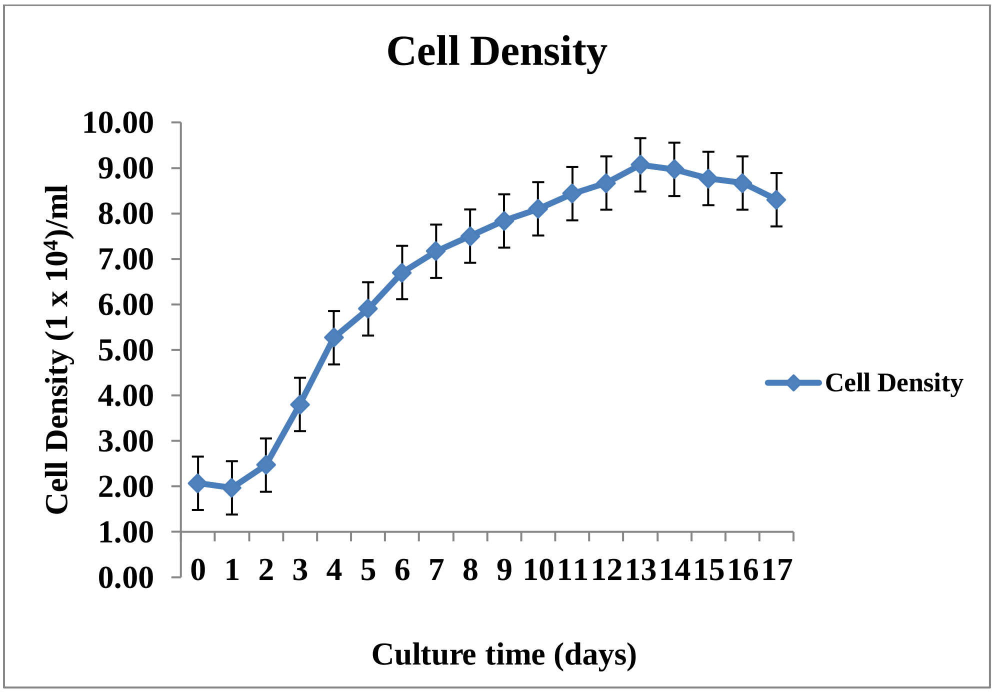


Figure 2.6: A Growth curve of SoMG cell line in Grace's medium containing 25% fetal bovine serum + 10pmol ecdysone+ vitamin mixture.
Significant level indicated by *= (p<0.05); **=(p<0.01); *=(p<0.001)**

Days	Cell Density	Days	Cell Density
0	2.07 ± 0.06	9	7.83 ± 0.23
1	1.97 ± 0.08	10	8.10 ± 0.23
2	2.47 ± 0.14	11	8.43 ± 0.17
3	3.80 ± 0.20	12	8.67 ± 0.14
4	5.27 ± 0.24	13	9.07 ± 0.23
5	5.90 ± 0.17	14	8.97 ± 0.20
6	6.70 ± 0.26	15	8.77 ± 0.06
7	7.17 ± 0.20	16	8.67 ± 0.03
8	7.50 ± 0.20	17	8.30 ± 0.15

Table 2.8: Shows the Cell growth density at 24 hrs interval on 9th passage
Significant level indicated by *= (p<0.05); **=(p<0.01); *=(p<0.001)**

Cell morphology

At the beginning cell growth was slow, but after few days the cell groups formed colonies loosely attached to the surface of the flask. There was also proliferation of cells in suspension. However, many small tissue pieces of midgut gradually attached to the bottom of the flask and many cells migrated from the tissue pieces and gradually spread out. The round cells grew faster than any other cells in the flask. Most cells were round, some cells appeared to be spindle- like when seeded, but gradually became round again as the density of the cells increased. Many cells aggregated to form multiple cell masses. The cell line named as SoMG after it had been successfully sub cultured for over 17 generations of subculture. The size of the SoMG cells was 16.4 ± 2.1 µm in diameter, the maximum density of cells was 3.45 ± 1.09 x 10^6 cells/ml, and the population doubling time during logarithmic growth phase was 72.3 ± 4.3 hrs at 28˚C.

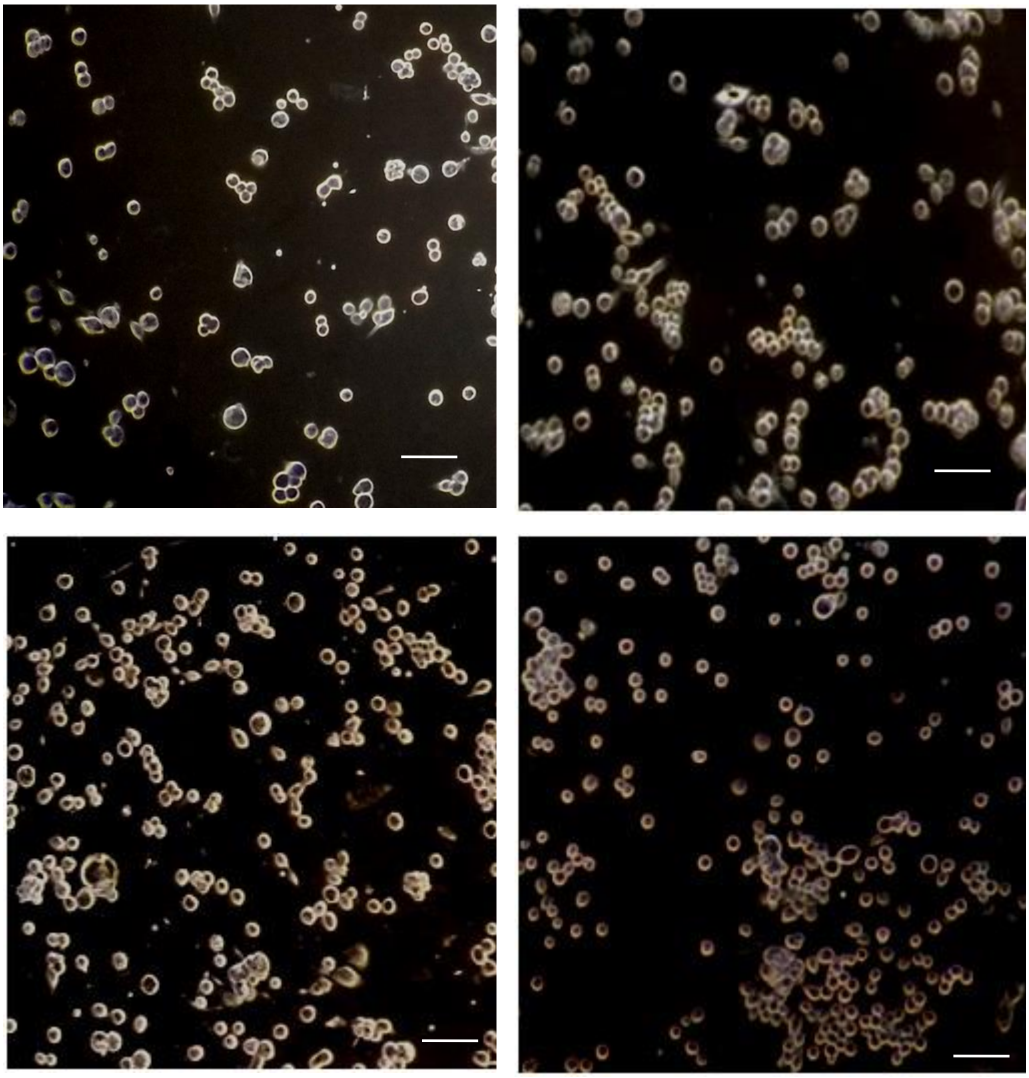


Figure 2.7: *S. oryzae* midgut cultured cell growth in Grace's media having 25% FBS +10pmol Ecdysone. A)at 24 hrs B) at 48 hrs C) at 72 hrs D) at 96 hrs (100X magnification, Bar=50µM)

Molecular Characterization

DNA sequencing of *Cytochrome oxidase I* (COI) and 16s rRNA was carried out to prove that the cell line belongs to *S. oryzae*. Sequence alignment and homology search was performed using MEGA 7 software. The obtained sequence was subjected to NCBI BLAST and was confirmed that there was 99.96% for COI and 96% for 16s rRNA homology of this cell line with *S. oryzae* with E value zero. COI and 16s rRNA sequence of *S. oryzae* on NCBI suggested that the cell line belongs to this species. Furthermore, to identify the nature of cell line, expression pattern of candidate genes of cuticle (*cup1* and *cup4*), *hmg176* which are known to be expressed in midgut cells (Shao *et al.,* 2013) was analyzed. The results revealed that the highest expression was of *hmg176* followed by *cup4* and *cup1* suggesting that the cell line showed the presence of epidermal markers as shown in Fig 2.10.

COI gel image

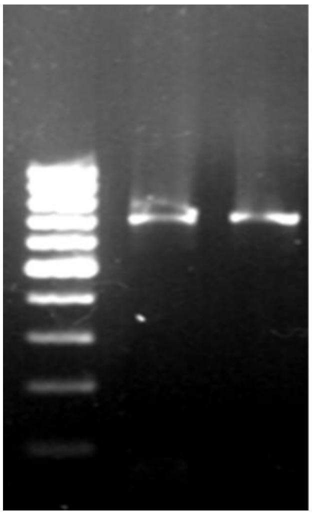


Figure 2.8: Gel image of COI, lane 1 DNA ladder, lane 2 tissue, Lane 3. SoMG cell line

**CCCAATCTTTATGATTTGTTGACAGGTTAACTTACAGGGCTGACCAAAAATATCACCA
ATATCTTTATGATTTGTTGACAAGGTAAAATTTCAGGTTGCCAAAAAATGCCCCATAAT
TTTGAGGATTGGTGGACAAATTCTTACTGCTCGTATAAAAAAGGTTATTCGTTCATATA
ATATTCCTGAGGGTCGTATATTATAGGCTGTTGTAATAAAATTAATAGCTCCTAGAATA
GATGAAATTCCTGCTATATGTAAACTGAAAATGGCCAGATCAACAGAAGCTCCTTCAT
GGGCAATATTGGATGAGAGCGGGGGGTAGACGGTTCATCCTGTTCCTGCTCCCTTTTC
AATAAATCTTCTTATTAGTAAAAGAGTTAAGGAGGGTGGAAGTAATCAAAATCTTATA
TTATTTAAACGGGGGAATGCTATATCTGGGGCTCCTAATATTAATGGGATTAATCAGT
TTCCAAATCCTCCAATTATAATTGGTATTACTATAAAGAAAATTATAATGAATGCATGT
GCTGTGACAATAGTATTATAAATTTGGTCATTTCCAATTAGTGATCCAGGATTTCCTAG
TTCTGCCCGAATTAGCAAACTTAAGGATGTACCTACTATTCCTGATCATGTTCCAAAAA
TAAAGTATAATGTTCCAATATCTTTATGATTTGTTGACCAAC**

16s rRNA

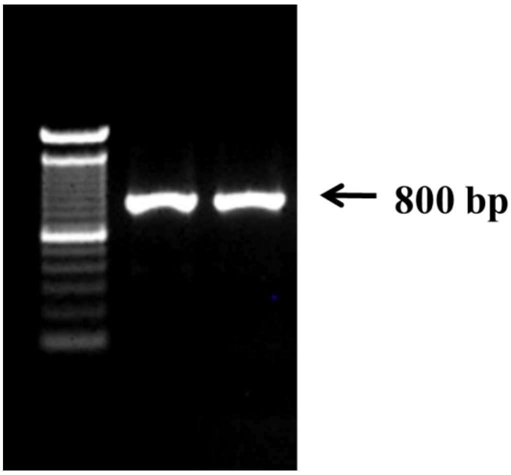
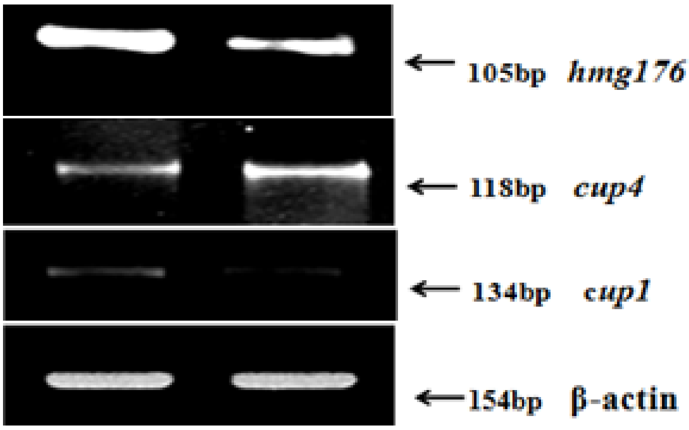


Figure 2.9: Gel image of 16s rRNA. lane 1 DNA ladder, lane 2 tissue, Lane 3. SoMG cell line

**CTTTTTGATTATAATTTAAAGTCTAACCTGCCCAATGATAAAATTTAAATGGCTGCGGT
ATTTTGACCGTACAAAGGTAGCATAATCATTAGTTTTTTAATTGAAAGCTGGAATGAAA
GGTTGCATGAAAAAATGACTGTCTCTATTTAAATTTAATTGAATTTTATTTTTAAGTAA
AAAACGCTTAAATTTTTCTAAAAGACGAGAAGACCCTATAGAGTTTTATAAATTTATTA
TAAGGAATTTTTAGGATTTAAAATAATTTATATTTAATTTATTTAATTGGGGTGATTGA
AAAATTTGTTAAACTTTTTTATTTATATTACATTAATTTATGAGTTTTTGATCCTTACTT
TAAGATTAAAAGATAAAATTACCTTAGGGATAACAGCGTTATGCGCATTGAGAGTTCA
AATCGAAAAAAAAGATTGCGACCTCGATGTTGGATTAAAATAAATTTTTGGTGTAGAA
GCTAAATTTTTGATTATAATTTAAAGTCTAACCTGCCCAATGATAAAATTTAAATGGCT
GCGGTATTTTGACCGTACAAAGGTAGCATAATCATTAGTTTTTTAATTGAAAGCTGGA
ATGAAAGGTTGCATGAATGCATGACTGTCTCTATTTAAATTTAATTGAATTTTATTTTT
AAGTAAAAAA**

A.



B.

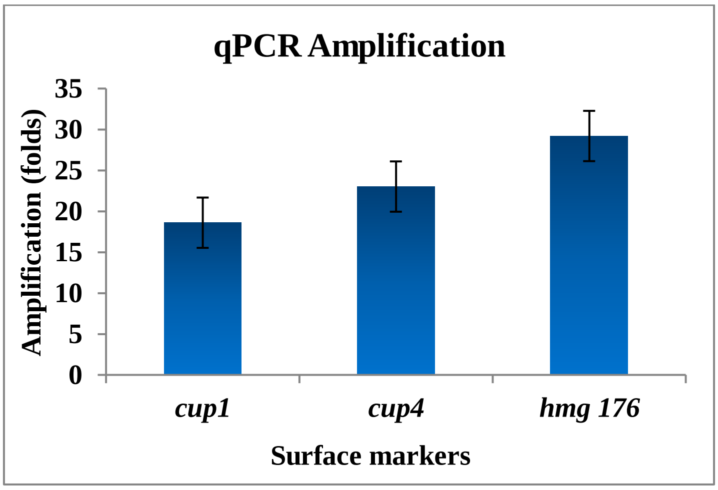


Figure 2.10: A. Semi-quantitative RT-PCR to compare gene expression patterns in SoMG cell line and tissues. B. Quantitative qPCR amplification for 3 surface marker.

Karyotype analysis

S. oryzae karyotype showed a total of 24 chromosomes (2n), which includes 11 pairs of autosomes and a pair of sex chromosomes. The results of chromosomal morphology revealed that 6, 8, 10, 11 were metacentric, 1, 7, X chromosomes were sub metacentric, 2, 3, 4, 5, 9 were acrocentric and the Y chromosome was Telocentric. The highest size of the chromosome was depicted by autosome no. 1 followed 6 and smallest was by autosome 10 followed by Y chromosome (Fig: 2.11) Male meiotic cells analysis suggested that the, a set of sex chromosome system of the Xyp type having the meioformulae n=11 + Xyp was determined by karyotype analysis (Fig: 2.11).

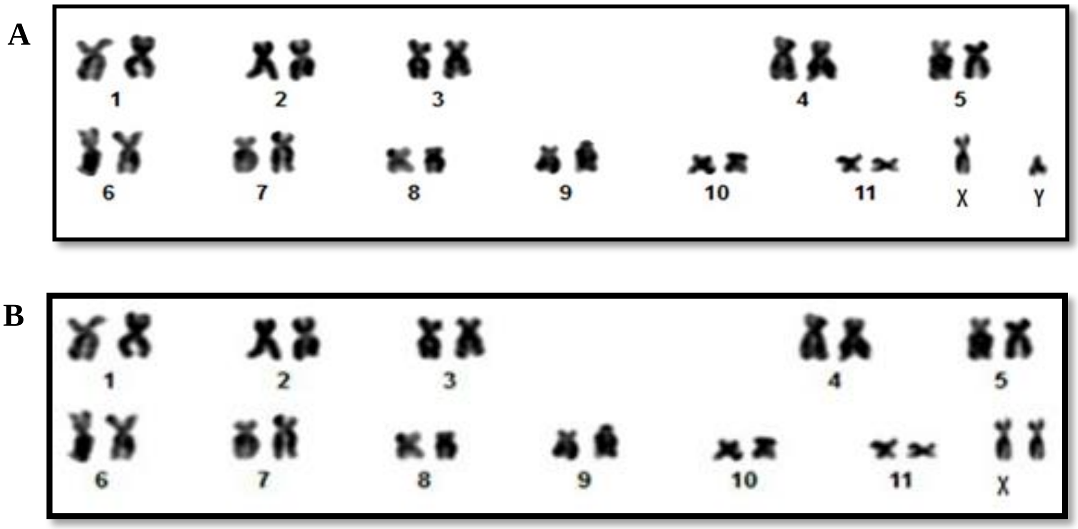


Figure 2.11: Karyotypes of SoMG cell line. A) Male B) Female (Magnification 10 x 100)

2.4 Discussion

Advances in insect science through cell line systems will be part of the modern vanguard of agricultural science necessary for safer production of healthier foods to meet the demands of a rapidly increasing human population. However, due to the heightened possibility of microbial contamination when isolating midgut cells, the culturing of midgut cells is more difficult than that of other tissues of insects. Therefore, cell line from midgut tissues are very limited in number (Garcia *et al.,* 2001; Pringle *et al.,* 2003; Goodman *et al.,*2004; Kharat *et al.,* 2010; Aljaber *et al.,* 2014; Li *et al.,* 2015).

One of the critical factors for successful initiation of primary cell line of insects is the optimization of the growth media (Aljabr *et al.,* 2014).Insect cells are readily amenable to suspension culture and the continuous improvement of cell culture media and additives has been reported to contribute and has been a reliable and robust scale-up practice for commercial applications (Mitsuhashi, 1989; Belloncik *et al.,* 1997; Agathos, 2007; 2010). In our study, among all the media analyzed, Grace's insect media was found to be more suitable and had resulted in optimum growth of the cells, which is in agreement to the previous study done by Aljabr *et al.,* 2014. However, in the present study it was reported that the concentration of FBS and ecdysone had a significant effect on cell growth. As reported earlier by Chittaranjan *et al.,* (2009) the insect steroid hormone ecdysone triggers as well as controls cell death and cell survival and have confirmed the presence of functional pro-survival genes using RNA interference in ecdysone-treated *Drosophila l(2)mbn* cells . The primary goal of the present work was to initiate and establish a new cell line form the midgut cell; hence the positive role of ecdysone as an additive in the media probably is helping in survival of the cells. However, RNAi studies will prove the dependency and potential function of ecdysone in cell growth regulation.

The time spent in cell growth and proliferation from midgut tissues of *S. oryzae* until the formation of the confluent monolayer was relatively short, similar to that reported by Segura *et al.*, (2012) and Cruz and Bello (2013). As reported by Zhang *et al.,* (2011) the formation of the cell monolayer and its further proliferation is dependent on the process of adaptation and on the nutritional, physical-chemical, and environmental conditions of the culture media. In the present study, FBS and the multivitamin mixture which were added to the Grace's insect media probably has fulfilled the necessary nutrients requirement. Further, trehalose and yeastolate, has possibly promoted the adhesion, growth, and spreading of the cells on the surface of culture flasks (Zhang *et al.,* 2011).

In the present study, we have characterized and established a new insect cell line (SoMG), from the adult mid gut of *S. oryzae,* which were mostly round cells and has been characterized as stem cells by many researchers and might be confirmed as stem cells upon closer inspection in the future. comparable, round stem cells have been isolated from the midgut of several insect orders including Coleoptera (Hakim *et al.,* 2007) and Lepidoptera (Hakim *et al.,* 2009) both from embryonic and adult tissues (Corley and Lavine, 2006). Midgut cells are regenerated during molting and show rapid cell turnover during active digestion (Loeb *et al.,* 2001;2003; 2010). Cells in the midgut epithelium culture developed in this study (SoMG) remained suspended in the medium, and the growth was slow exhibiting a distinct lag phase and a stable plateau phase. These results suggest that SoMG cells possess growth characteristics similar to those of normal rather than transformed cells as reported by Aljabr *et al*. (2014) who have established cell line from *R. ferrugineus* beetle and Li *et al.,* 2015b who has developed cell line from *H. armigera*. Thus from the present work unlike the previous studies where the establishment of new cell line has been from various tissues as well as with the mixture of adult and larval tissues, we have successfully initiated primary cell line from non-embryonic tissues from the midgut cells of *S. oryzae*.

Characterizing cell lines on the basis of phenotype or gene expression is potential authentication technique. After the successful survival of the cells two DNA fragments (COI and16S rRNA) for cell line identification were used as COI sequences are easier to align and the sequencing quality of 16S sRNA is comparatively higher (Folmer *et al.,* 1994; Simon *et al.,* 1994; Levy *et al.,* 2002; Ratnasingham and Hebert, 2007; Nunez-Valdez et al., 2008; Tanak *et al.,* 2012; Lv *et al.,* 2014; Jianghuai *et al.,* 2015). The efficiency of the two DNA fragments was evaluated by BLASTn method. The obtained target fragment sequences were aligned with the published sequences in the database. The homology sequence database results revealed 100% similarity between SoMG cell lines and its host *S. oryzae*. Further, it also confirms that

DNA barcoding is a strong tool which replaces isoenzyme analysis for the identity of cell line characterization. Furthermore, we characterized the cells using the marker genes of cuticle: *cup1*, *cup4* and *hmg176* to confirm the origin of the cell line. We found that all the three genes *cup1, cup4, hmg176* were expressed in midgut cells supplied with Grace's Insect media having 25% FBS +10pmol ecdysone suggesting the presence of epidermal cells in the midgut. The expression of these genes thus implicate that the SoMG cell is epidermal in origin which is in agreement with the studies of Shao *et al.*, (2008) where they have established HaEpi cell line from *H. armigera* and have proved that the cell line is an epidermal cell line based on its unique gene expression pattern.

Karyotyping confirms cell line identity and species of origin. Comparative cytogenetics to derive the phylogenic relationship among *Sitophilus* grain weevils (Coleoptera, Curculionidae, Dryophthorinae) has been studied by Silva *et al.*, (2018) but no studies have dealt with the karyotyping of *S. oryzae* cell line, hence, we have compared and analysed the karyotype obtained from *S. oryzae* insect. The study showed 2n=22 of chromosome number, the parachute configuration, and the dominance of metacentric type of chromosomes found in midgut cells of *S. oryzae* which resembles the characteristics already described in most species of Curculionidae (Fornelli *et al.*, 2004; Lachowska *et al.*, , 2006, 2008, Rozek *et al.*, 2009; Holecová *et al.*, 2013). Similar studies on the karyotypes of *S. oryzae,* that have 2n=22 chromosomes, suggest they could have originated as a result of pericentric inversions in small pairs followed by fusions between them. Therefore, we predict 2n=22 represent the ancestral chromosomal number for other Curculionidae species (Smith and Virkki, 1978; Holecová *et al.*, 1995; Lachowska *et al.*, 1998). As c-banding pattern was not required for the present study, hence we cannot comment on heterochromatin and euchromatin region.

The cytogenetic analysis unravelled the possible differences among the karyotype in the position and size of the autosomes and sex chromosomes. For instance, Silva *et al.,* (2018) suggested that karyotype of *S. granaries* and *S. linearis* had submetacentric X chromosome but the Y chromosome was subtelocentric, respectively. While, *S. oryzae* and *S. zeamais* showed metacentric X chromosomes, while, the Y chromosome in *S. zeamais* was punctiform that of *S. oryzae* was telocentric. In *S. linearis*, the X chromosome represents the longest element in the karyotype. Together, these characteristics facilitate the identification of this particular species. Our results are in accordance to the above described characteristics of autosomes and sex chromosomes of *S. oryzae, S. granaries* and *S. linearis*.

We also predict the phenomena of translocation due to which there is difference in the size of the chromosomes which is also been reported in several other insect species (Bárcenas-Ortega, 1992; Macaisne *et al.,* 2006; Dutrillaux and Dutrillaux, 2007; Kaiser and Bachtrog, 2010; Dutrillaux *et al.,* 2013). Similar studies conducted on the *R. ferrugineus* by Al-Qahtani *et al.,* (2014) classified autosomes into five pairs of metacentric and five pairs of submetacentric with chromosome Y dot-shaped a and chromosome X acrocentric with meioformula of n♂ =11+Xyp which was in agreement with ours and most of the previously reported studies (Smith and Virkki, 1978; Tucic and Mesaros,1992; Holecova *et al.,* 1997; Lachowska *et al.,* 1998; Lachowska and Holecova, 2000; Holecova *et al.,* 2002; Dutrillaux *et al.,* 2008; Lachowska *et al.,* 2008; Holecova *et al.,* 2013). However, further studies will be necessary to confirm the mechanism, banding pattern of autosomal pairs involved in the process of chromosomal rearrangements concerning the cells of SoMG.

Hence, it is proved that the midgut cell line expresses the epidermal genes, however, the exact function of these genes in the cells are yet to be discovered. Therefore, further studies are needed to carry out the mechanistic action and their role in these cells.

2.5 Conclusion

From the present study, we can conclude that the newly established adult *S. oryzae* mid gut cell line has its epidermis roots and the cell line responded well in Grace’s insect media with 25% FBS + 10 pmol ecdysone + vitamin mixture + antibiotic. This opens up new avenue presenting a new cell line model of store grain pest for investigating the hormonal and other signaling transduction pathways in Coleoptera. However, further studies are needed for making the cell line immortal by transfecting it with bacculovirus.